Comparison of the Amide I/II Intensity Ratio of Solution and Solid-State Proteins Sampled by Transmission, Attenuated Total Reflectance, and Diffuse Reflectance Spectrometry

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The absolute and relative differences in amide I and amide II band intensities of albumin, β-lactoglobulin, and myoglobin as measured by attenuated total reflection infrared (ATR-IR) spectrometry, transmission of aqueous solutions, and KBr disks and diffuse reflectance (DR) spectrometry are compared. The amide I/II intensity ratios of the proteins sampled by ATR, DR, and transmission spectrometry of KBr disks were similar and were significantly different from the intensity ratios of the proteins in solution. The absolute amide II band intensity of dissolved proteins did not vary significantly with changes in pH. The difference in amide I/II intensity ratios between solution and adsorbed proteins was attributed to differences in secondary and possible tertiary structure. The thickness of each protein film was estimated with the use of the absorptivities calculated from the amide II band intensities of the KBr disk spectra. pH had a significant influence on the thickness of the adsorbed films. Differences in film thickness were attributed to a difference in the orientation of the protein molecules at the surface of the germanium internal reflection element.

Index Headings: ATR; Adsorbed protein; Solid-state and solution spectra of proteins; Amide I/II intensity ratio; Absorptivities of proteins.

INTRODUCTION

Research in the area of protein adsorption on solid interfaces has increased dramatically over the past 15 years as evidenced by three books[1-3] and many reviews. Many techniques have been used to study the adsorption of proteins including ellipsometry,[4] circular dichroism,[5] fluorescence,[6] and radiolabeling.[7] Attenuated total reflection infrared spectrometry has proven to be a particularly powerful method of studying adsorption phenomena at aqueous/solid interfaces because of its capability of investigating proteins in their natural environment, as well as the ease with which liquid samples can be handled. Much of this work has been directed toward understanding the clotting of blood that contacts solid surfaces. The changes in protein secondary structure that occur upon adsorption to a solid surface have been of particular interest.[8,9] From a qualitative standpoint, shifts in peak locations and band intensities in derivative and deconvolved spectra in the amide I and amide II region of the spectrum have been correlated to changes in the secondary structure of proteins. Recently, Jackson and Mantsch[10] described the artifacts associated with the determination of protein secondary structure by ATR spectroscopy. Far fewer studies have involved quantitative studies of adsorbed proteins. In this paper, we compare the absolute and relative difference in the amide I and amide II band intensities of adsorbed proteins as measured by ATR as well as transmission spectrometry of aqueous solutions and KBr disks and DR spectrometry of proteins dispersed in KBr.

EXPERIMENTAL

Bovine serum albumin (BSA), fraction V, β-lactoglobulin, and horse heart myoglobin were purchased from Sigma (St. Louis, MO). Aqueous solutions of myoglobin were filtered through polyvinylidene difluoride (hydrophilic) membrane filters of 0.45-μm pore size that were pretreated with a 100-mL aliquot of protein solution to minimize the losses associated with protein binding to the membrane. BSA and β-lactoglobulin were used without further treatment. Protein solutions were prepared in 0.15F NaCl (saline), and the pH was adjusted with HCl or NaOH.

DR and KBr Disks. Protein samples and KBr were ground with a mortar and pestle. Diffuse reflectance spectra (1.0% w/w) were collected with a Spectra-Tech (Stamford, CT) "Collector" diffuse reflectance sampling accessory. Fifty-milligram aliquots of 1.0 wt % protein in KBr were compressed into 7-mm-diameter disks approximately 0.5 mm thick. A 25-mg sample of myoglobin was used because the absorption bands were so intense. Protein spectra were ratioed against a KBr background spectrum.

Aqueous Solution Spectra. An 11.9-μm-pathlength cell with calcium fluoride windows was used. Saline reference spectra were recorded at pH 4.8, 7.0, and 9.0. The transmission cell was flushed with saline solution of the appropriate pH in between measurements of each protein spectrum. A saline reference spectrum of the appropriate pH was digitally subtracted from each protein spectrum to yield the spectrum of the solute.

