Raman Spectral Analysis in the C−H Stretching Region of Proteins and Amino Acids for Investigation of Hydrophobic Interactions

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Raman spectra of amino acids showed complexity in the C−H stretching region (2800−3100 cm^{-1}) attributed to diversity of CH, CH_2, and CH_3 groups in the side chains, ionization state, and microenvironment. The involvement of specific amino acids in the C−H stretching region of selected proteins, namely, lysozyme, α-lactalbumin, *β*-lactoglobulin, and their binary mixtures, was investigated by deconvolution using maximum likelihood techniques. The main protein band near 2940 cm^{-1} was attributed not only to aromatic and aliphatic amino acids but also to many other amino acids. A band near 3065 cm^{-1} was assigned to aromatic residues, whereas bands near 2880 and 2900 cm^{-1} corresponded primarily to aliphatic amino acids. Heating at 90 °C increased relative intensity near 2940 cm^{-1} and decreased relative intensity at 2895−2902 cm^{-1} for lysozyme and its mixtures with α-lactalbumin or *β*-lactoglobulin. Additional bands at 2812 or 2838 and 3003 cm^{-1} were observed after heating or in 8 M deuterated urea, reflecting changes upon denaturation.

Keywords: *Raman spectroscopy; amino acids; proteins; denaturation; hydrophobic interactions*

INTRODUCTION

The importance of hydrophobic interactions in functionality of proteins has been well-documented. However, methodology is still lacking to monitor changes in surface or effective hydrophobicity that may occur in concentrated samples, precipitates, or gels. This problem is particularly pronounced with respect to the investigation of the role of aliphatic amino acid residues in hydrophobic interactions. However, even in the case of aromatic amino acid residues, most of the methods that are used (e.g., ultraviolet, fluorescence, near-UV circular dichroism, or resonance Raman spectroscopy) primarily apply to the analysis of proteins in dilute solutions.

Visible laser Raman spectroscopy is a branch of vibrational spectroscopy that may be used to advantage as a probe of the molecular structure of food proteins, due to its applicability to studying molecules in concentrated aqueous solutions as well as in solid phase including films, precipitates, or gels. Although the basis for Raman spectroscopy was first discovered in 1928 by Sir Chandrasekhra Venkata Raman, practical applications of the technique have only been realized by the development of suitable laser sources of excitation and the current explosion of computer technology for data analysis (Li-Chan et al., 1994). Current and future trends in the application of Raman spectroscopy in food science are illustrated in a recent review (Li-Chan, 1996). The potential to study food protein structure in situ has been demonstrated for surimi from Pacific whiting (Bouraoui et al., 1997), frozen aggregates of cod myosin (Careche and Li-Chan, 1997), and whey protein and ovalbumin gels (Nonaka et al., 1993; Howell and Li-Chan, 1996; Li-Chan and Qin, 1998). Advances in instrumentation for Fourier transform (FT) Raman spectroscopy (Chase and Rabolt, 1994) are also expected to generate rapid growth in applications, including the rapid classification of foods such as oils and fats (Baeten et al., 1998).

In the study of proteins, the potential information that can be obtained by Raman spectroscopic analysis is based on the fact that both the relative intensity and the frequency of vibrational motions of the amino acid side chains and polypeptide backbone are sensitive to chemical changes and the microenvironment around those functional groups. Thus, for example, Raman spectral analysis can provide estimates of protein secondary structure fractions based on amide I, amide III, and skeletal stretching modes related to the polypeptide backbone. Similarly, changes in the conformation around the disulfide bond of cystinyl residues, the involvment of phenolic group of tyrosinyl residues in hydrogen bonding, or the degree of exposure or buriedness of aromatic residues are reflected in characteristic Raman bands related to S−S stretching or the aromatic ring vibrational modes (Tu, 1986; Li-Chan et al., 1994).

Amino acids, peptides, and proteins also exhibit C−H stretching vibrational bands in the 2800−3100 cm^{-1} region of the Raman spectrum, which has generally been considered to be related to hydrophobic groups. Over 20 years ago, Verma and Wallach (1976) reported that the Raman literature contained little information about scattering in the C−H stretching region of amino acids other than glycine; these authors thus reported some important features that they observed for the crystalline form of alanine, valine, leucine, isoleucine, methionine, and threonine. However, the implications of the C−H region with regard to protein structure have not been extensively studied, despite indications that

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some bands may indeed have structural implications (Tu, 1986).

Aside from proteins, many other constituents of biological systems may contribute to the C−H band, and the study of lipids and membrane systems has received particular focus [e.g., Aslanian et al. (1986), Carmona et al. (1987), Devlin and Levin (1990), Mikkelsen et al. (1978), Verma and Wallach (1976, 1977a), and Verma et al. (1975)]. These investigations have indicated dramatic changes in the C−H stretching region with state transitions of both biological membranes and model systems, as well as with changes in the polarity of the environment around methylene (CH_2) and methyl (CH_3) groups.

The difficulty in interpreting observed features of the C−H stretching region in the Raman spectrum of both proteins and lipids stems in large part from the questions that remain unanswered as to band assignments for these complex systems (Verma and Wallach, 1977a). The many C−H bands are due to fundamental C−H stretching vibrations, overtones of various C−H stretching vibrations, and Fermi resonance between C−H stretching vibrations and the first overtones of C−H deformation bands (Tu, 1986; Mátrai et al., 1990).

In theory, discrete vibrational frequencies should be assigned for symmetrical and asymmetrical vibrations of the CH_2 and CH_3 moieties of saturated alkyl groups and for ═CH groups of unsaturated or aromatic groups. In practice, as with other spectroscopic methods, several factors complicate both the resolution and the line width or broadness of the peaks or bands observed in a Raman spectrum. In complex molecules, the large number of CH groups in conjunction with the heterogeneity of other substituents and the surrounding microenvironment lead to overlapping of vibrations and general broadening of bands. However, even simple molecules can produce broad bands, especially when dynamic, rapid interconversion between different molecular states or conformations occurs. Differences in the Raman spectra also may arise from the influence of configuration on microenvironment of the C−H groups; for example, three bands at 2869, 2932, and 2955 cm^{-1} were observed for the homopolypeptide polyglycine I, compared to two bands at 2979 and 2940 cm^{-1} for the same homopolypeptide in a different configuration, polyglycine II (Tobin, 1972).

Mathematical deconvolution and curve-fitting techniques for resolution enhancement of "intrinsic" (molecular) broadening have become increasingly popular to analyze spectra comprising overlapping bands. The technique based on Fourier deconvolution or the Fourier self-deconvolution algorithm has been applied to resolve the components of the amide I Raman band of proteins, which are related to secondary structural conformation (Susi and Byler, 1988). However, the conceptual simplicity of the Fourier self-deconvolution technique must be counterbalanced by a number of failings, including the negative intensities that may result from the finite Fourier transform and the great sensitivity of the technique to noise (Friesen and Michaelian, 1991).

