Adsorbed Protein Secondary and Tertiary Structures by Circular Dichroism and Infrared Spectroscopy with Refractive Index Matched Emulsions

Fiona A. Husband, Martin J. Garrood, Alan R. Mackie, Gary R. Burnett, and Peter J. Wilde*

Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, United Kingdom

The secondary structure of protein adsorbed at the emulsion interface has been studied in refractive index matched emulsions using the techniques of circular dichroism (CD) and Fourier transform infrared spectroscopy. Bovine serum albumin (BSA) and bovine β-lactoglobulin (βlg) stabilized emulsions were studied, and the refractive index was altered by the addition of glycerol or polyethylene glycol. The effect of additive on the solution and adsorbed protein structure in addition to the effect of adsorption time was considered. Both adsorption and glycerol addition alter protein secondary structure; however, the majority of secondary structure remains. Small changes are observed in the secondary structure of adsorbed protein with time. Near-ultraviolet CD studies showed the effect of glycerol and adsorption on the aromatic groups. BSA showed small changes both upon the addition of glycerol to protein in solution and upon adsorption. βlg showed slightly larger changes upon the addition of glycerol to protein in solution and a larger change upon adsorption.

Keywords: *Emulsion; secondary structure; β-lactoglobulin; BSA; CD; refractive index matched emulsion*

INTRODUCTION

The structure of adsorbed proteins has been of interest for some years and has been approached by many different techniques, including fluorescence, ellipsometry, and spectroscopy. Each of these techniques yields different information concerning the amount or structure of the protein adsorbed at an interface. Ellipsometry has been used mainly to quantify the amount of adsorbed protein (*1*), although some evidence of conformational change upon adsorption has also been obtained (*2*). Both front-face steady-state fluorescence (FFSF) (*3*) and total internal reflectance fluorimetry (TIRF) studies have focused on conformational changes by analyzing changes in fluorescence. However, these fluorometry techniques either provide limited information on specific amino acids (i.e., FFSF) or have inherent drawbacks such as the lack of a reliable calibration method (i.e., TIRF) (*4*). Fourier transform infrared spectroscopy (FTIR) has frequently been used for investigating the conformation of proteins adsorbed to solid surfaces and more recently to β-lactoglobulin-stabilized emulsions (*5*). However, quantifying secondary structure from FTIR can be rather unreliable (*6*) due to the need to subtract solvent contributions.

Circular dichroism (CD) was originally used to study proteins adsorbed to flat solid substrates (*7*), but analysis was difficult due to sample orientation effects. This problem was overcome by Kondo (*8*) by the adsorption of protein to 15 nm diameter colloidal silica. Light scattering was minimized by the use of silica, which was sufficiently small to minimize scattering and had a refractive index (RI) similar to that of water. This approach was extended recently by Maste (*9*), who adsorbed protein to 215 nm diameter latex beads. However, both colloidal silica and latex differ significantly in surface characteristics and size from oil droplets in a protein-stabilized emulsion. Size, curvature, and surface characteristics as well as surface coverage may play important roles in determining the degree of conformational change of the protein at an interface.

Proteins adsorbed at an oil-in-water emulsion interface are more difficult to study because the emulsion droplets (diameter = 1−10 μm) produce a significant amount of light scattering. This makes measuring secondary structure of adsorbed protein by CD difficult because this technique relies upon the relative adsorption of right and left circularly polarized light. Therefore, to overcome the scatter problem, the RI of the continuous phase (phosphate buffer in this case) was adjusted, by the addition of a higher RI liquid, so that its RI matched that of the oil phase (*n*-tetradecane).

RI additives, for far-ultraviolet (UV) CD use, must have a high RI, have good water solubility, have low absorbance from 260 to 190 nm, be nonhalogenic, be non-denaturing to protein structure, and be preferably not chiral. Glycerol and polyethylene glycol fulfill these criteria, and both have been used as refractive index additives in this paper. Glycerol has previously been used to stabilize the native structure of proteins (*10*), whereas polyethylene glycol (PEG) has been used for a protein crystallizing agent (*11*).

It is the aim of this paper to show the applicability of CD, with supporting FTIR measurements, to measure adsorbed protein secondary structure in a protein-stabilized emulsion, as well as to compare the effects of RI additives and adsorption time on protein structure. It is also the aim to show the possibilities of examining tertiary structure in a refractive index matched emulsion (RIME) system.

* Author to whom correspondence should be addressed [telephone +44 (0)1603 255258; fax +44 (0)1603 507723; e-mail Peter.Wilde@bbsrc.ac.uk].