ATR Spectra. ATR spectra were measured with a CIRCLE® cell (Spectra-Tech, Stamford, CT) with a cylindrical (3.25 × 0.25 in.) germanium internal reflection element (IRE). The flow cell was initially filled with saline at the appropriate pH. A saline reference spectrum was measured and stored for use with the saline subtractions. Protein solutions (0.01% w/v) were flowed over the IRE at 0.5 mL/min. After 4 h, saline at the appropriate pH was substituted for the protein solution. Spectra consisting of 175 coadded scans were collected every 5 min the first hour and then every 10 min for the next 3 h. The data acquisition protocol was repeated for the 4-h saline rinse. The temperature in the sample compartment of the spectrometer was maintained at 21°C. Only the final saline-subtracted spectra at each pH collected at $t = 8$ h are displayed in this paper.
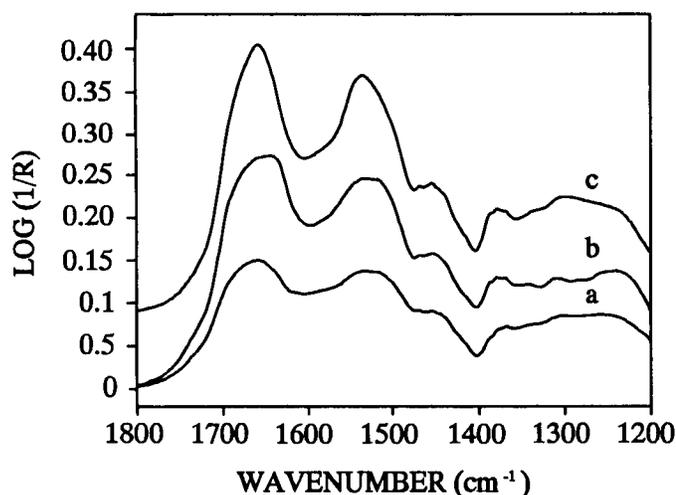
FIG. 1. Diffuse reflectance spectra of (a) albumin, (b) β-lactoglobulin, and (c) myoglobin dispersed in KBr at a concentration of 1.0% (w/w).
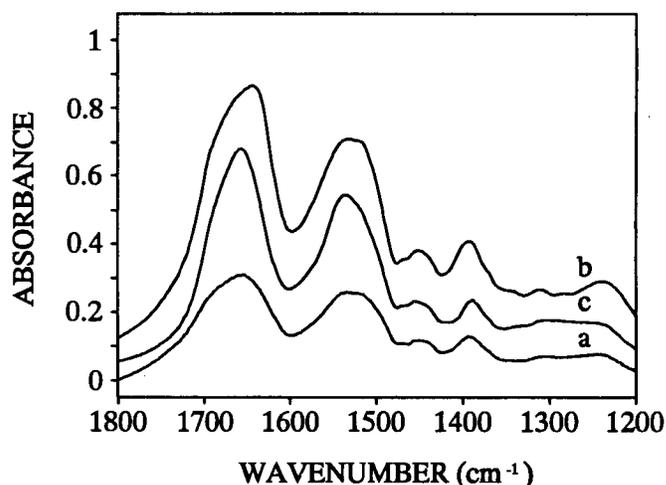

FIG. 2. KBr disk spectra of (a) albumin, (b) β-lactoglobulin, and (c) myoglobin at concentration of 1.0% (w/w). Note that the myoglobin disk was approximately half the thickness of the albumin and β-lactoglobulin disks.

ATR spectra were measured at 4 cm⁻¹ resolution on a Niciolet 740 FT-IR spectrometer equipped with a medium-range mercury-cadmium-telluride detector. Transmission, diffuse reflectance (DR), and KBr disk spectra were measured at 4 cm⁻¹ resolution on a Digilab FTS-40 FT-IR spectrometer equipped with a deuterated triglycine sulfate detector. All spectra were transferred to a network of personal computers. Spectral manipulations were carried out with SpectraCalc software (Galactic Industries, Salem, NH). Solution and ATR spectra were baseline-corrected at 2000, 1900, 1710, 1480, 1330, and 1190 cm⁻¹ as described by Lenk et al.[11] KBr disk and DR spectra were baseline corrected at 2000 and 900 cm⁻¹.

