Infrared and Circular Dichroism Spectroscopic Characterization of Structural Differences between β-Lactoglobulin A and B[†]

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ABSTRACT: Structural differences between two genetic variants of bovine β-lactoglobulins (type A and B) in aqueous solutions were characterized using Fourier transform infrared and circular dichroism spectroscopies. To probe differences in structural dynamics, the effects hydrogen−deuterium exchange were also compared for the two proteins. The infrared spectra recorded in H_2O solution for the two proteins were nearly identical in the conformationlly sensitive amide I region. The only exceptions were small differences at the band ascribed to a high-wavenumber β-sheet component near 1693 cm^{-1} and the band assigned to turns at 1684 cm^{-1}. In contrast, when the proteins were prepared in D_2O solution, marked spectral differences were observed at all regions ascribed to β-sheet and turn structures. These differences are consistent with the structural differences of the two variants at amino acid residues 64 and 118, which are located at a turn and a β-sheet structure, respectively, as revealed by X-ray crystallographic studies [Monaco et al. (1987) *J. Mol. Biol. 197*, 695−706]. The circular dichroism spectra for the two proteins were essentially identical, both before and after hydrogen−deuterium exchange. Therefore, hydrogen−deuterium exchange did not alter the proteins' secondary structure. The enhancement of the amide I spectral difference upon hydrogen−deuterium exchange was ascribed to the differences in the structural mobility of the two proteins. Since the rate of exchange was greater for variant A, it was concluded that this variant has greater structural mobility than variant B. These findings indicate that the combination of infrared spectroscopy and hydrogen−deuterium exchange has great potential in characterization of even subtle structural differences in proteins induced by naturally occurring point mutations and/or site-directed mutagenesis.

The dramatic developments and extensive applications of genetic engineering techniques, such as site-directed and/or random mutagenesis, in the past decade have resulted in numerous protein products that have single or multiple amino acid residue mutations. However, the structural effects of most of these mutations have not been adequately characterized. This is because there has not been a suitable technique that is sensitive even to subtle structural differences, and also can be easily carried out with good reproducibility and accuracy in a short period of time. In this study, our goal was to demonstrate the utility of using Fourier transform infrared (FT-IR)[1] spectroscopy, coupled with hydrogen−deuterium (H−D) exchange, to characterize and magnify the otherwise subtle structural differences in proteins induced by genetic variation.

FT-IR spectroscopy has been used extensively in studies of secondary structural composition (Susi & Byler, 1986;

Surewicz & Mantsch, 1988; Dong et al., 1990; Prestrelski et al., 1992; Surewicz et al., 1993; Arrondo et al., 1993) and conformational changes in proteins. Protein structural information is obtained mainly from the analysis of the conformationally sensitive amide I band, which is due primarily to the C=O stretching vibrations of the protein backbone. The frequency of the amide I vibration is determined by the nature of hydrogen bonds involving the C=O and N−H groups of the peptide linkage and the geometry of the protein backbone (Miyazawa & Blout, 1961; Krimm & Bandekar, 1986) and, thus, is sensitive to changes involving the secondary structure of proteins.

Alterations in protein conformation induced by temperature (Byler & Purcell, 1989; Surewicz et al., 1987; Casal et al., 1988; Ismail et al., 1992; Görne-Tschelnokow et al., 1993), pH (Casal et al., 1988), pressure (Le Tilly et al., 1992; Wong & Armstrong, 1992; Howlett et al., 1992; Takeda et al., 1995), lyophilization (Prestrelski et al., 1993a,b), chemicals (Jackson & Mantsch, 1991, 1992), and changes in oxidation (Dong et al., 1992b, 1995) and enzymatic states (Arrondo et al., 1987) have been studied in the past few decades. Also, FT-IR spectroscopy in combination with H−D exchange has been used for years to probe alterations in protein conformational mobility induced by extrinsic factors and by alterations in the peptide backbone, such as those resulting from limited proteolysis (Harris et al., 1986). The kinetics and extent of exchange can be readily followed, due to large alterations in infrared spectra that occur as the protein is
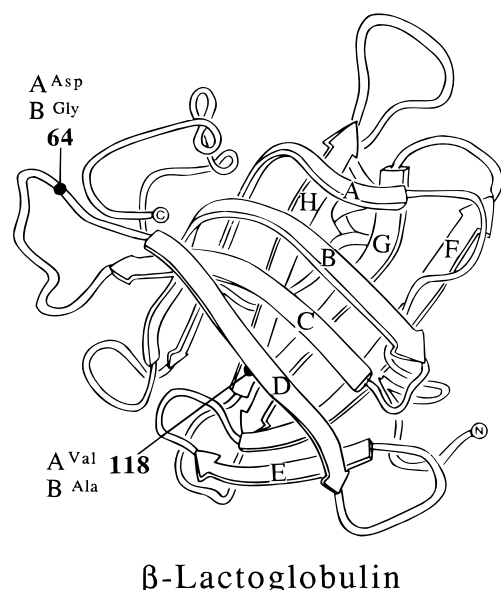
β-Lactoglobulin