Maximum likelihood techniques have been recommended for spectral restoration or deconvolution when the signal-to-noise ratio is low or when the exact outcome with regard to number of bands after deconvolution is unknown. In contrast to curve-fitting or modeling techniques, in which the number of peaks to be fitted must be specified by the user, often subjectively, the maximum likelihood restoration process objectively determines the most likely number of peaks present. Unlike Fourier deconvolution, which ignores noise, maximum likelihood deconvolution takes noise into consideration. Furthermore, maximum likelihood methods provide the added assurance that the result is always the most probable solution (Mendel, 1990; DeNoyer and Dodd, 1991). A maximum entropy method was applied by Ni and Scheraga (1985) for resolution enhancement of the complicated Raman spectra of proteins, and the technique was suggested to be suitable for spectral estimation of a short-time record of noisy data, minimizing the necessary exposure time of protein solutions to the intense laser beam. However, to our knowledge, no information has been reported on the application of maximum likelihood resolution to the C−H stretching region of proteins.

The objective of the present study was to investigate the potential of using the C−H stretching region of the Raman spectra to study hydrophobic interactions of aliphatic and aromatic amino acid residues of proteins. Because the O−H stretching band of water occurs near the C−H stretching region and has a line width in excess of 500 cm^{-1}, samples were prepared as deuterated solutions to minimize the effect of the water signal on the Raman spectra. Using maximum likelihood deconvolution to enhance resolution of the component bands, the Raman spectra of three proteins and their binary mixtures were compared to the measured spectra of individual amino acids. The effects of heat or urea treatment on the C−H stretching vibrations of the selected proteins were also investigated.

MATERIALS AND METHODS

Materials. Deuterium oxide (D-4501, 99.9 atom % D), deuterated urea (U-4877, urea-d_4, 98+ atom % D), and analytical reagent grade L-amino acids were from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide (MD-175, minimum 99.9 atom % D) from MSD Isotope (Montreal, Canada) was also used. α-Lactalbumin (L-5385, from bovine milk, type 1, containing 1−2 mol of calcium/mol of protein), β-lactoglobulin (L-0130, from bovine milk, 3× crystallized and lyophilized), and lysozyme (L-6876, from chicken egg white, 3× crystallized, dialyzed, and lyophilized) were from Sigma Chemical Co.

To study the effect of heat treatment and interactions between proteins, 15% (w/v) solutions in D_2O (apparent pD 6.8) were prepared of the three individual proteins as well as the binary mixtures (1:1 weight ratio) of α-lactalbumin−lysozyme, β-lactoglobulin−lysozyme, and α-lactalbumin−β-lactoglobulin, as described by Howell and Li-Chan (1996). As reported earlier by Howell et al. (1995), white precipitates were formed in the mixtures of whey protein with lysozyme, whereas the mixture of the two whey proteins remained soluble. The solutions and pellets obtained after centrifugation of mixtures at 10000*g* were used for analysis. One set of the individual protein samples and their mixtures was heated at 90 °C for 30 min and then cooled in an ice−water bath for 5 min, followed by storage at 5 °C overnight, prior to analysis (Howell and Li-Chan, 1996).

To study the effect of denaturation by urea, samples were dissolved to a final protein concentration of 5% (w/v) in 0.1 M deuterated sodium phosphate buffer [apparent pD 7.5, prepared according to the method of Dennison (1988)] in the absence or presence of 8 M deuterated urea.

Raman Spectral Analysis. Samples were excited at 488 nm using an argon ion laser and a Spectra Physics model 168B power supply (Spectra Physics, Mountain View, CA). The Raman scattering of samples placed in hematocrit capillary tubes (Nichiden-Rika Glass Co. Ltd., Japan) was measured

Table 1. Raman Shifts in the C−H Stretching Region (2800−3100 cm^{-1}) of Various Amino Acids in Relation to Bands Associated with Proteins Deconvoluted by Maximum Likelihood Techniques Using Lorentzian and Gaussian Peak Shape Assumptions[a]

protein band wavenumber (cm^{-1}) after deconvolution		
Lorentzian	Gaussian	amino acids (wavenumber, cm^{-1})
2812	2812−2815	
	2821−2827	
2838	2842−2859	Met (2848 w)
2876−2879	2874−2879	His (2873 w), Leu (2883 s), Lys (2884 m), Ile (2885 s), Val (2888 m−s)
	2889−2897	Thr (2892 w−m), Pro (2898 m)
2895−2903	2901−2909	Arg (2905 m−s), Ala (2906 w), Ser (2906 m−s), Ile (2911 s), Val (2913 s), Leu (2913 s)
2922−2924	2914−2927	Thr (2923 w), Pro (2925 w), Ser (2929 w), Ile (2932 s)
2939−2944	2934−2945	Met (2935 s), Trp (2936 s), Arg (2939 s), cystine (2941 s), Leu (2941 s), Lys (2941 s), Ile (2948 s), Phe (2951 m), Tyr (2951 m), Glu (2952 s), Thr (2952 s), Val (2953 s), Pro (2954 s), His (2955 s), Asp (2955 s), Asn (2956 s)
2963−2979	2956−2977	Ala (2961 s), cystine (2963 m), Cys (2966 s), His (2969 s), Ser (2970 s), Leu (2976 s), Trp (2976 s)
2980−2983	2981−2989	Gly (2981 s), Lys (2981 m), Ile (2984 m−s), Val (2985 s)
3003−3004	3001−3003	Thr (2994 m), cystine (2995 w), Met (3002 w), Ala (3005 w), Pro (3005 s), Cys (3008 w)
	3007−3009	
3012	3016−3018	Gly (3019 w)
	3024−3057	Tyr (3025 w)
3063−3065	3061−3068	Tyr (3074 s), Phe (3076 s), Trp (3076 w)
	3078, 3087	
		His (3160 m−s)

[a] All samples were in deuterium oxide, as described in the text. Relative peak intensities for the amino acid bands are indicated by the following letters after the wavenumber: s, strong; m, medium; w, weak. Abbreviations for amino acids are as given by Dodd (1986).

at ambient temperature on a Jasco model NR-1100 laser Raman spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), using the following conditions: laser power, 200 mW; slit height, 2 or 4 mm; spectral resolution, 5.0 cm^{-1} at 19000 cm^{-1}; sampling speed, 120 cm^{-1} min^{-1} with data collected every cm^{-1}. Frequency calibration of the instrument was performed daily using the 1050 ± 2 cm^{-1} band of a standard solution of 1 M potassium nitrate; optical alignment was also monitored by checking the relative intensity of this standard solution. The data presented are based on the signal average of 10 scans for each sample used in the study of the effects of heat treatment and interactions, and a minimum of eight scans in duplicate for each protein sample used in the study of urea denaturation and for the amino acids.