RESULTS AND DISCUSSION

DR and KBr Disk Spectra. The DR and KBr disk spectra of BSA, β-lactoglobulin, and myoglobin in the region between 1800 and 1200 cm⁻¹ are displayed in Figs. 1 and 2, respectively. As noted above, the bands in the spectrum of a 50-mg sample of myoglobin at a concentration of 1.0% were so intense that it was necessary to reduce the sample quantity by one half. The amide I/II intensity ratio for the proteins was approximately 1.1 for the DR spectra and 1.2 for the KBr disk spectra (Table

I). Castillo et al.[12] recorded the same amide I/II intensity ratio (1.2) for a KBr disk of human serum albumin, while Lenk et al.[11] reported an amide I/II intensity ratio of 1.6 for a KBr disk of BSA. While the amide I/II intensity ratios were nearly the same for each protein, the absolute amide II band intensities of the DR and KBr spectra were significantly different even though the protein concentrations (wt %) were the same (see Table II). Intuitively, one would have expected the absolute amide II band intensity to remain constant from protein to protein for both DR and KBr disk spectra, since each sample on average would theoretically contain approximately the same number of peptide bonds that contribute equally to the amide II vibrational band. The calculated absorptivities of the amide II band of these proteins, based on the KBr disk spectra, varied by more than a factor of 2.5 (see Table II), with the value for albumin being significantly less than that of β-lactoglobulin and of myoglobin. The unit of absorptivity shown in this table is cm⁻¹, as the protein concentration was expressed as a unitless fractional percent, and the pathlength was the thickness of the disk in cm.

TABLE I. Amide I/II intensity ratio.

		Method of analysis			
Protein	pH	ATR[a]	Aqueous transmission	DR	KBr disk
Albumin	4.8	1.30 ± 0.13	2.00		
	7.0	1.08 ± 0.23	1.85	1.09	1.15
	9.0	1.50 ± 0.62	2.74		
β-Lactoglobulin	4.8	1.22 ± 0.04	2.24		
	7.0	1.17 ± 0.08	1.99	1.11	1.23
	9.0	1.12 ± 0.04	2.99		
Myoglobin	4.8	1.16 ± 0.17	2.32		
	7.0	1.27 ± 0.16	1.94	1.13	1.26
	9.0	1.38 ± 0.31	2.63		

[a] Avg ± SD, n = 2.

TABLE II. Amide II intensity and absorptivity.

	DR spectra	KBr disk		Solution spectra		
Protein	Amide II intensity (AU)	Amide II intensity (AU)	Absorptivity (cm⁻¹)	pH	Amide II intensity (mAU)	Absorptivity (cm⁻¹)
Albumin	0.137	0.334	710	4.8	11.2	1880
				7.0	10.2	1710
				9.0	10.7	1800
β-Lactoglobulin	0.246	0.672	1450	4.8	8.8	1480
				7.0	8.9	1500
				9.0	9.8	1650
Myoglobin	0.279	0.526[a]	2010	4.8	11.8	1980
				7.0	11.7	1970
				9.0	14.7	2470

[a] Disk was approximately half the thickness of the albumin and β-lactoglobulin disks.
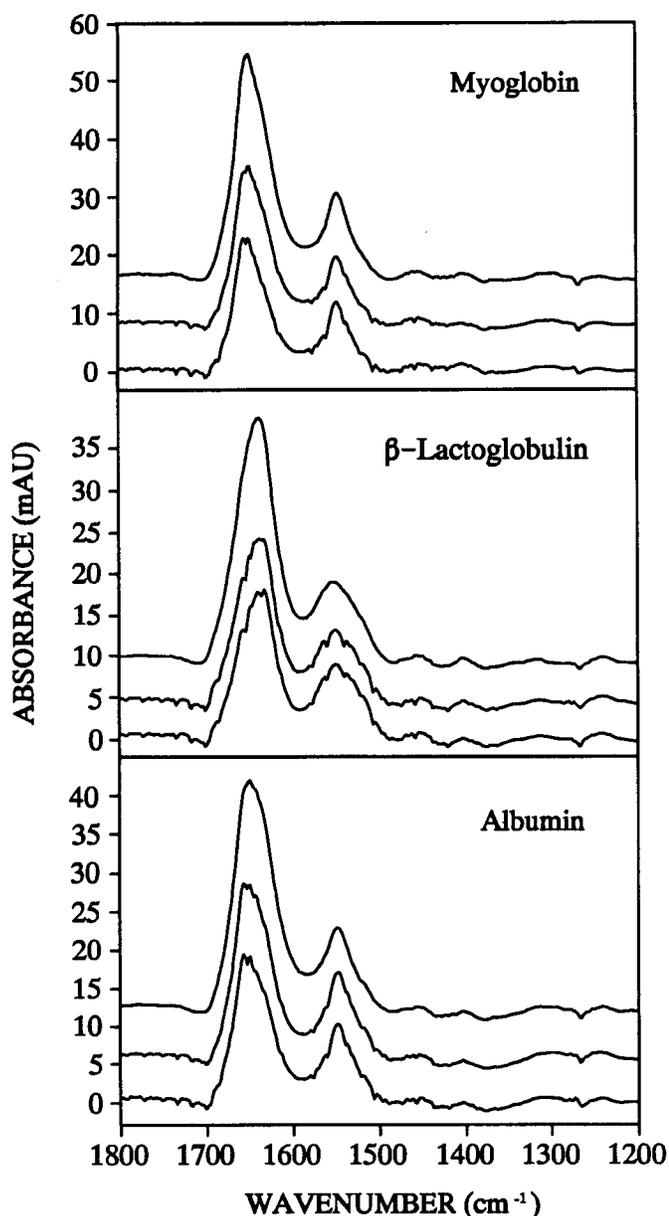
Fig. 3. Transmission spectra of 0.5% (w/v) solutions of protein in saline at pH 9.0 (top), pH 4.8 (middle), and pH 7.0 (bottom) in a 11.9-μm-pathlength cell with CaF$_2$ windows.