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Table 1. Qualitative Band Assignments for FTIR Spectra (*5*, *13*)

wavenumber (cm^{-1})	assignment
1622	β-sheet (exposed/intermolecular)
1632	β-sheet (antiparallel)
1645	random
1649	α-helix + random
1678	β-sheet
1682	intermolecular β-sheet, β-turns

MATERIALS AND METHODS

A 20% (v/v) *n*-tetradecane emulsion stabilized by 3 mg/mL bovine β-lactoglobulin (βlg) (Sigma, L-0130, lot 91H7005) and a 16.7% (v/v) *n*-tetradecane emulsion stabilized by 4.5 mg/mL bovine serum albumin (BSA) (Sigma, A7030, lot 19F0091) were used. The emulsions were formed by passing a premix through a valve homogenizer (Avestin) at 4000 psi. Particle sizing was obtained from an LS-230 (Coulter), with emulsions prior to the addition of the RI additive. The amount of adsorbed protein was assayed by centrifuging the emulsion at 13000 rpm (11600*g*) for 15 min and measuring the absorbance, with a λ-9 spectrophotometer (Perkin-Elmer), of the subnatant at 280 nm after correction for scatter, using extinction coefficients of 0.96 and 0.66 mg mL^{-1} cm^{-1} for βlg and BSA, respectively.

The absorbance of the RIME was monitored, at 280 nm with a 1 cm path length, to check for any changes in protein adsorption within the time taken for the CD experiments to be carried out. Changes in protein adsorption could occur if the emulsion was creaming.

RI matching was carried out immediately prior to measurement of the CD spectra. The emulsions were index matched with either glycerol or polyethylene glycol. The addition of glycerol was by weight rather than volume and resulted in a pH change of <0.1 pH unit. Protein concentrations of 1 mg/mL βlg and 1.44 mg/mL BSA were used for CD measurements.

CD spectra were recorded using a JASCO J-700 spectropolarimeter, under the following conditions: 50 nm/min scan speed, bandwidth = 1 nm, response = 0.5 s, 5 points/nm, and 4 accumulations. Far-UV and near-UV CD spectra were recorded with a 0.1 mm and 1 cm path lengths, respectively. The spectropolarimeter was calibrated using camphorsulfonic acid. Limited analysis of far-UV CD spectra was carried out using the Selcon method (*12*). In the case of RIMEs, the spectral contribution of unbound protein has been subtracted prior to spectral analysis. The far-UV and near-UV CD unsmoothed spectra are represented as molar CD, based on the average molecular weight of an amino acid of 113.

FTIR spectra were recorded using a Nicolet 860 spectrometer, with an MCT detector and an ATR cell with a ZnSe crystal; 256 scans were accumulated per spectrum at a resolution of 2 cm^{-1}. Fourier self-deconvolution (FSD) was carried out, using OMNIC 5 software with a bandwidth of 16 cm^{-1} and an enhancement factor of 2. The emulsions were washed to remove unbound protein, by centrifuging at 13000 rpm (11600*g*) for 10 min and exchanging the subnatant layer for fresh buffer. This was repeated three times. The final cream layer obtained was used for FTIR studies. Deuterated phosphate buffer and deuterated glycerol were used for all FTIR measurements. For qualitative analyses, the IR spectra were Fourier self-deconvolved to enhance the bands due to individual secondary structure motifs. Band assignments were based on those described previously (*5*, *13*) and are shown in Table 1.

Glycerol (spectrophotometric grade) and PEG 300 (catalog no. 20,239-8) were obtained from Aldrich Chemical Co. Tween 20 was obtained from Pierce and Warriner. D-Glycerol [glycer-(ol-d_3)] and D-phosphate buffer salts were prepared by contacting the protonated forms with equal volumes of D_2O for 30 min and then removing the solvent by rotary evaporation. This was repeated for three aliquots of D_2O. All other chemicals were obtained from Sigma (Poole, Dorset, U.K.). The water (surface tension = 72.6 mN m^{-1}) used in this study was cleaned using an Elga Elgastat UHQ water purification system.

Table 2. Characteristics of the Protein-Stabilized Emulsions Used in This Study

protein	aqueous concn (mg/mL)	$d_{(3,2)}$ (μm)	SSA (cm^2/mL)	coverage (mg/m^2)	adsorbed amount (%)
βlg	3.0	1.10	55500	2.1	77
BSA	4.5	1.27	47000	3.4	60

Experiments were carried out in 10 mM sodium phosphate buffer, pH 7.0, or pD 7.0 for FTIR, at 20 °C.

RESULTS

Table 2 shows the basic characteristics of the βlg and BSA emulsions. The model emulsion recipes created emulsions with a monomodal size distribution and similar mean droplet diameters. Seventy-seven percent of the protein was adsorbed on the βlg emulsion, which corresponds to a surface coverage of 2.1 mg m^{-2}. No significant change in UV absorbance of the emulsions was observed within 10 min, suggesting little or no creaming occurred over the time scale of the CD measurement.

Figure 1 shows the effect on the far-UV CD spectra of index matching βlg-stabilized emulsions with glycerol. The spectra shown are unsmoothed. If the emulsions were not or only partially refractive index matched, the spectra were both noisy and positively displaced from the *x*-axis at 260 nm. The spectra were truncated at lower wavelengths due to poor signal-to-noise ratio. Addition of 58% (v/v) glycerol was required to totally refractive index match an emulsion; the absorbance of βlg-RIME at 350 nm with a 1 mm path length accounting for the absorbance of 58% glycerol was 0.007.

Figure 2 shows the effect of addition of 58% (v/v) glycerol on adsorbed and bulk βlg. CD analysis suggested that the addition of 58% (v/v) glycerol to βlg in buffer slightly increased the α-helix content (2%). A larger increase in α-helix (8%) was observed in the case of adsorbed βlg.