FIGURE 1: Schematic representation of the secondary structure of BLG. Modified from Monaco et al. (1987).

deuterated [for a review, see Englander and Kallenbach (1984)].

Recently, FT-IR spectroscopy has been used to probe, in single solvent systems, structural changes in proteins induced by site-directed mutagenesis. Bowler and colleagues (Bowler et al., 1993, 1994; Herrmann et al., 1995) studied the effects of a single mutation at Lys73 on conformation and denaturation properties of yeast iso-1-cytochrome *c* in H_2O solution. Prestrelski and Arakawa (1991) studied the effects of a double mutation (Cys69 → Asp and Cys101 → Arg) on structure and conformational dynamics of tumor necrosis factor-α in D_2O solution.

We selected bovine β-lactoglobulins (BLG) A and B as model proteins for this study. BLG A and B are natural genetic variants that are commonly found in the milk of various breeds of cows and have been studied extensively by infrared (Timasheff & Susi, 1966; Susi & Byler, 1986; Casal et al., 1988), circular dichroism (Townend et al., 1967), optical rotatory dispersion (Timasheff et al., 1966), and nuclear magnetic resonance (Pessen et al., 1985) spectroscopies, calorimetry (Griko & Privalov, 1992; Azuaga et al., 1992), and X-ray crystallography (Papiz et al., 1986; Monaco et al., 1987). The monomers of the proteins consist of 162 amino acid residues in a single polypeptide chain, which differ only at positions 64 and 118, where an Asp and a Val in variant A are substituted by a Gly and an Ala in variant B (Braunitzer et al., 1973), respectively. The three-dimensional crystal structure has shown that the core of BLG is made of a short α-helical segment and eight strands of antiparallel β-sheet, which wrap around to form an antiparallel β-barrel (Figure 1). The amino acid residues at positions 64 and 118 are located on a turn and a β-sheet structure, respectively. However, no significant structural differences between the two variants were found by comparison of the crystal structures determined at 2.5 Å resolution (Monaco et al., 1987), nor were structural differences discerned by the spectroscopic methods in single-solvent systems. On the other hand, significant differences were detected in the pH-dependent conformational transition in the pH range of 4−9 (Timasheff et al., 1966) and the gelation properties (Huang et al., 1994) of the two variants.

As will be shown in the current study, FT-IR spectroscopy is very sensitive to subtle changes in protein secondary structure resulting from genetic variation. The secondary structural units, in which the two different amino acids in BLG A and B (out of 162 total residues) are located, were assignable on the basis of differences in the proteins' infrared spectra in H_2O solution. These subtle structural differences between the two variants were greatly enhanced by H−D exchange and indicated that variant A had greater conformational mobility than variant B. Also, we found that the overall blue shifting of the amide I band of both BLG variants, as a result of H−D exchange, could be accounted for fully by absorbance redistribution within the red-shifted individual component bands.