Aqueous Solution Spectra. The amide I and amide II bands of the transmission spectra of aqueous solutions of BSA, β-lactoglobulin, and myoglobin were narrower and more defined in structure than the corresponding spectra of the proteins prepared as KBr disks (Fig. 3). pH had a significant effect on the amide I/II intensity ratio. The highest ratios were observed at pH 9.0 and the lowest at pH 7.0, independent of the identity of the protein. Lenk et al.[11] reported the amide I/II intensity ratio of a transmission spectrum of aqueous BSA (30 mg/mL) at pH 6.8 to be about 2. We observed similar amide I/II intensity ratios at pH 7.0 from all three proteins and at a much lower protein concentration (Table I). While the amide I/II intensity ratios changed, the amide II band intensity essentially remained constant with changes in pH (Table II). Given that the concentration (w/v) of each protein solution was the same, these results indicate that

the intensity of the amide II band (which is usually assigned to mixing of the N-H bending mode with the C-N stretch) of the proteins in solution was unaffected by the changes in pH used in this study. The absorptivities of the three proteins at pH 4.8, 7.0, and 9.0 are displayed in Table II. Absorptivity was again reported with units of cm^{-1}, allowing for comparison with the corresponding data from KBr disk spectra. The changes in the amide I band intensities with pH are not a result of incorrect water subtractions and are probably caused by changes in secondary structure and possibly tertiary structure. For example, Jakobsen et al.[13] observed an increase in peak height and band area of the amide I band combined with a shift to higher frequency and a pronounced narrowing of the amide I vibration when albumin was exposed to ethylene glycol. The intensity increase was later attributed to an increase in the helical secondary structure of albumin.[14] High pH has also been reported to favor the formation of helix structure.[8] Kato et al.[15] reported a correlation between α-helix content and integrated amide I/II intensity ratio for transmission spectra of dried BSA films. A high amide I/II intensity ratio (near 1.7) was correlated to a high α-helix content. These results are in agreement with our observations at pH 9.0, where higher amide I/II intensity ratios were observed than at pH 7.0 or 4.8.

The calculated absorptivities as determined from KBr disk and solution spectra were similar for β-lactoglobulin and myoglobin; however, the absorptivity of BSA as determined from the KBr disk spectra was approximately 2.5 times less than the absorptivity determined from the solution spectra. The results from the KBr disk spectra were reproducible, and we cannot explain why the two absorptivity values for BSA are so different.