Far-UV CD spectra showing the effect of PEG on βlg, adsorbed and in solution, are shown in Figure 3. βlg in solution showed an approximate additional 10% helix in the presence of PEG 300. Index-matching βlg emulsions with PEG 300 resulted in a dramatic change in the shape of the spectra, with a peak shift in the minima from 206 to 213 nm and an increase in the peak maxima at lower wavelengths. PEG 400 and 600 produced similar changes in the shape of the spectra; however, the intensity of the spectra at 222 nm increased with increasing PEG chain length. No attempt was made to analyze the CD spectra of RIMEs prepared with PEG by the Selcon method. PEG absorbs UV light strongly below 191 nm, and hence the spectra were truncated. Data truncation, to 191 nm, results in larger errors when analyzed by Selcon. In the presence of glycerol, a shift in peak minima was also observed. However, in comparison to PEG 300, there were significantly smaller increases in both the peak minimum and maximum in the presence of glycerol. Consequently, glycerol was chosen for further refractive index matching experiments because the effect of glycerol on the structure of βlg in solution was much less than the effect of PEG 300.

The FTIR spectra (Figure 4a) and FSD spectra (Figure 4b) show the effect of glycerol on secondary structure of βlg in solution. The bands present at 1632 and 1678 cm^{-1} are similar in the presence and absence

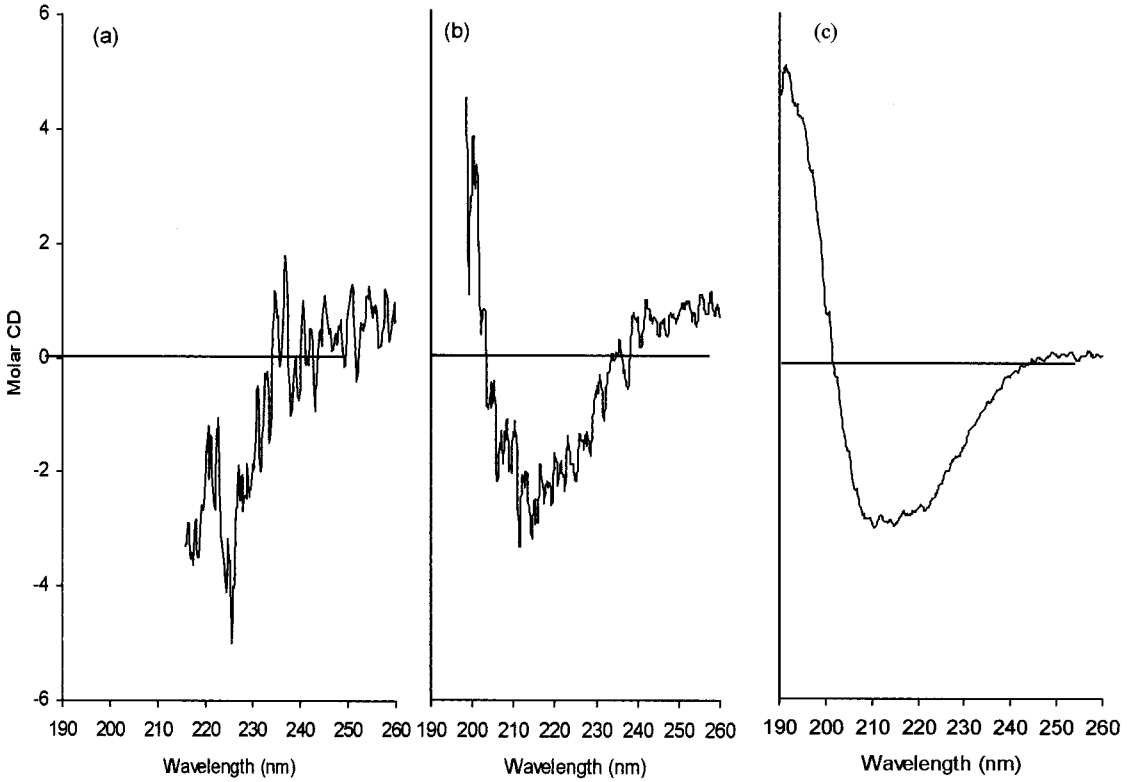

Figure 1. Far-UV CD spectra of βlg emulsions with (a) 0% (v/v) glycerol, (b) 35% (v/v) glycerol, and (c) 58% (v/v) glycerol.