MATERIALS AND METHODS

Sample Preparations. Bovine β-lactoglobulins A and B were purchased from Sigma and used without further purification. For H_2O solutions, the proteins were dissolved in 10 mM potassium phosphate/H_2O buffer (pH 7.4) at a concentration of 20 mg/mL. Three different H−D exchange techniques were used to prepare the proteins in D_2O solutions. With the first technique, the dried proteins were dissolved directly in 10 mM potassium phosphate/D_2O (99.9%, Sigma) buffer (pD 7.0) at a concentration of 2 mg/mL and kept at room temperature for 24 h. Then, the protein solutions were divided into two samples. One was concentrated to about 10 mg/mL using a Centricon 10 microconcentrator (Amicon) at 4000*g*. The other was incubated at 50 °C for 1 h, cooled to room temperature, and then concentrated as described above. The heat treatment should promote H−D exchange of the proteins but was not sufficient to induce thermal denaturation (Casal et al., 1988). With the second H−D exchange technique, the proteins were first dissolved in the H_2O buffer at a 20 mg/mL concentration and then diluted 10-fold with D_2O buffer. The mixtures were concentrated by centrifugation using a Centricon 10 microconcentrator at 4000*g*. This procedure was repeated four times during a 3 h period. The samples were then kept at room temperature for an additional 21 h. At this point, the protein solution was divided into two samples. One sample was used to acquire infrared spectra, and the other was heat treated as discussed above. The final concentrations of proteins were about 10 mg/mL. Finally, a third technique was used to determine the effects of maximum H−D exchange. The proteins were dissolved in 10 mM phosphate/D_2O buffer (pD 7.0) at a 2 mg/mL concentration and kept at room temperature overnight. Then, the protein solutions were lyophilized using Labconco 4.5 Freeze Drier. The procedure was repeated twice to ensure a maximum H−D exchange. The final lyophilized protein powder was rehydrated in 10 mM phosphate/D_2O buffer at a concentration of 2 mg/mL. To foster additional exchange, aliquots were incubated at 70 °C for 1 and 3 h and cooled to room temperature, prior to spectral acquisition. This treatment allowed H−D exchange in the thermally unfolded proteins (Casal et al., 1988). As the infrared results will demonstrate, the protein did not aggregate during this thermal treatment and refolded upon cooling to room temperature at the concentration studied.

Infrared Measurement and Amide I Spectra Analysis. Protein solutions were prepared for infrared measurement in a CaF₂ cell (Beckman FH-01) with a 6 μm spacer for
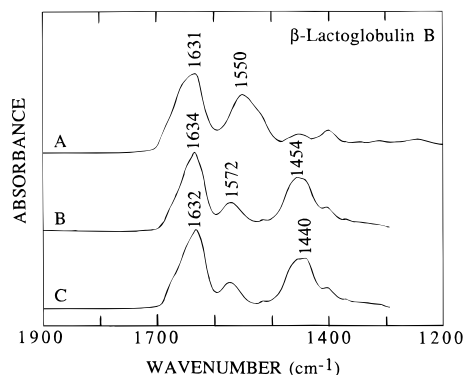
FIGURE 2: Infrared spectra of BLG B in H_2O and D_2O solutions. (A) Spectrum (20 mg/mL) in 10 mM phosphate/H_2O buffer (pH 7.4). (B) Spectrum (10 mg/mL) in 10 mM phosphate/D_2O buffer (pD 7.0). (C) Spectrum in D_2O after the sample was heated at 50 °C for 1 h and then cooled to 25 °C. The spectral contributions of the buffer and atmospheric water vapor were subtracted from the protein spectra as described Materials and Methods.

H_2O solution and a 25 μm spacer for D_2O solution. Freeze-dried proteins were ground into a fine powder with KBr and pressed into discs, as previously described (Prestrelski et al., 1993b). Infrared spectra were recorded at 25 °C with a Magna Model 550 spectrometer (Nicolet) with a dTGS detector. For each spectrum, a 512-scan interferogram was collected in the single-beam mode with a 4 cm^{-1} resolution. Reference spectra were recorded under identical scan conditions with only the corresponding buffer in the cell. The spectra of liquid and gaseous water were subtracted from the observed protein spectra according to previously established criteria (Dong et al., 1990) and the double-subtraction procedure (Dong et al., 1992b). The final protein spectrum was smoothed with a seven-point Savitsky−Golay smooth function to remove the white noise. Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet). The inverted second-derivative spectra were obtained by factoring by −1 and then curve fitted with Gaussian band profiles (Dong et al., 1992a). In cases where bands in the second-derivative spectrum were not clearly resolved, the fourth-derivative spectrum was calculated. With a high-quality original spectrum, the fourth-derivative spectrum can be used to determine the number and positions of bands to which curves are fitted in the second derivative spectrum. Fourier self-deconvolution (FSD) was carried out using a half-band width (full width at half-height) of 23 cm^{-1} and an enhance factor (*K* value) of 2.7, over the frequency range of 2000−1300 cm^{-1}.