ATR Spectra. ATR spectra of BSA, β-lactoglobulin, and myoglobin adsorbed on a germanium IRE are displayed in Fig. 4. These spectra represent the protein that remained after the 4-h saline rinse. Very little or no change was observed in the amide I and amide II band intensities during the saline rinse, indicating that the protein was firmly bound to the Ge surface. The mean amide I/II intensity ratios from two sets of data are displayed in Table I. The ratios varied from a high of 1.38 to a low of 1.08 and were closer to those observed from the DR and KBr disk spectra than the corresponding ratio of the solution spectra. A specific trend in the amide I/II intensity ratio was not evident with changes in pH, the identity of the protein, or the quantity of the protein adsorbed. When the BSA concentration in the solution from which the protein was adsorbed (pH 7.0) was dropped to 0.001% (w/v), the amide I/II intensity ratio dropped to approximately 1.0. Raising the protein concentration to 0.1% (w/v) and repeating the experiment resulted in an amide I/II intensity ratio of 1.22 after the saline rinse. At the higher concentration, both the amide I and amide II band intensities dropped approximately 2.5 milliabsorbance units (mAU) to 15.8 mAU and 13.0 mAU, respectively, during the 4-h rinse period, indicating that some of the BSA contributing to the spectrum initially was reversibly bound and/or present in bulk solution.

The mean amide I/II intensity ratio for BSA, β-lactoglobulin, and myoglobin was 1.24 ± 0.23 (mean ± stan-

dard deviation, $n = 18$). The mean amide I/II intensity ratio (1.08 ± 0.23, $n = 2$) from the spectra of adsorbed BSA at pH 7.0 was significantly lower than the corresponding ratio observed by Lenk et al.[11] for a BSA film adsorbed on a Ge IRE from a pH 6.8 solution. These authors reported a mean amide I/II intensity ratio of 1.76 ± 0.15 ($n = 3$) for adsorbed films and a ratio near unity for bulk solution protein. Several experimental parameters differed between the two experiments. Our solutions were held at 21°C (compared to 33°C), the solution concentration was much lower (0.1 mg/mL, compared to 30 mg/mL), and the flow rate was lower (0.5 mL/min as compared to 75 mL/min in the work of Lenk et al.[11]). Finally, our exposure time of the protein to the Ge substrate prior to the saline rinse was longer (4 h as compared to 2 h). These differences in experimental parameters apparently had a strong influence on the protein film with respect to the secondary structure and the observed amide I/II intensity ratio.

Protein Film Thickness. One of the goals of this investigation was to determine the thickness of each protein film that remained after the saline rinse. The protein films were much thinner (tens of nm) than the depth of penetration, d_p (386 nm at 1650 cm^{-1} and 411 nm at 1550 cm^{-1}); thus, the electric field can be assumed to be constant over the film thickness. The effective thickness, as described by Harrick[16], is given by:

$$d_e = \frac{n_{21}E_0^2 d}{\cos \theta} \tag{1}$$

where the n_{21} is the ratio of the refractive index of the optically rare medium (water) to that of the IRE, E_0 is the electric field intensity at the interface, d is the film thickness, and θ is the angle of incidence. The electric field amplitudes in the thin film for perpendicular and parallel polarization, respectively, are given by:

$$E_\perp = \frac{2 \cos \theta}{(1 - n_{31}^2)^{1/2}} \tag{2}$$

where n_{31} is the ratio of the refractive index of water to that of the IRE, and

$$E_\parallel = \frac{2 \cos \theta[(1 + n_{32}^4)\sin^2\theta - n_{31}^2]^{1/2}}{(1 - n_{31}^2)^{1/2}[(1 + n_{31}^2)\sin^2\theta - n_{31}^2]^{1/2}} \tag{3}$$

where n_{32} is the ratio of the refractive index of water to that of the protein. Inserting the values of electric field in Eq. 1 gives the effective thickness for perpendicular and parallel polarizations:

$$d_{e\perp} = \frac{4n_{21}d \cos \theta}{(1 - n_{31}^2)} \tag{4}$$

and

$$d_{e\parallel} = \frac{4n_{21}d \cos \theta[(1 + n_{32}^4)\sin^2\theta - n_{31}^2]}{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2\theta - n_{31}^2]}. \tag{5}$$