The recorded spectra were analyzed using LabCalc software (Galactic Industries Corp., Salem, NH) for baseline correction and smoothing. The baseline-corrected spectra were smoothed and deconvoluted using the MSmooth (E-Smooth or maximum entropy method) and SSRes (spectral restoration) algorithms, respectively, in the add-on Square Tools software (Spectrum Square Associates, Ithaca, NY), which are based on maximum likelihood techniques. Deconvolutions using Lorentzian or Gaussian peak shape and full width at half-maximum (fwhm, in cm^{-1}) of 20−40 cm^{-1} were compared to ensure good resolution of peaks without overfitting. Maximum entropy deconvolution sometimes causes deviation of the relative peak heights from the original spectrum; however, the spectral intensity is always proportional to the area under a peak, rather than the height (Ni and Scheraga, 1985). Selection of peaks and calculations of peak areas as a measure of spectral intensity were performed by maximum likelihood peak fitting using the DataFit algorithm in Square Tools. For five replicate spectra of ovalbumin, each spectrum being the average of 12 scans, the mean coefficients of variation for peak positions and peak areas were 10.5 and 8.3%, respectively (Arteaga, 1994).

RESULTS AND DISCUSSION

Amino Acids. Typical Raman spectra in the C−H stretching region (2800−3100 cm^{-1}) for a range of the amino acids are illustrated in Figures 1−3, and the bands for the 20 amino acids are grouped according to their Raman shift in Table 1. Overlapping of vibrations usually occurs, causing broadness of bands; many of the amino acids studied gave more than one band in this region. This can be attributed to the fact that the C−H stretching vibration is affected by the chemistry of the functional group and its surrounding environment. Amino acid side chains may contain the C−H moiety in methyl or methylene groups, in alkyl chains, and in aromatic or heterocyclic rings. Even the C−H moiety on the α-carbon will not be in the same microenvironment, due to the differences in the side chains.

Because assignments for all amino acids and proteins in the C−H stretching region have not been reported hitherto, we have attempted to relate some of these bands to the assignments published in the literature for alkanes and cyclopropane or cyclobutane derivatives as shown in Table 2 (Lin-Vien et al., 1991). The frequencies of the Raman shifts for acyclic alkanes have been reported to be characteristic for the CH_3 and CH_2 groups, and no more than ±10 cm^{-1} variation is expected in these stretching modes for groups attached to another saturated carbon in acyclic alkanes; however, the Raman shifts increase to higher values when the groups are in a strained ring (such as cyclopropane or cyclobutane) or adjacent to oxygen or nitrogen atoms (Lin-Vien et al., 1991). Assignments similar to those shown in Table 2 were also reported by Terpinski (1987). In addition, the phenyl group with five aryl C−H bonds usually shows one strong Raman band at 3030−3070 cm^{-1} (Lin-Vien et al., 1991), whereas heteroaromatic compounds with five-membered rings show C−H stretching bands at higher frequencies, in the 3100−3150 cm^{-1} region (Terpinski, 1987).

On the basis of the comparison to the alkanes, tentative assignment of the bands in the protein spectra may be made. For example, for the protein spectra deconvoluted by the Gaussian peak shape assumption, vibrations attributed to CH_2 symmetrical stretching may be assigned to bands near 2842−2859 cm^{-1}, CH_3 symmetrical stretching and R_3C-H stretching to bands near 2874−2897 cm^{-1}, CH_2 asymmetrical stretching to bands at 2914−2927 and 2935−2945 cm^{-1}, CH_3 asymmetrical stretching and α-C−H stretching to bands at