Circular Dichroism Measurement and Spectral Analysis. The far-UV CD spectra were recorded at 25 °C with an Aviv Model 62DS spectrometer (Lakewood, NJ) in the wavelength range of 180−260 nm using a 0.01 mm path length quartz cell. Spectra were background corrected and converted to mean residue ellipticity (deg cm^2/dmol). Concentrations were determined by absorbance at 278 nm with an E_{1cm} of 0.96 mL/mg cm and a mean residue weight of 113 g/mol.

RESULTS

The initial spectroscopic assessment of the influence of H−D exchange was made with samples prepared by direct dissolution of the protein powders in D_2O solution (i.e., the first technique described in Materials and Methods). Figure 2 shows the spectra of BLG B in H_2O and D_2O, with and
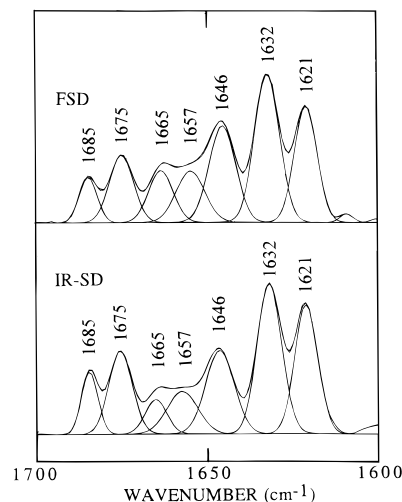

FIGURE 3: Comparison of the curve-fitted Fourier self-deconvoluted and inverted second-derivative spectra of BLG B in D_2O for 23 h at room temperature, for 1 h at 50 °C, and then cooled to 25 °C. (Upper) The curve-fitted FSD spectrum. (Lower) The curve-fitted inverted second-derivative spectrum.

without heat treatment at 50 °C for 1 h. The spectra of BLG A acquired under the same conditions were essentially identical (data not shown) to those shown for BLG B. In H_2O solution, both proteins exhibit absorbance maxima for their amide I and II bands at 1631 and 1550 cm^{-1}, respectively, indicating a predominantly β-sheet structure (Susi & Byler, 1986; Dong et al., 1990). Upon H−D exchange, the amide I bands of both proteins shift about 2 cm^{-1} to a higher wavenumber. Although the amide I frequencies changes as a result of H−D exchange at protein backbones have been reported extensively (Byler & Susi, 1986; Susi & Byler, 1986; Surewicz & Mantsch, 1988), it is rarely seen that the amide I absorbance maximum of a protein shifts to a higher wavenumber upon H−D exchange. For most, if not all, proteins studied to date, the amide I bands are generally shifted to a lower wavenumber when the protein is dissolved in D_2O solutions.

Prior to heat treatment, the amide II bands, which arise mainly from an out-of-phase combination of N−H in-plane bending and C−N stretching vibrations of peptide linkages (Krimm & Bandekar, 1986), are red-shifted by about 100 cm^{-1} from 1550 to 1454 cm^{-1}. The direction and magnitude of this shift are typical for proteins during H−D exchange. After heat treatment at 50 °C for 1 h, the absorbance maximum of amide II′ is further red-shifted to near 1440 cm^{-1}, indicating that additional H−D exchange has occurred. Similar amide II′ band shifts were also observed in variant A. However, identical amide I/amide II intensity ratios (3.03) were observed for both proteins in D_2O solution, before and after heat treatment. Finally, the band near 1572 cm^{-1} remaining in the amide II region after H−D exchange may arise from the ionized carboxylate side chains of the glutamate and/or aspartate residues (Chirgadze et al., 1975).