It can be seen from Eqs. 4 and 5 that the effective thickness for thin films is proportional to the true film thickness. As a result, the relative intensities of absorption bands in the spectra of thin films are the same as they are in the transmission spectra of bulk materials. Therefore, the relative intensities of bands in the internal
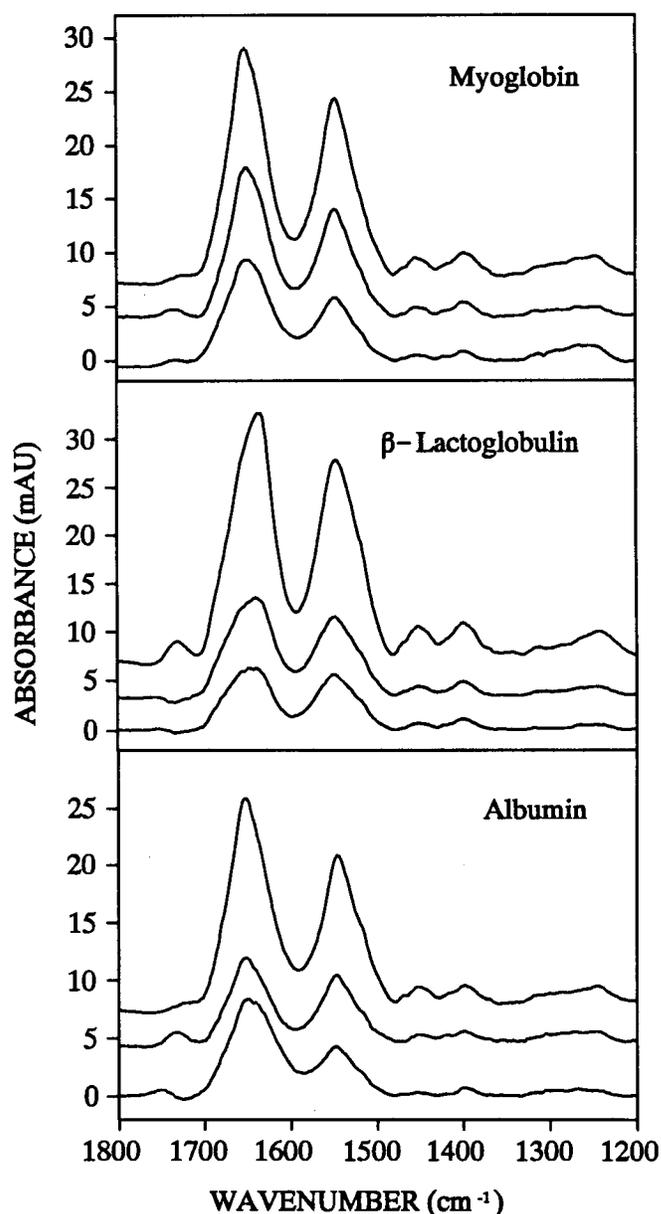

FIG. 4. ATR spectra of proteins adsorbed on a germanium IRE from flowing 0.01% (w/v) solution in saline at pH 4.8 (top), pH 7.0 (middle), and pH 9.0 (bottom).

reflection spectra of the protein films should be equal to those obtained by transmission spectrometry, assuming no change in conformation.

Assuming that the secondary structure of the proteins measured by KBr disk and ATR are similar (i.e., based on the similarity of the amide I/II intensity ratio), the effective thickness of the protein films can be calculated with the use of the absorptivities from the KBr disk spectra. As bulk protein was adsorbed on the IRE, the protein concentration was assigned a value of unity. With the ten reflections that occur within the Ge IRE taken into account, the calculated effective thickness, d_e, for albumin, β-lactoglobulin, and myoglobin are displayed in Table III.

A three-phase system of stratified media was defined as Ge ($n_1 = 4.0$), protein ($n_2 = 1.5$), and water ($n_3 = 1.33$). Substituting these optical constants into Eqs. 4 and 5 at

TABLE III. Effective and true thickness of thin protein film.

| | | ATR Amide II intensity (mAU) | | KBr disk | $d_e/10$ (nm) | | d (nm) | |
| | | Trial 1 | Trial 2 | Absorptivity (cm^{-1}) | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
Protein	pH							
	4.8	12.6	14.0		17.7	19.7	12.0	13.4
Albumin	7.0	5.8	7.5	710	8.2	10.6	5.6	7.2
	9.0	4.3	7.0		6.1	9.9	4.1	6.7
	4.8	20.2	17.7		13.9	12.2	9.5	8.3
β-Lactoglobulin	7.0	8.2	7.6	1450	5.7	5.2	3.9	3.6
	9.0	5.7	6.8		3.9	4.7	2.6	3.2
	4.8	16.4	12.2		8.2	6.1	5.6	4.1
Myoglobin	7.0	10.0	14.4	2010	5.0	7.2	3.4	4.9
	9.0	5.8	12.1		2.9	6.0	2.0	4.1