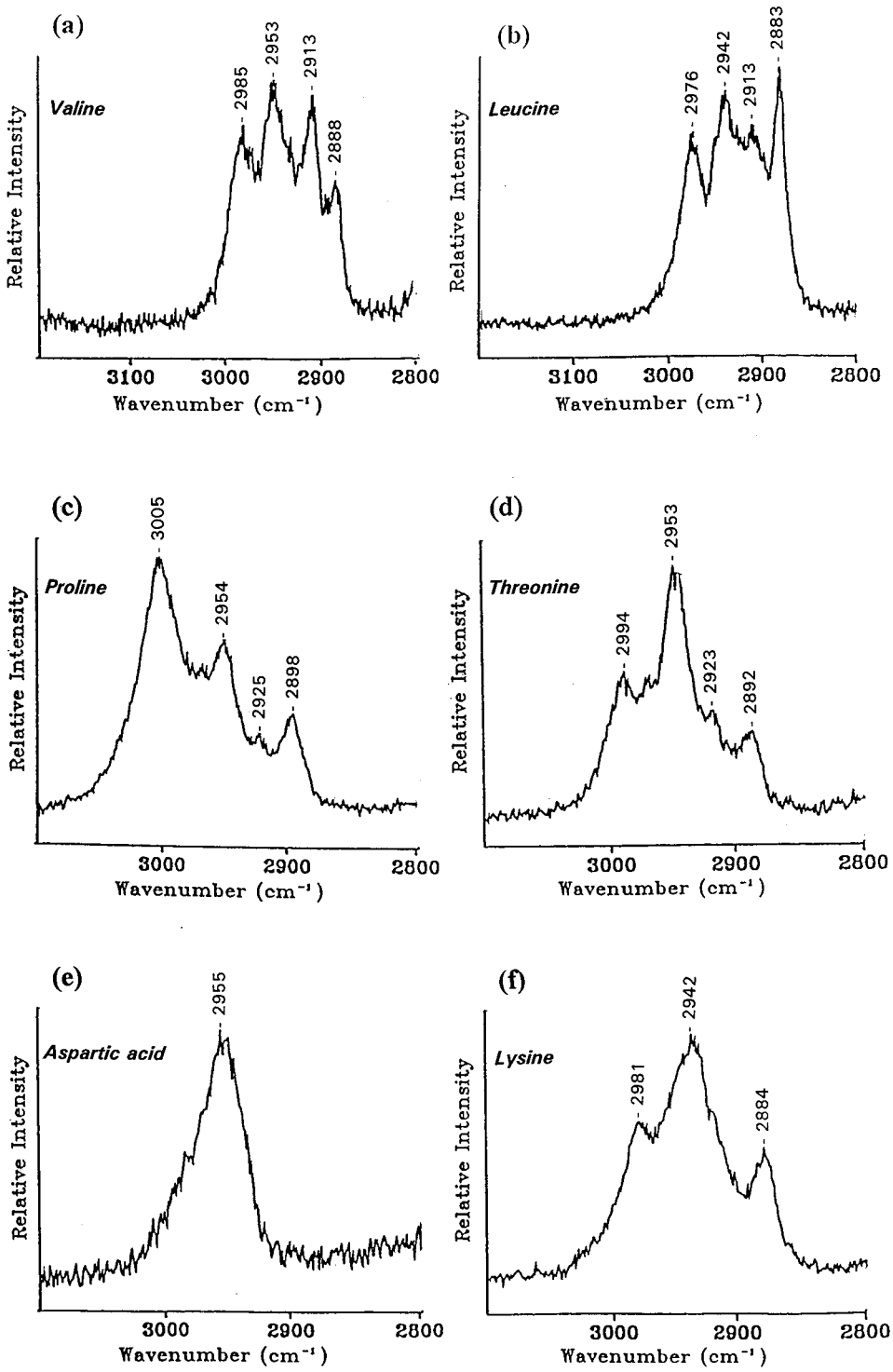

Figure 1. Raman spectra of some amino acids dissolved in 1 M DCl in D_2O: (a) 0.2 M valine solution; (b) 0.2 M leucine solution; (c) 1 M proline solution; (d) 1 M threonine solution; (e) 1 M aspartic acid solution; (f) 1 M lysine solution. The original spectra (no baseline correction or smoothing) are shown.

2956−2977 and 2980−2989 cm^{-1}, and =C−H stretching to bands at 3061−3068 cm^{-1}. It is interesting to note that Verma and Wallach (1977b) suggested that the intensity at 2930 cm^{-1} was due to the CH_3 group vibrations, although evidence for this was not presented.

Most of the bands were associated with several amino acids. The aromatic and aliphatic amino acids had prominent C−H stretching bands, particularly under the main band near 2935−2955 cm^{-1} (Figures 1a,b, 2, and 3). However, it was also clear that a number of other amino acids including the charged amino acids such as lysine and glutamic and aspartic acids, as well as proline, threonine, and histidine, also produced signals in the C−H stretching region due to the presence of CH groups and either CH_3 or CH_2 groups (Figure 1c−f and Table 1). In addition, in this study it was noted that a strong Raman band was observed at 3005 cm^{-1} for proline, whereas weaker bands at 3002−3008 cm^{-1} were observed for alanine as well as the sulfur-containing amino acids, methionine, and cysteine. Only tyrosine produced a signal at 3025 cm^{-1}. The aromatic amino acids, namely, phenylalanine, tyrosine, and tryptophan,

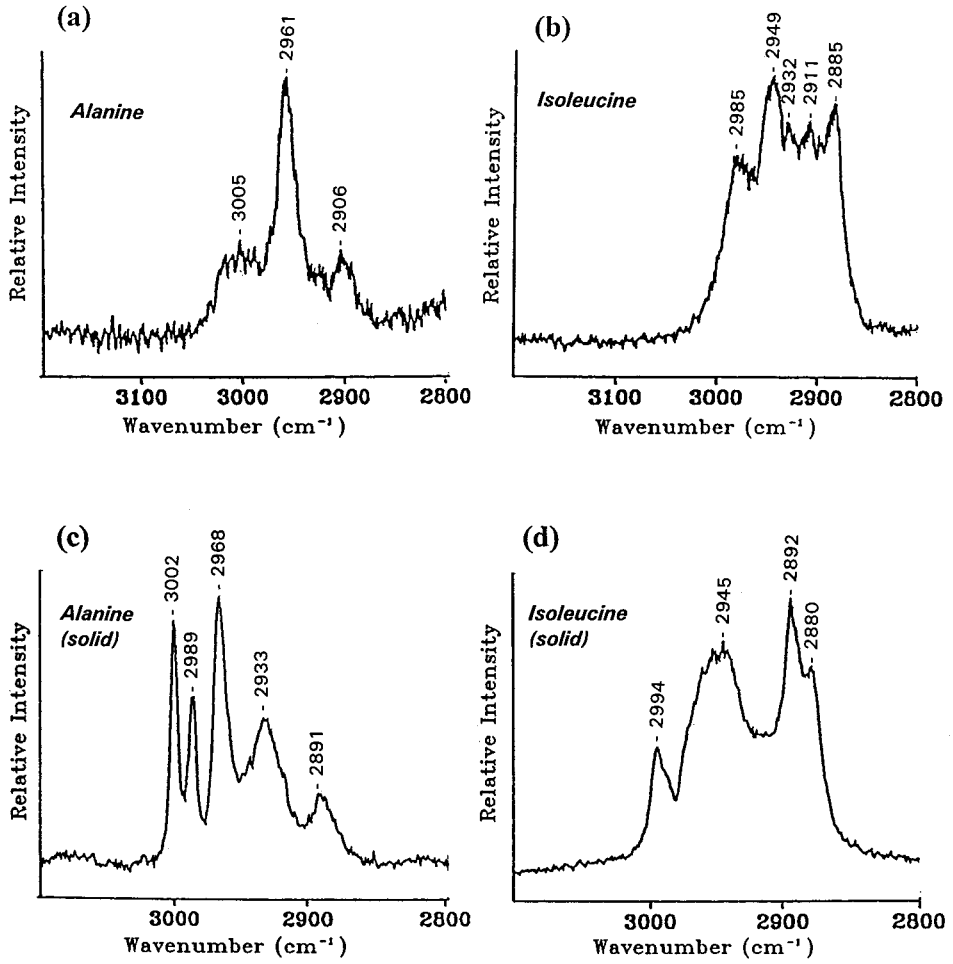

Figure 2. Raman spectra of the aliphatic amino acids alanine and isoleucine, dissolved in 1 M DCl in D_2O or in the solid phase: (a) 1 M alanine solution; (b) 0.2 M isoleucine solution; (c) solid alanine; (d) solid isoleucine. The original spectra (no baseline correction or smoothing) are shown.