To resolve and quantitate the relative areas of the overlapping band components under the amide I contour, two independent mathematical band-narrowing techniques, namely Fourier self-deconvolution and second-derivative, in combination with curve-fitting analysis were carried out. As an example, Figure 3 shows a comparison of the curve-fitted FSD and second-derivative spectra of BLG B in D_2O solution after heat treatment. An overall similarity between the spectra

Table 1: Assignments and Relative Band Areas of Infrared Second-Derivative Amide I Components of β-Lactoglobulin A in H_2O and D_2O Solutions[a]

H_2O		D_2O		D_2O/heat		
ν (cm^{-1})	area (%)	ν (cm^{-1})	area (%)	ν (cm^{-1})	area (%)	assignment
1628	29.2	1623	23.9	1622	22.4	β-sheet
1641	16.6	1633	30.4	1632	25.2	β-sheet
1652	9.0	1648	18.1	1646	16.1	unordered
1656	9.4	–	–	1656	8.6	α-helix
1661	8.8	1662	10.4	1664	6.8	turn
1671	8.4	–	–	–	–	turn
1683	10.2	1676	14.0	1675	13.0	turn
1692	8.4	1690	3.2	1683	7.9	β-sheet

[a] The band component at 1613 cm^{-1}, which arises from side chain vibration, in the spectrum of the H_2O solution is not included in the calculation.

Table 2: Assignments and Relative Band Areas of Infrared Second-Derivative Amide I Components of β-Lactoglobulin B in H_2O and D_2O Solutions

H_2O		D_2O		D_2O/heat		
ν (cm^{-1})	area (%)	ν (cm^{-1})	area (%)	ν (cm^{-1})	area (%)	assignment
1628	28.1	1621	20.5	1621	21.1	β-sheet
1639	18.7	1634	30.8	1632	26.1	β-sheet
1652	10.4	1648	18.5	1646	16.9	unordered
1656	9.7	–	–	1657	9.4	α-helix
1663	7.2	1664	13.1	1665	5.3	turn
1671	7.6	–	–	–	–	turn
1684	11.5	1678	13.0	1675	14.0	turn
1693	6.8	1692	4.1	1685	7.2	β-sheet

of FSD spectrum and those of the inverted second-derivative spectrum is observed. A total of eight band components in H_2O solution and seven band components in D_2O solution was revealed. Detailed parameters obtained from curve-fitting analysis for BLG A and B are listed Tables 1 and 2. On the basis of previous infrared studies of other proteins in H_2O solutions (Dong et al., 1990; Dong & Caughey, 1994), the bands at 1693, 1639, and 1628 cm^{-1} can be assigned to β-sheets, the band at 1656 cm^{-1} to an α-helix, the bands at 1684, 1671, and 1663 cm^{-1} to turns, and the band at 1652 cm^{-1} to an unordered structure. Assignments of the bands for the proteins in D_2O solution can also be made on the basis of earlier studies of proteins in this solvent (Byler & Susi, 1986; Susi & Byler, 1986) and are shown in Tables 1 and 2. The 1693 cm^{-1} band in H_2O and the 1685 cm^{-1} band in D_2O after heat treatment indicate that the β-sheet structures in the proteins are antiparallel β-sheet structure (Krimm & Bandekar, 1986; Chirgadze & Nevskaya, 1976; Susi & Byler, 1986). The band near 1613 cm^{-1} has been generally assigned to side chain vibration of amino acid residues (Chirgadze et al., 1975). We must point out that the technique of quantitative analysis of protein secondary structure using the infrared amide I band is a matter of debate (Surewicz et al., 1993; Dong et al., 1995a,b; Jackson & Mantsch, 1995). It is noteworthy, however, that the overall secondary structural compositions estimated using the second-derivative spectra by the method of Dong et al. (1992a) and the FSD spectra by the method of Susi and Byler (1986) for variants A and B (Table 3) are essentially the same, in view of the approximately 5% error in estimation of secondary structural content by either method (Susi & Byler, 1986; Surewicz et al., 1993; Dong et al., 1995a,b). The secondary

structural compositions estimated by both methods agree well with those reported by X-ray crystallographic analysis (Papiz et al., 1986; Monaco et al., 1987) and indicate that there is essentially no difference in the secondary structure of the two variants. Also, H–D exchange does not alter the contents of secondary structural elements. It is noteworthy that a band component assignable to an α-helix, which is present in the spectra for the proteins in H_2O, cannot be clearly identified in the spectra of either protein for partially H–D-exchanged state (Tables 1 and 2). However, an α-helix band is present after greater H–D exchange.