an angle of 45° gives the relationship between the effective thickness and the true thickness

$$d_{e\perp} = 1.19d \qquad (6)$$

and

$$d_{e\parallel} = 1.87d \qquad (7)$$

for perpendicular- and parallel-polarized radiation. If we assume that the polarized radiation of the FT-IR spectrometer in our lab, which is 61% perpendicular and 39% parallel, was scrambled to the same extent at each internal reflection at the surface of the cylindrical IRE, the total effective thickness is:

$$d_e^{total} = 0.61d_{e\perp} + 0.39d_{e\parallel} \qquad (8)$$

and thus the true thickness is given by

$$d = 0.685d_e^{total}. \qquad (9)$$

The film thicknesses for BSA, β-lactoglobulin, and myoglobin calculated in this way are displayed in Table III. The greatest film thicknesses were observed at pH 4.8, independent of the identity of the protein; however, myoglobin adsorption in trial 2 seemed to be unaffected by changes in pH. At this time, we have no explanation of why this occurred. The increase in film thickness observed at pH 4.8 may be attributed to changes in the orientation of protein molecules at the Ge surface. Philips[17] observed that the surface shear viscosity of BSA adsorbed at an oil/water interface passed through a maximum at the isoelectric pH (pI) of BSA. This observation indicates that the protein adsorption layer must be very compact at the pI due to the collapse of the protein structure. At pH 4.8, albumin has a net charge of zero and β-lactoglobulin a net charge near zero. Under these conditions, the proteins may be in a more compact conformation due to reduction of charge repulsion at or near the isoelectric point, allowing for closer packing of individual protein molecules at the surface while maintaining monolayer coverage. Albumin has been described as a prolate ellipsoid with dimensions of approximately 4.0 × 14.0 nm.[5,18] Assuming a monolayer of BSA formed at the surface of the IRE, the film thickness data indicate that the ellipsoidal protein was possibly standing on end with its long axis perpendicular to the surface of the IRE. The calculated thickness of 12–13 nm was remarkably close to the reported length of 14 nm. Thicknesses of 5.6

and 7.2 nm were calculated for the protein adsorbed at pH 7.0 and 4.1 and 6.7 nm at pH 9.0 (Table III). These values suggest that BSA was lying on its side when pH > pI. The small differences at pH 7.0 and 9.0 may be attributed either to experimental variability or to charge repulsions leading to slightly lower surface coverage at pH 9.0 compared to pH 7.0. Myoglobin is a globular protein with dimensions of 4.5 × 3.5 × 2.5 nm.[19] Again, the calculated film thicknesses (around 4 nm) were very close to the thickness of a film that would be formed by a monolayer of adsorbed myoglobin molecules. The film thickness (2.0 nm) at pH 9.0 in trial 1 suggests that the adsorbed film was less than a monolayer.

CONCLUSIONS

Good agreement between the absorptivities of the amide I and amide II bands of β-lactoglobulin and myoglobin as sampled as KBr disk and in aqueous solutions was obtained, although this was not true for albumin. We have no explanation of why the absorptivity of albumin measured by KBr disk was not similar to the value measured as protein in solution. The amide I/II intensity ratios of solid albumin, β-lactoglobulin, and myoglobin as sampled by DR of a loose dispersion or as a pressed KBr disk were similar to the corresponding ratio of films adsorbed on a Ge IRE, whereas the amide I/II intensity ratios of the proteins in solution were significantly different. The solid-state proteins and the adsorbed protein films may possess similar secondary structure, as evidenced by the similarities of these amide I/II intensity ratios. Individual results from ATR spectra of solution proteins[14] and transmission spectra of dried protein films[15] suggest that the amide I/II intensity ratio may be associated with changes in secondary structure. The adsorbed protein films are thin enough ($d < d_p$) that the wavelength dependence of the relative band intensities associated with ATR of bulk materials is not a factor in these measurements. The differences in amide I/II band ratios between ATR spectra and solution spectra should probably be attributed to differences in the secondary and possibly tertiary conformation of surface adsorbed proteins and proteins in solution. Absorptivities obtained from KBr disk spectra used to calculate the thicknesses of the adsorbed protein films resulted in calculated thicknesses that could be rationalized in terms of

a monolayer of protein on the Ge surface with the orientation of the molecules being dependent on the pH.

ACKNOWLEDGMENTS

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