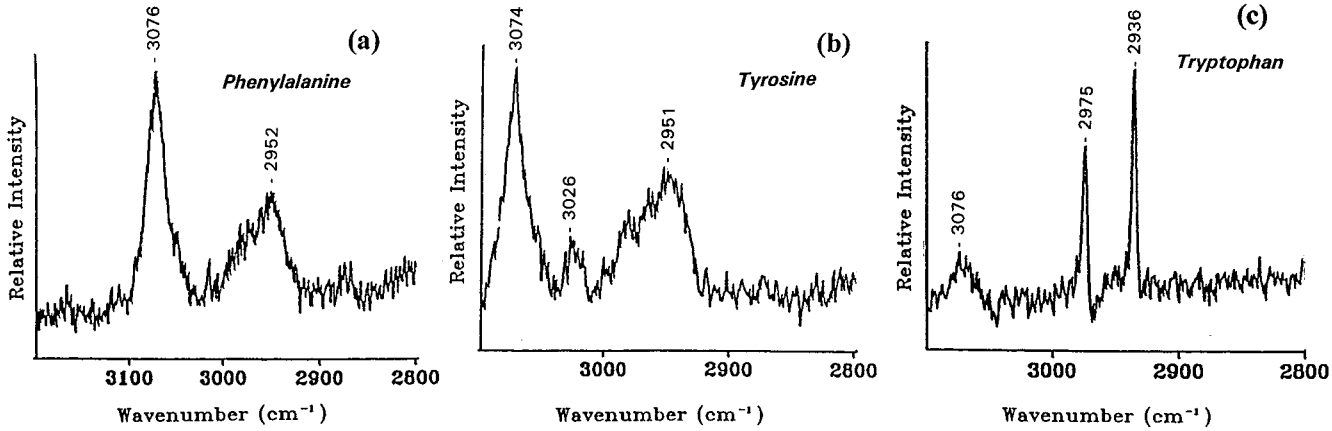

Figure 3. Raman spectra of the aromatic amino acids dissolved in 1 M DCl in D_2O: (a) 0.2 M phenylalanine solution; (b) 0.2 M tyrosine solution; (c) 0.2 M tryptophan solution. The original spectra (no baseline correction or smoothing) are shown.

produced bands at higher Raman shifts of 3075 cm^{-1}, whereas histidine had a characteristic band at 3160 cm^{-1}.

Changes in the spectra were observed when solid samples of amino acids were compared to the amino acid solutions prepared in 1 M DCl. The solid-state spectra of alanine and isoleucine showed greater resolution than the corresponding solution phase (Figure 2). New or intensified bands in the region near 2995−3010 cm^{-1} were also observed in the Raman spectra of several of the amino acids in solid state, including glycine, aspartic acid, cysteine, histidine, isoleucine, phenylalanine, and valine (data not shown). Sharpening and increased resolution of the spectral bands in solid samples may be related to a reduction in the dynamic interconversion of different molecular conformations that may exist in solution form. Raman spectra of aqueous solutions and solid-state samples of L(+)-glutamic acid and its salts were reported by Shurvell and Bergin (1989). The solid-state spectra of monosodium glutamate, glutamic acid, and the acid hydrochloride all contained many more resolved features than the corresponding solution phase.

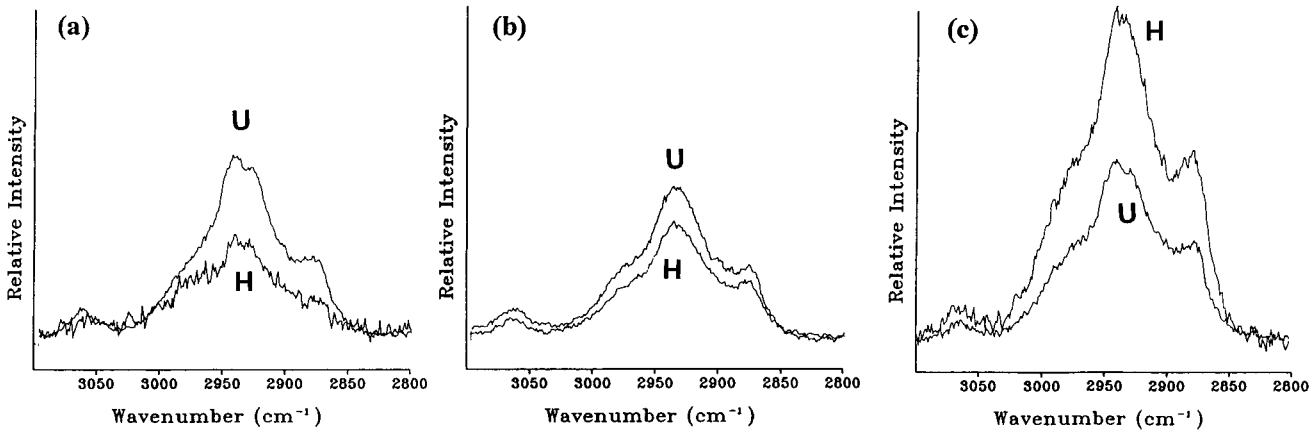

Figure 4. Effect of heat treatment (90 °C, 30 min) on the Raman spectra of lysozyme, α-lactalbumin, and β-lactoglobulin (15% w/v in D_2O, apparent pD 6.8): (a) lysozyme; (b) α-lactalbumin; (c) β-lactoglobulin; H, heated; U, unheated. Spectra were baseline corrected and normalized to the intensity of the phenylalanine band at 1005 ± 2 cm^{-1}.

Table 2. Raman Shift (Wavenumber, cm^{-1}) Associated with C−H Stretching Vibrations of *n*-Alkanes, Alkanes, and Cyclopropane and Cyclobutane Derivatives [Adapted from Lin-Vien et al. (1991)][a]

C−H stretching vibration	wavenumber, cm^{-1}		
	alkanes	cyclopropane derivatives	cyclobutane derivatiaves
CH_3 (asym)	2972−2952 (vs)		
CH_3 (sym)	2882−2862 (vs)		
CH_2 (asym)	2936−2916 (vs)	3105−3085 (m−w)	2990−2970 (var)
			2956−2945 (s)
CH_2 (sym)	2863−2843 (vs)	3040−3020 (m)	2985−2975 (s)
		3020−3000 (s)	2970−2955 (m)
R_3C−H	2890 (w)		
α-CH		3060−3020 (m−s)	∼2970 (w)

[a] Sym refers to symmetrical and asym refers to asymmetrical. Relative intensities are given in parentheses as follows: vs, very strong; s, strong; m, medium; w, weak; var, variable.

Whereas the aqueous solutions showed a very strong but broad band at 2925 cm^{-1}, the solid samples had several strong bands near 2970, 2934, 2917, and 2880 cm^{-1}. Similar observations have been made in the C−H stretching spectra of liquid and solid forms of fatty acids (Verma and Wallach, 1977a).

In addition to the differences arising from solid versus solution phase, the C−H stretching vibrations may also be affected by ionization of the amino acids. For example, a CH_2 group in a saturated *n*-alkane system shows two stretching frequencies, a symmetrical one near 2850 cm^{-1} and an asymmetrical one near 2925 cm^{-1}. In glycine, the observed vibrations were reported by Takeda et al. (1958) to be dependent on the state of ionization; the anionic species gave only one broad line near 2940 cm^{-1}, whereas the cation and dipolar ionic species each showed two vibrations—at 2974 and 3017 cm^{-1} for the cation and at 2970 and 3015 cm^{-1} for the dipolar ion. In contrast, the amino acid alanine, which contains no CH_2 groups, gave three strong bands near 2890, 2950, and 3000 cm^{-1}, and these were not affected by ionization (Takeda et al., 1958). In this study, the amino acids were dissolved in 1 M DCl in D_2O; due to the acidity of the solvent, the amino acids were likely to exist in the cationic form. Alanine in this solution showed a strong band at 2961 and weaker bands at 2906 and 3005 cm^{-1}, whereas the solid form of alanine had broad bands of moderate intensity at 2891 and 2933 cm^{-1} and strong, sharp bands at 2968, 2878, and 3001 cm^{-1} (Figure 2).