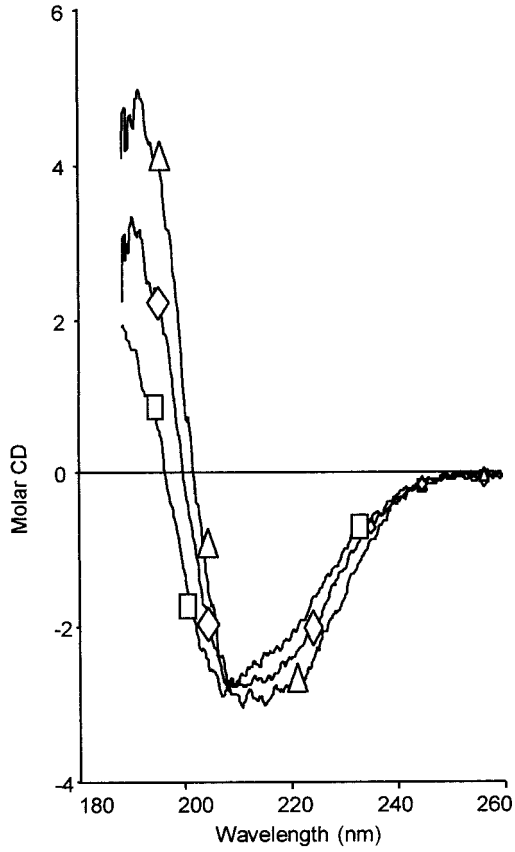

Figure 2. Far-UV CD spectra of βlg in buffer (□), βlg in buffer/glycerol (◇), and βlg-RIME with 58% glycerol (△).

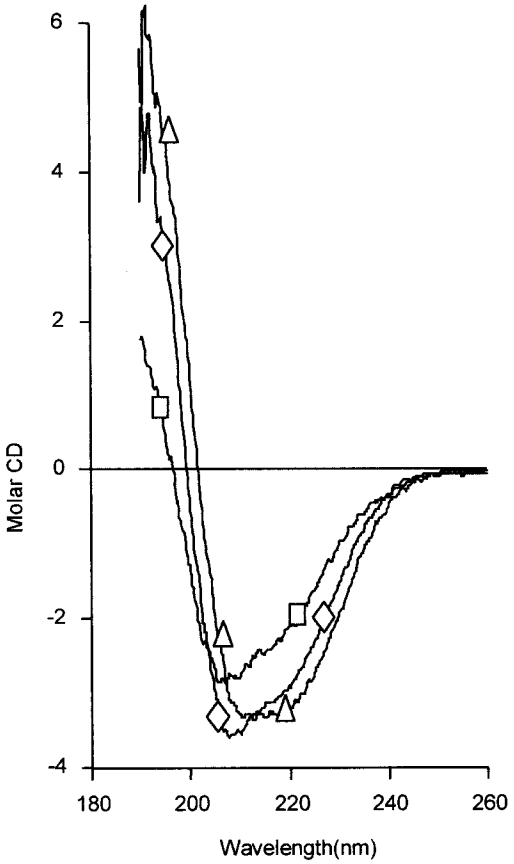

Figure 3. Far-UV CD spectra of βlg (□), βlg/PEG 300 (◇), and βlg/PEG 300-RIME (△).

of glycerol, indicating similar amounts of β-sheet; however, there are more obvious differences in the 1650−1640 cm^{-1} region. In βlg solution without glycerol there are two bands within this region, which are assigned as helix and random, whereas in the presence of glycerol, there is only one band present in the same region, indicating changes in the α-helix and aperiodic structures.

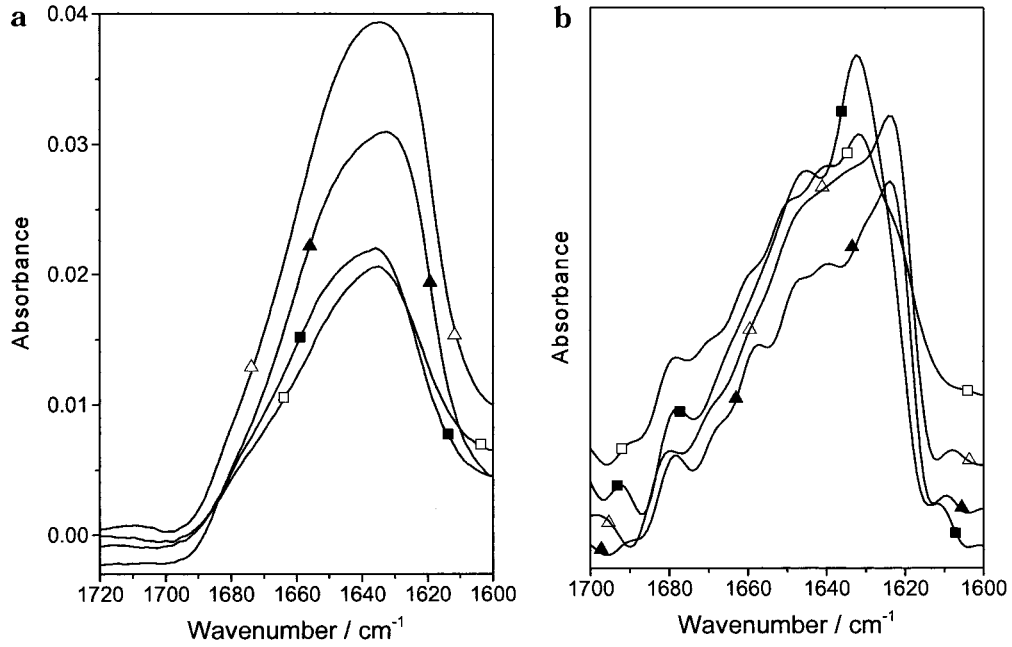

Figure 4. (a) FTIR and (b) FSD spectra of βlg (□), βlg + glycerol (■), βlg emulsion (△), and βlg-RIME (▲).