Figure 4 shows comparisons of the second-derivative spectra of variants A and B in the amide I region. The spectra recorded in H_2O solution are nearly identical, except for small differences at the band ascribed to a high-wavenumber β-sheet component near 1693 cm^{-1} and the band ascribed to a turn at 1684 cm^{-1}. In contrast, marked spectral differences between the two variants were observed in D_2O solution after 24 h of H–D exchange. The bands assigned to a high-wavenumber β-sheet component (1692 cm^{-1}) and to a turn structure (1678 and 1664 cm^{-1}) for the B variant were found at about 3 cm^{-1} higher than those for the A variant. Large differences in frequencies and intensities were also observed for the bands assigned to low-wavenumber β-sheet components near 1633 and 1621 cm^{-1}. After heat treatment at 50 °C for 1 h, the spectral differences between the two variants were diminished in the region assigned to the turn structure but remained in regions assigned to high- and low-wavenumber β-sheet structures. It should be noted that, for both variants, the heat treatment resulted in further red shift at the band assigned to the high-wavenumber β-sheet component. Such changes are apparently due to increased H–D exchange at the β-sheet structure of the proteins even though the amide I/amide II ratio remains unchanged.

To rule out the possibility that the spectral differences between the two variants in D_2O solution were due to different degrees of unfolding in the dried state [cf. Prestrelski et al. (1993a,b)] and a resulting difference in the exposure of various residues to the solvent during rehydration, we examined (1) the spectra of the dried proteins lyophilized from H_2O solution or from D_2O solution after two H–D exchange cycles, (2) the spectra of proteins initially dissolved in H_2O-based buffer and then exchanged with D_2O-based buffer, and (3) the spectra of proteins in D_2O solution after two lyophilization and H–D exchange cycles, followed by heating at 70 °C. As will be shown by the infrared data, the second method allows the protein to refold prior to the H–D exchange and the third method allows maximum H–D exchange.

Figure 5 shows an overlay of the second-derivative spectra of BLG A and B in the dried state. The spectra of the dried state differ markedly from the spectra of the aqueous state, indicating that the conformations of the proteins were altered significantly in the dried solid [cf. Prestrelski et al. (1993a,b)]. However, the spectra of BLG A and B in the dried state, lyophilized from either H_2O or D_2O solutions, are very similar, indicating a similar degree of unfolding. There are only small differences at bands near 1695 and 1632 cm^{-1} for proteins lyophilized from H_2O solution and at bands near 1691 and 1632 cm^{-1} for proteins lyophilized from D_2O solution. Both band regions are assignable to β-sheet structure (Dong & Caughey, 1994). Also for the proteins

Table 3: Comparison of the Secondary Structures of β-Lactoglobulin A and B Estimated by Infrared Spectroscopy and X-ray Crystallography

medium	secondary structure (%)				methods	reference
	α-helix	β-sheet	turn	unordered		
H$_2$O						
BLG A	9.4	54.2	27.4	9.0	IR-SD[a]	this work
	10.8	51.8	27.1	10.3	FSD/CF[b]	this work
	7	53	—	—	X-ray	c
BLG B	9.7	53.6	26.3	10.4	IR-SD	this work
	10.1	51.5	27.2	11.2	FSD/CF	this work
	7	53	-	—	X-ray	c,d
D$_2$O/heat treated						
BLG A	8.6	55.5	19.8	16.1	IR-SD	this work
	9.9	51.5	22.5	16.0	FSD/CF	this work
BLG B	9.4	54.4	19.3	16.9	IR-SD	this work
	10.5	50.7	20.4	18.5	FSD/CF	this work

[a] Infrared second-derivative, which is the method of Dong et al. (1992a). [b] Fourier self-deconvolution and curve fitting, which is the method of Susi and Byler (1986). [c] Monaco et al. (1987). [d] Papiz et al. (1986).
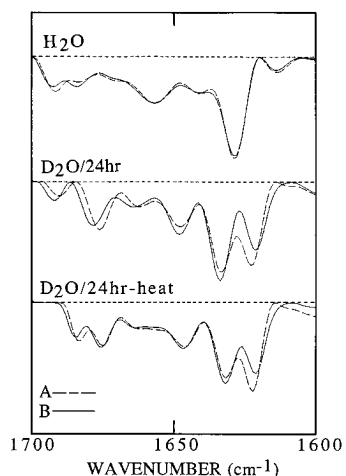