Proteins and Effects of Heating or Denaturation by Urea. The effects of heating on the Raman spectra of lysozyme, α-lactalbumin, and β-lactoglobulin are shown in Figure 4. Because the efficiency of collection of the Raman scattering signal is dependent on the sample characteristics, the spectrum in the C−H region for each sample was normalized to the intensity of the phenylalanine band at 1005 ± 2 cm^{-1} for that sample, prior to comparison of the relative intensities and areas between different sample spectra. Figure 4 illustrates that the area under the broad C−H stretching envelope was decreased after heating of lysozyme (Figure 4a) and α-lactalbumin (Figure 4b) but was increased significantly after heating of β-lactoglobulin (Figure 4c).

Further investigation of the contribution of specific amino acids in the C−H stretching region was performed in the current study by mathematical deconvolution of the broad protein bands using maximum likelihood techniques and the application of Lorentzian and Gaussian peak shape assumptions. Figure 5 shows typical original and deconvoluted spectra for β-lactoglobulin, whereas Table 1 shows the Lorentzian and Gaussian wavenumber range for all protein samples studied. Preliminary results from deconvolution of the β-lactoglobulin spectrum based on the Lorentzian peak shape using fwhm values of 20, 25, and 30 cm^{-1} resulted in the resolution of 7−8 bands, whereas fwhm values of 35 and 40 cm^{-1} resulted in the resolution of only 4 bands. Deconvolution based on the Gaussian peak shape using fwhm of 20 cm^{-1} yielded 10−11 bands. Thus, it is clear that the Lorentzian assumption resulted in fewer peaks than the Gaussian assumption and may be preferred to avoid overfitting. Both the Lorentzian (fwhm of 25 cm^{-1}) and Gaussian (fwhm of 20 cm^{-1}) assumptions gave bands for proteins which corresponded well with those of the amino acids (Table 1).

Susi and Byler (1988) reported that the overall shape of the amide I Raman band of proteins was found to be nearly Gaussian or composed of Gaussian components and that Fourier deconvolution assuming Gaussian peak shape yielded more detailed results than those obtained with Lorentzian approximation. On the other hand, Mátrai et al. (1990) used pure Lorentzian curves for band envelope fitting of the C−H stretching region of cyclohexane. These latter authors indicated that although the Lorentzian curves may give only rough approximations to the different branches of rovibrational bands, the wavenumber values of the branch maxima were more precisely determined by this ap-

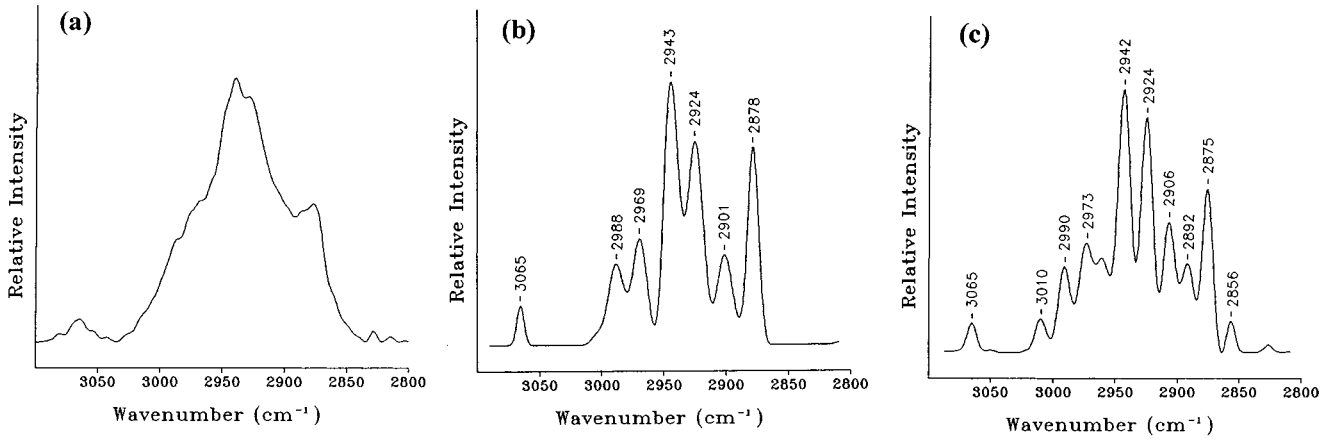

Figure 5. Raman spectrum of β-lactoglobulin (15% w/v in D_2O, apparent pD 6.8): (a) original spectrum after baseline correction and smoothing; (b) deconvoluted spectrum using Lorentzian peak shape and fwhm of 25 cm^{-1}; (c) deconvoluted spectrum using Gaussian peak shape and fwhm of 20 cm^{-1}.

Table 3. Relative Areas of the Raman Bands in the C−H Stretching Region (2800−3100 cm^{-1}) of Deconvoluted Spectra (Lorentzian Peak Shape, fwhm of 25 cm^{-1}) for Unheated and 90 °C Heated Lysozyme (L), α-Lactalbumin (A), and β-Lactoglobulin (B) and Their Binary Mixtures (AL, BL, AB)[a]

	relative peak area (%)											
wavenumber (cm^{-1})	L	L90	A	A90	B	B90	AL	AL90	BL	BL90	AB	AB90
2812	–	–	–	–	–	–	–	–	–	–	–	–
2838	–	4	–	–	–	–	–	–	–	–	–	–
2877−2879	17	5	19	22	26	39	16	20	22	9	22	26
2895−2902	8	4	9	5	9	2	7	2	4	4	4	9
2921−2924	22	sh	sh	sh	17	4	20	5	20	sh	sh	14
2939−2944	30	49	57	57	30	31	28	38	28	63	61	32
2962−2969	10	sh	–	4	11	–	12	9	10	9	2	6
2975−2979	–	–	9	–	–	12	–	–	–	–	–	–
2982−2989	7	22	–	–	3	3	9	9	10	9	5	7
3003	–	4	–	2	–	–	–	–	–	–	–	–
3012, 3024	–	2	–	–	–	–	–	–	–	–	–	–
3057−3066	5	10	6	9	4	8	8	16	6	5	6	6
3083	–	–	–	–	–	–	–	0.5	–	0.5	–	–

[a] sh, shoulder; –, not detected.

proach and the deconvolution was able to aid in understanding the underlying component band structure in the case of overlapping bands (Mátrai et al., 1990). Ni and Scheraga (1985) reported that in Raman spectroscopy, it has been shown both theoretically and experimentally that spectral profiles can be approximated by a Lorentzian, or a convolution of a Lorentzian with a Lorentzian or with a Gaussian, which is generally the instrumental slit function.