Figure 4b also shows that the effects of adsorbing βlg to the oil/water interface were considerably greater than those caused by the addition of glycerol. Emulsified βlg shows an increase in the 1680 and 1624 cm^{-1} bands (relative to solution βlg) and a corresponding decrease in the 1640 and 1650 cm^{-1} region. This suggests an increase in intermolecular β-turns or bends, whereas the intramolecular β-sheet remains similar in the emulsion and in solution. Furthermore, the addition of glycerol appears to have few structural effects on the βlg adsorbed onto the emulsion interface. In addition, the intensity ratio of the amide I to amide II band was constant for each protein in all four IR spectra (i.e., buffer, glycerol, emulsion, and RIME). This demonstrates that the denaturation of the protein upon emulsification does not result in further H to D exchange (which would cause structure-independent band changes).

CD spectra showing the effects of glycerol and adsorption upon BSA secondary structure are shown in Figure 5. The addition of glycerol resulted in minimal changes to the secondary structure (Selcon analysis suggests a 1% decrease in α-helix). However, upon adsorption to oil droplets in the presence of glycerol, there was a decrease in α-helix (9%), an increase in β-structures (14%), and a decrease in aperiodic structure.

Parts a and b of Figure 6 show the FTIR and FSD spectra of BSA adsorbed and in solution in the presence and absence of glycerol. Addition of glycerol to BSA in solution caused minimal changes in the spectrum. Upon adsorption, the FSD spectra showed a decrease at 1653 cm^{-1} as well as an increase at 1633 cm^{-1}, suggesting a decrease in α-helix as well as an increase in β-sheet structure. Addition of glycerol to the BSA emulsion appeared to have few effects on secondary structure.

Parts a and b of Figure 7 show the effect of adsorption time on the secondary structure of βlg and BSA, respectively. βlg showed the majority of changes at 222 nm up to 270 min, with only a small change between 270 and 1440 min. At 208 nm, the signal decreased significantly with time. The signal at 190 nm (data not shown) showed no trend over a similar time, and small

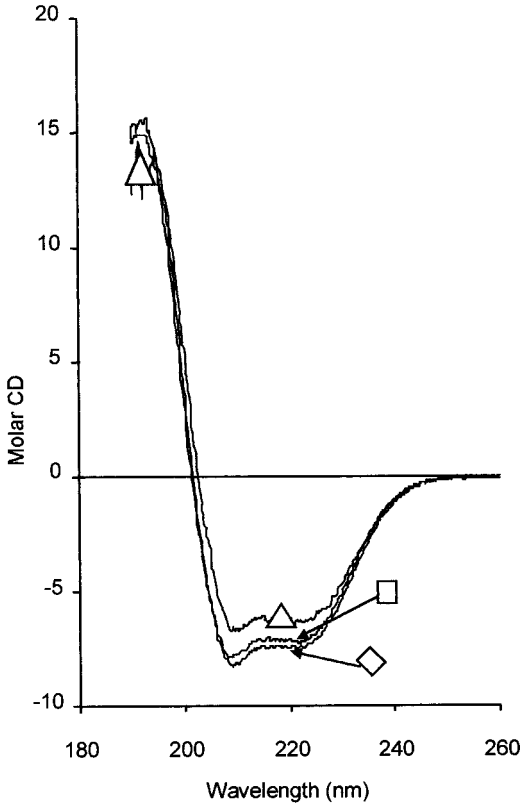

Figure 5. Far-UV CD spectra of BSA in buffer (□), BSA + glycerol (◇), and BSA-RIME (△).

changes (<0.5 nm) were observed in the negative to positive crossover point. These experiments were carried out by adding glycerol to an aliquot of emulsion immediately prior to measurement. A similar final spectrum (24 h sample) was also observed if the emulsion was aged in the presence of glycerol over 24 h (results not shown); however, the kinetics appeared to be slightly slower.

Adsorbed BSA, shown in Figure 7b, showed a progressive increase in signal at 222 nm with time, up to 290 min. At 208 nm, there was a slight increase in