It should be noted that the signals for amino acids would not be expected to match the exact location in proteins. It has been suggested that molecular interactions weaken some bonds, decreasing the force constant and hence the Raman shift; thus, a decrease in the Raman shift is observed for molecules in liquid compared to gaseous phase and for hydrogen-bonded versus free functional groups (Terpinski, 1987). Conversely, the addition of water or deuterium oxide to simple organic solvents such as alcohols, dioxane, and dimethyl sulfoxide caused shifts of the Raman C−H stretching area to higher wavenumbers (Arteaga, 1994). Hence, it might be expected that amino acid side chains exposed to a polar environment would behave like and show similar Raman shifts as the free amino acids, whereas those which are involved in interactions would be at a lower wavenumber shift. This was observed in the present study, in comparing the C−H stretching region of the amino acids and proteins. For example, the main bands at 2935−2956 cm^{-1} for amino acids are seen at 2934−2945 cm^{-1} for proteins, whereas the band at 3075 cm^{-1} for the aromatic amino acids is clearly identified at 3061−3068 cm^{-1} for proteins.

For lysozyme, α-lactalbumin, and β-lactoglobulin the main band at ∼2940 cm^{-1} may involve C−H stretching contributions from most of the constituent amino acids (Table 1). On heating at 90 °C, increases in the relative peak area of this band were noted for lysozyme and for mixtures of lysozyme with either α-lactalbumin or β-lactoglobulin (Table 3). In contrast, the corresponding band in the spectra of α-lactalbumin and β-lactoglobulin did not increase in relative peak area on heating; in fact, a decrease was observed in peak area of this band for a mixture of the two whey proteins when heated. The difference between lysozyme and whey proteins may reflect differences in the unfolding of the proteins and relative amounts of amino acids. It is interesting to note that the changes or lack thereof in the relative area of the ∼2940 cm^{-1} band are not immediately apparent without careful investigation of the original spectra (Figure 4), due to the more pronounced changes in the total area of the broad band envelope. These results illustrate the advantage of applying deconvolution analysis for more detailed investigation of this broad band.

Verma and Wallach (1976, 1977b) and Mikkelsen et al. (1978) reported large increases in the Raman scat-

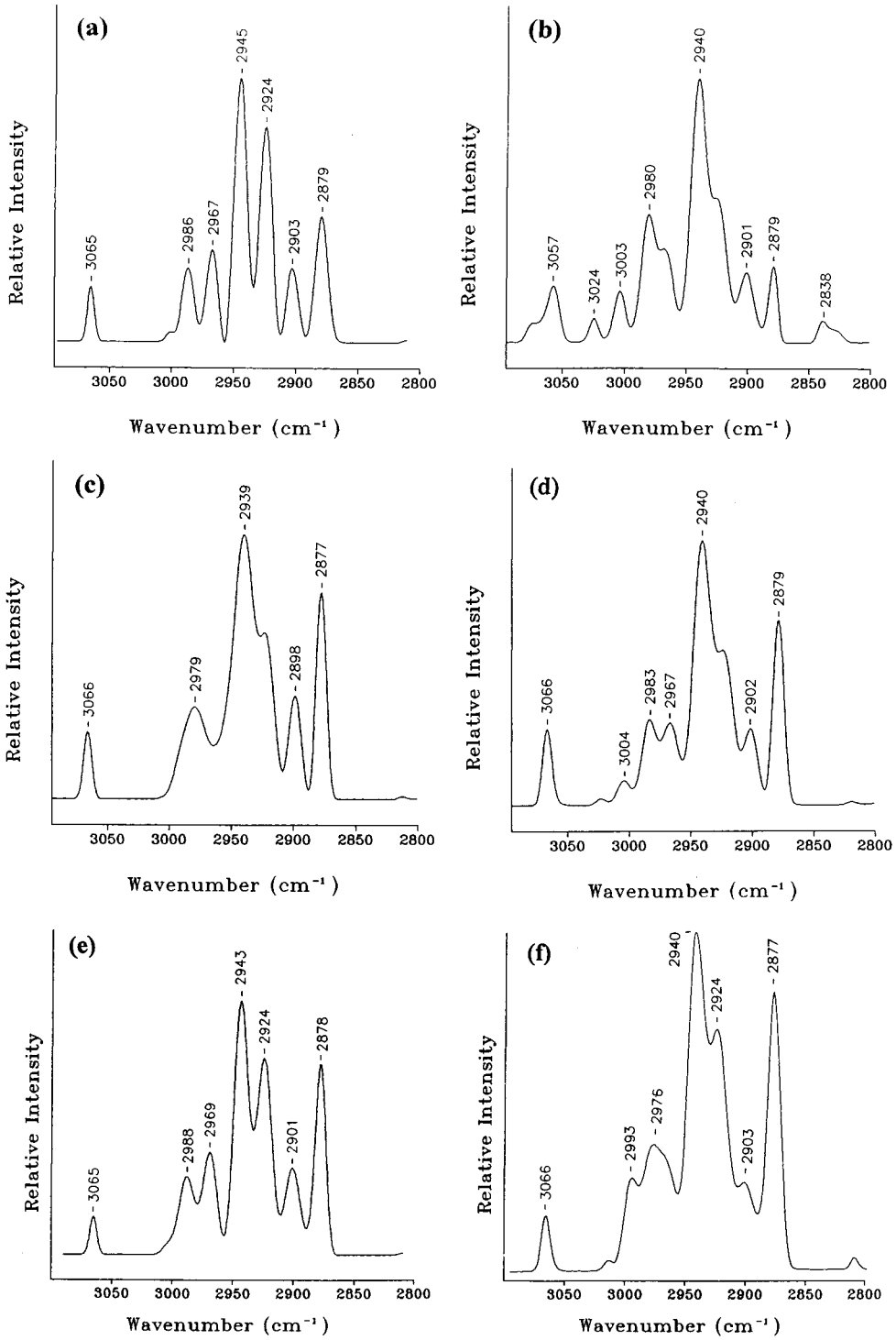

Figure 6. Effect of heat treatment (90 °C, 30 min) on the deconvoluted Raman spectra of lysozyme, α-lactalbumin, and β-lactoglobulin (15% w/v in D_2O, apparent pD 6.8): (a) lysozyme; (b) heated lysozyme; (c) α-lactalbumin; (d) heated α-lactalbumin; (e) β-lactoglobulin; (f) heated β-lactoglobulin. Spectra were deconvoluted assuming Lorentzian peak shape and fwhm of 25 cm^{-1}.