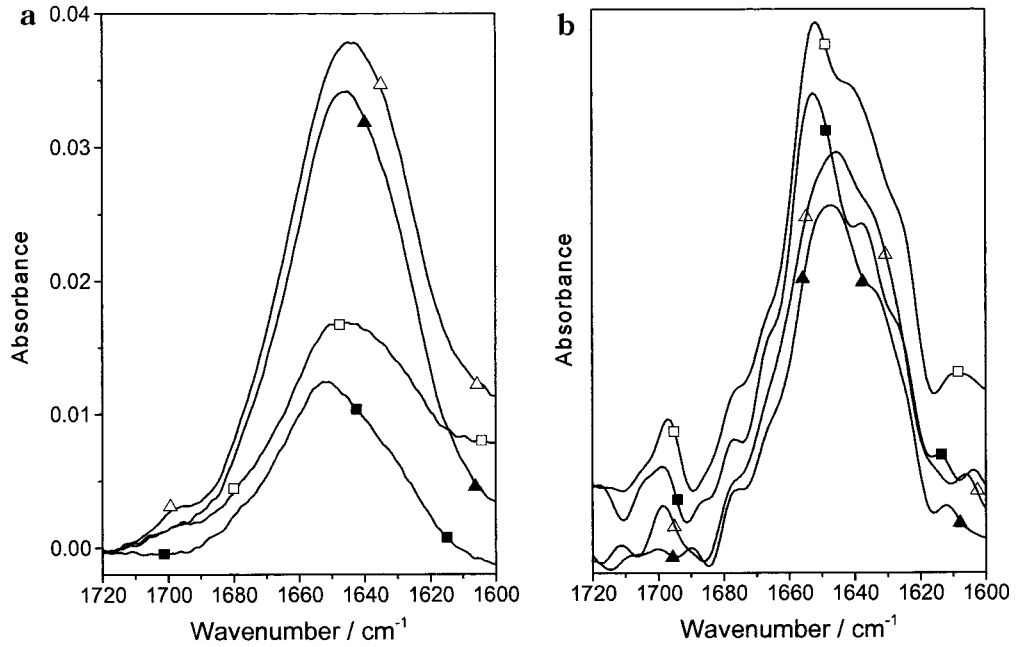

Figure 6. (a) FTIR and (b) FSD spectra of BSA (□), BSA + glycerol (■), BSA emulsion (△), and BSA-RIME (▲).

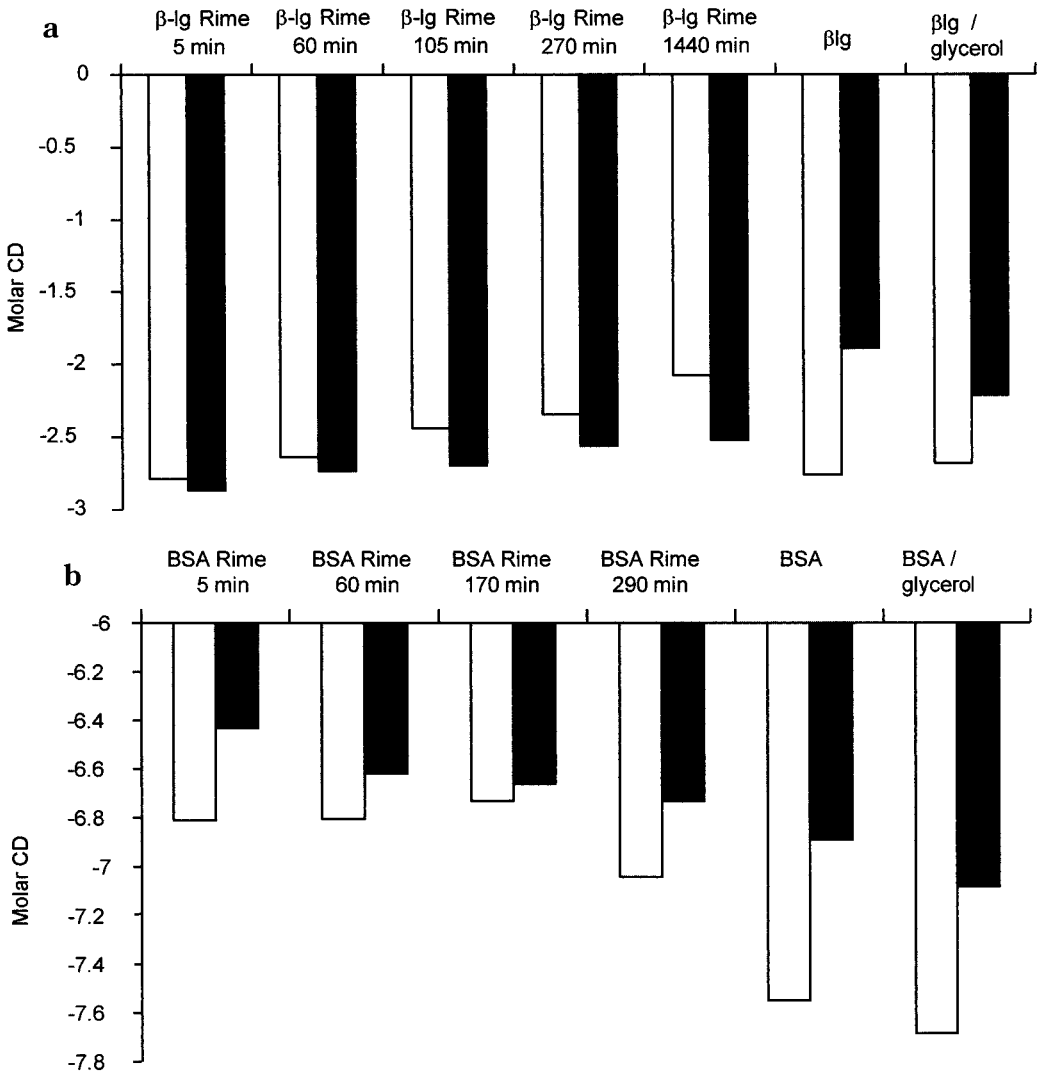

Figure 7. Effect of adsorption time on secondary structure of (a) βlg and (b) BSA molar CD at 208 (□) and 222 nm (■).

signal over 290 min. The signal intensity at 222 and 208 nm of BSA RIMEs appeared to be becoming more similar to the signal of protein in buffer/glycerol, although differences still existed.