tering intensity at 2930 cm^{-1} of erythrocyte membranes and ribonuclease as a function of increasing temperature. At an apparent pD of 2.72, a broad transition was observed in the intensity of the 2930 cm^{-1} band of ribonuclease between 26 and 44 °C, with a midpoint at 38 °C (Verma and Wallach, 1977b), which corresponded to the position and width of transition detected by differential thermal calorimetry. These increases in intensity at 2930 cm^{-1} were suggested to arise from the unfolding of the protein molecules and the insertion of previously buried, aliphatic amino acid residues such as valine, leucine, and isoleucine into water. The position of the band did not change detectably. Increases in relative intensity of the 2930 cm^{-1} band in relation to other C−H stretching vibrations of lipid systems have also been proposed to reflect increasing polarity of environment of CH_2 and CH_3 groups of the lipid hydrocarbon chains (Larsson and Rand, 1973). These interpretations are supported by observations of the changes in the spectral features upon addition of water to solvents such as 1-propanol (Larsson and Rand, 1973) and *tert*-butyl alcohol, methanol, and dimethyl sulfoxide (Verma and Wallach, 1977b). In contrast, decreases in the normalized intensity and peak area of the 2930−

2940 cm^{-1} band after processing of Pacific whiting surimi into the heat-set kamaboko gel were reported (Bouraoui et al., 1997).

As mentioned above, the present study confirmed that changes in the band near 2930−2940 cm^{-1} do occur and that these changes may involve either an increase or a decrease in the relative intensity or area of the band. However, our study also clearly suggests that a number of amino acids other than aliphatic or aromatic amino acids contribute to the Raman signal in this region, and therefore direct interpretation of changes in relation to hydrophobic interactions is not clearly justified.

Another prominent band at 2877−2879 cm^{-1} showed a decrease in relative intensity for lysozyme and increases for α-lactalbumin and β-lactoglobulin after heating. The area of the band at 2885 cm^{-1} was reported to be correlated with the hydrophobicity values of eight proteins as measured by the fluorescent probes 1,8-anilinonaphthalenesulfonate and *cis*-parinaric acid (Arteaga, 1994). The position (Table 2) of the band suggests symmetric CH_3 vibrations may be involved. The aliphatic amino acids (alanine, isoleucine, leucine, and valine), threonine, and methionine contain terminal methyl groups on their side chains. Although histidine and lysine also show bands in this region, those signals were weak or medium in intensity compared to the strong signals of leucine, isoleucine, and valine, and thus the band may be related predominantly to the exposure of aliphatic amino acids.

The behavior of mixtures of α-lactalbumin or β-lactoglobulin with lysozyme on heating differed from those observed in the individually heated proteins; this supports findings by Howell and Li-Chan (1996), who found that interactions of lysozyme with either whey protein resulted in experimental spectra differing from the theoretically calculated average spectra of the component individually heated proteins. In their study, related to the Raman spectra in the 450−1900 cm^{-1} region, both the lysozyme−α-lactalbumin and lysozyme−β-lactoglobulin complexes showed the involvement of hydrophobic interactions by intensification of spectral bands assigned to the CH and CH_2 bending vibrations and a decrease in the intensity of bands assigned to tryptophan residues in a nonpolar environment.

In the present study, heating considerably increased the relative peak areas of the main band at 2939−2944 cm^{-1} for mixtures of lysozyme and α-lactalbumin or lysozyme and β-lactoglobulin, with a corresponding decrease in the peak area of the 2921−2924 cm^{-1} band. In contrast, mixtures of α-lactalbumin and β-lactoglobulin had a larger peak area for the unheated sample compared with the heated sample and vice versa for the 2921−2924 cm^{-1} band (Table 3). These results confirm the differences observed in the previous studies (Howell and Li-Chan, 1996; Howell et al., 1995) between heated lysozyme−whey protein mixtures and heated α-lactalbumin−β-lactoglobulin mixtures. Because the main band near 2940 cm^{-1} may be attributed to charged amino acids in addition to hydrophobic amino acids (Table 1), this band may reflect the electrostatic interactions in addition to hydrophobic interactions in the lysozyme−whey protein complexes reported previously (Howell and Li-Chan, 1996; Howell et al., 1995).

In the present study, aromatic amino acids, namely, phenylalanine, tyrosine, and tryptophan, produced bands at higher Raman shifts of 3075 cm^{-1} compared with the proteins which were at 3065 cm^{-1}. Positive correlation was obtained between the position of the band at 3065 cm^{-1} and hydrophobicity measured by fluorescent probe methods (Arteaga, 1994). On heating, an increase in the relative peak area of the band near 3065 cm^{-1} was noted for the proteins and protein mixtures in this study, with the exception of the mixtures containing β-lactoglobulin, confirming the involvement of the aromatic groups in conformational changes resulting from heating. Heating of α-lactalbumin and lysozyme also produced additional bands near 3003 and 3024 cm^{-1} (Figure 6). The latter band may be assigned to tyrosine residues, whereas the former band may be indicative of proline, or possibly alanine and the sulfur-containing amino acids. It is also interesting to note that new bands appeared near 3000 cm^{-1} for some of the amino acids in the solid state, compared to the corresponding amino acids dissolved in deuterium oxide.

Similar spectral changes were observed for α-lactalbumin and lysozyme in the presence of 8 M urea (Arteaga, 1994). In the presence of urea, lysozyme and α-lactalbumin showed additional bands at 2831 and 2826 cm^{-1}, respectively, which were also observed for heated lysozyme (2838 cm^{-1}) and heated α-lactalbumin (2826 cm^{-1}). In addition, β-lactoglobulin and α-lactalbumin in the presence of deuterated urea showed new bands at 3020 and 3012 cm^{-1}, respectively, which also appeared in heated protein samples, reflecting the exposure of hydrophobic groups on unfolding and denaturation of proteins.

Conclusions. Raman C−H stretching vibrations of amino acids indicated several bands for each amino acid; these bands corresponded to the CH, CH_2, and CH_3 moieties of saturated alkyl groups and to the =CH groups of unsaturated or aromatic groups. Maximum likelihood deconvolution, using both the Lorentzian and Gaussian assumptions, of the C−H stretching region of selected proteins, namely, lysozyme, α-lactalbumin, β-lactoglobulin, and their mixtures, reported for the first time, confirmed the presence of several bands that corresponded well with those identified for the amino acids. The main band at 2939−2944 cm^{-1} is not only due to the aromatic and aliphatic amino acids, as previously assumed, but also due to the charged amino acids and proline, threonine, and histidine. In contrast to the band near 2940 cm^{-1}, the bands near 2880 and 3065 cm^{-1} may reflect predominantly aliphatic and aromatic groups, respectively. A number of features in the C−H stretch region changed during the heating of proteins and their binary mixtures as well as in the presence of denaturing agent 8 M urea. These changes may be used to monitor hydrophobic interactions, denaturation, and conformational changes of proteins.

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