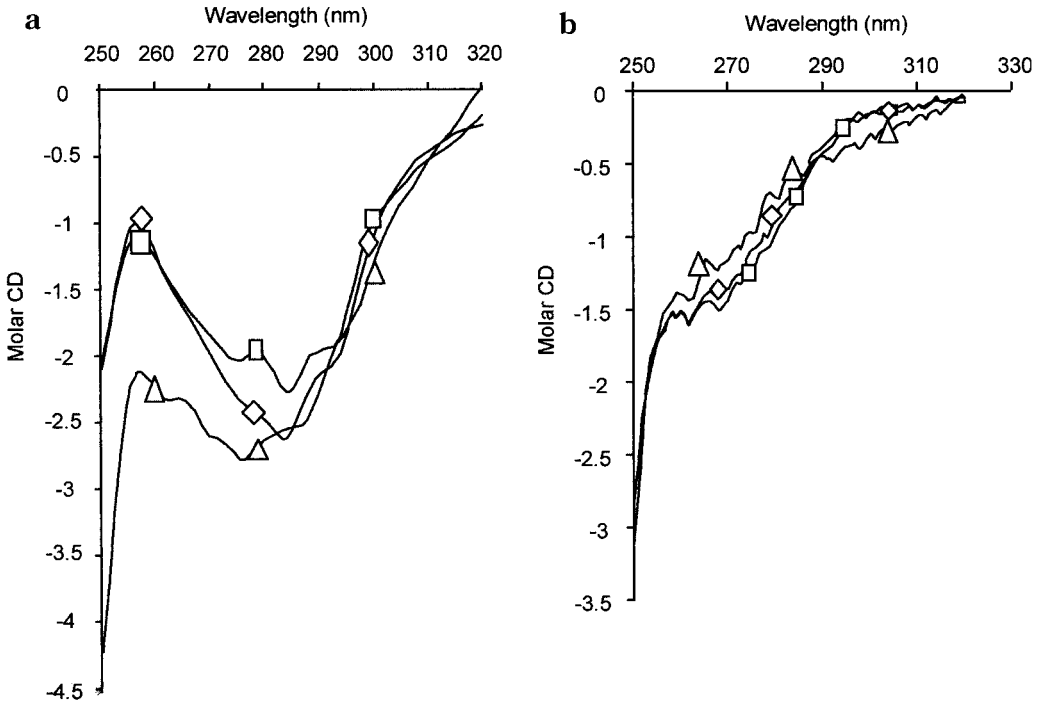

Figure 8. (a) Near-UV CD spectra of βlg (□), βlg/glycerol (◇), and βlg/glycerol RIME (△). (b) Near-UV CD spectra of BSA (□), BSA/glycerol (◇), and BSA/glycerol-RIME (△).

Near-UV CD of βlg and BSA in emulsions are shown in parts a and b, respectively, of Figure 8. βlg in solution showed a change in intensity of spectra around 278 nm in the presence of glycerol. Upon adsorption at an emulsion interface, βlg showed large differences between 250 and 290 nm, with some smaller differences above 290 nm.

BSA, in solution, showed small changes in the presence of glycerol, compared to BSA in buffer. Larger changes in the near-UV CD spectra were observed when BSA was adsorbed onto an emulsion. BSA showed smaller changes than βlg both in the presence of glycerol and upon adsorption to an emulsion.

DISCUSSION

The RIME method presented here allows for the measurement of the secondary structure of adsorbed protein in an emulsion system by the use of CD spectropolarimetry.

An increase in helical content of βlg in solution was suggested from Selcon analysis of CD spectra, in the presence of glycerol and PEG. This phenomenon has previously been reported for a variety of solvents (*14*, *15*).

Glycerol has been widely used as a stabilizing agent on protein structure and is widely used in the storage of enzymes (*16*). Cryopreservation usually involves the addition of ~50% (v/v) glycerol. At lower concentrations [up to 35% (v/v) glycerol], very little change in secondary structure was observed (results not shown). This agrees with the findings of Gekko and Timasheff (*10*), who proposed that in the presence of up to 40% (v/v) glycerol, the protein appears to be preferentially hydrated. However, at higher concentrations glycerol appears to cause an increase in α-helix (from CD experiments). The structural changes observed may be due to increased penetration of the glycerol into the preferential hydration layer and therefore increased formation of contacts with the protein. Alternatively, the high glycerol concentration may cause dehydration of the inner core of the protein (*17*).

Similar to glycerol, proteins are preferentially hydrated in the presence of PEG (*18*, *19*). The change in secondary structure of βlg in solution, at the high concentrations of PEG used here, is again attributed to increasing penetration of the protein hydration layer by PEG.

Secondary structure differences were observed between addition of PEG/glycerol to protein in solution and adsorbed protein (RIME). Protein is thought to unfold upon adsorption, although the extent of unfolding probably reflects the available space at the interface for each protein to unfold. The unfolding of protein will increase the exposure of nonpolar groups to the solvent. Bhat and Timasheff (*18*) proposed that increasing the exposure of nonpolar groups to solvent would result in a decrease in preferential exclusion of PEG. PEG is hydrophobic in nature and can therefore interact with the additional protein groups exposed on unfolding (*20*), thereby facilitating PEG's penetration of the hydration layer of the protein (*18*).

FTIR results show trends upon the adsorption of βlg to an emulsion interface similar to those reported previously by Fang and Dalgleish (*5*), that is, alteration of the β-sheet structure. FTIR also demonstrates that the effect of glycerol on solution βlg secondary structure is small in comparison to the effect of adsorbing the protein.

Broad agreement exists between the CD and FTIR results, in that the majority of the main structural elements are retained upon adsorption. CD analysis suggests an increase in α-helix (βlg in solution with glycerol). FTIR shows a decrease in 1640 cm^{-1}, which can be assigned to aperiodic structure (*5*). However, comparison of the FTIR spectra of βlg/glycerol and βlg-RIME suggests changes in β-structure, and CD analysis suggests changes in helical structure. This may be explained by two factors: (a) The CD signal from the

intermolecular β-sheet peak at 217 nm is usually dwarfed by the α-helix signal. However, in some cases, the absorbance of the β-forms can increase as reported previously (*15*). (b) CD analysis overestimates α-helix in β-sheet-rich βlg, when analyzed from 190 to 260 nm (*6*). Closer agreement with the X-ray structure is obtained when CD spectra are analyzed from 170 to 260 nm (*6*). In the case of βlg, the spectral changes (i.e., crossover from negative to positive and peak position) are more indicative of increases in β-sheet structure (*21*) than the suggested increase in α-helix from Selcon analysis of CD spectra.

Glycerol addition has little effect on the structure of BSA in solution, whereas upon emulsification there is a 9% decrease in α-helix by Selcon analysis. This agrees with the results from the FTIR spectra. The amount of helix estimated from the CD spectra of BSA is higher than would be expected. This is believed to be an artifact of the commercial method of preparation; a discussion on the effect of different purification methods is detailed elsewhere (*22*). Similar results have previously been reported for defatted BSA (*23*). In this case, BSA is specified as containing low concentrations of fatty acids (<0.02%).

Studying a βlg emulsion with time shows an initial increase in intensity at 222 nm followed by a decrease with time. This overshoot phenomenon has previously been reported for βlg in refolding experiments (*24*). In this case, the changes in structure take place over several hours as opposed to the seconds time scale previously observed (*24*), possibly due to the relatively restrained state of the adsorbed form.

In the case of BSA, there is an initial decrease in the amount of α-helix upon emulsification. The amount of α-helix tends toward that observed for BSA in solution, with time. BSA-stabilized emulsions show only slight differences in structure with time. This is perhaps not so surprising as the structure is stabilized by 13 disulfide bonds.

The near-UV CD spectra of βlg shown in this study are similar to spectra reported previously (*25*). The peaks at 293 and 285 nm have previously been attributed to tryptophan, with peaks at 277 and 265 nm being ascribed to tyrosine (*26*). The presence of glycerol has resulted in an increase in intensity of the tryptophan and tyrosine bands. This can be explained by the presence of easily available aromatic group(s) on the exterior of βlg. Adsorbed βlg shows a further dramatic change in the CD spectra around 260 nm; this area is dominated by contributions from phenylalanine and tryptophan.

The near-UV CD of BSA demonstrated that there had been small changes in the environment of the aromatic groups (48 in total) both in the presence of glycerol and upon adsorption. The changes are mainly within the tyrosine and tryptophan bands. This agrees with front face steady-state fluorescence measurements (*3*), which observed an increase in intrinsic fluorescence due to tyrosine and tryptophan, as well as a λ_{max} peak shift, upon adsorption to a dodecane-in-water emulsion.

The choice of RI additive must be made with care, as even chemicals that were not thought to produce changes in structure (e.g., glycerol) can do so at the higher concentrations used here to index-match emulsions. The presence of glycerol affects the structure of the adsorbed protein differently from that of the protein in solution.

The high surface coverage of the emulsions by the protein, in this case, may have a direct effect on the amount of secondary structure changes observed in the protein upon adsorption. Atomic force microscopy studies have previously shown (*27*) that at similar surface coverages, complete monolayers of protein are formed. The formation of a complete monolayer may affect the ability of the protein to unfold.

CONCLUSIONS

The RIME system presented here allows for the measurement of the secondary structure of adsorbed protein in an emulsion system by CD. There appear to be only small changes in the secondary structure of the proteins examined, an α-helix-rich and β-sheet-rich protein, upon adsorption in this system. The tertiary structure appears to be more drastically affected by the adsorption, as evidenced by the changes in the environments of the aromatic residues.

ABBREVIATIONS USED

FFSF, front-face steady-state fluorescence; TIRF, total internal reflectance fluorometry; CD, circular dichroism; RI, refractive index; FTIR, Fourier transform infrared; PEG, polyethylene glycol; RIME, refractive index matched emulsion; βlg, β-lactoglobulin; BSA, bovine serum albumin; FSD, Fourier self-deconvolution; SSA, specific surface area.

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