FIGURE 4: Comparisons of second-derivative spectra of BLG A and B in H$_2$O and D$_2$O solutions. The H−D exchange was carried out by direct dissolution of the proteins in D$_2$O-based buffer. (Top) Spectra in H$_2$O solution. (Middle) Spectra in D$_2$O solution. (Bottom) Spectra in D$_2$O solution after the sample was heated at 50 °C for 1 h and then cooled to 25 °C.
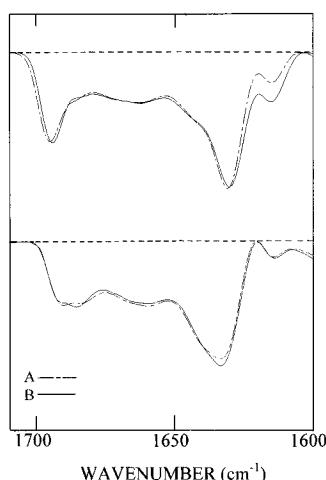

FIGURE 5: Comparison of second-derivative spectra of BLG A and B in dried states. (Upper) The proteins were lyophilized from H$_2$O solution. (Lower) The proteins were lyophilized from D$_2$O solution after two cycles of H−D exchange. The spectra were recorded from protein/KBr pellets.

lyophilized from H$_2$O, there is an intensity difference at the band near 1613 cm^{-1}, which is due to side chain vibrations (Chirgadze et al., 1975). It is likely that the differences in β-sheet bands are the result of differences in the amino acid
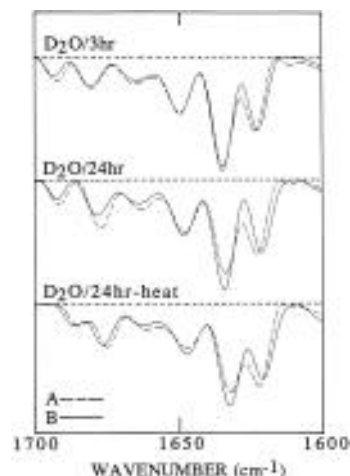

FIGURE 6: Comparisons of second-derivative spectra of BLG A and B dissolved initially in a H$_2$O-based buffer and then exchanged to a D$_2$O-based buffer. The spectra (10 mg/mL) were recorded at 3 and 24 h of H−D exchange.

residue at position 118. Despite these minor spectral differences, the overall similarity between the spectra of the proteins in the dried solid indicates that the degree of unfolding is very similar for both proteins. Hence, differences in H−D exchange during rehydration probably were not responsible for the large differences between the spectra of the two proteins in D$_2$O solution.

This suggestion is confirmed by examination of the spectra for BLG A and B prepared with two other commonly used H−D exchange techniques. Figure 6 shows comparisons of the spectra of proteins dissolved initially in H$_2$O-based buffer and then exchanged to D$_2$O-based buffer. The spectral differences between the proteins are maintained after 24 h in D$_2$O at room temperature and after subsequent heating for 1 h at 50 °C. Figure 7 shows comparisons of the spectra of proteins in D$_2$O solution after two lyophilization and H−D exchange cycles and after the solution was heated at 70 °C. It is noteworthy that, at a concentration of 2 mg/mL, no irreversible aggregation was observed in either BLG A or B after 70 °C heat treatment for 3 h. Spectral differences between BLG A and B at regions assignable to β-sheet and turn structures similar to those noted in Figure 4 were still observed after these treatments, which should maximize H−D exchange.

On the basis of the time- and temperature-dependent spectral changes in D$_2$O solutions (Figures 4, 6, and 7), the
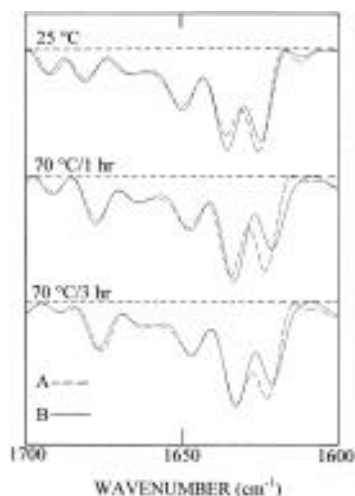
FIGURE 7: Comparisons of second-derivative spectra of BLG A and B in D_2O solution after two H−D exchange and lyophilization cycles. All spectra (2 mg/mL) were recorded at 25 °C with or without prior heat treatment at 70 °C for 1 or 3 h.
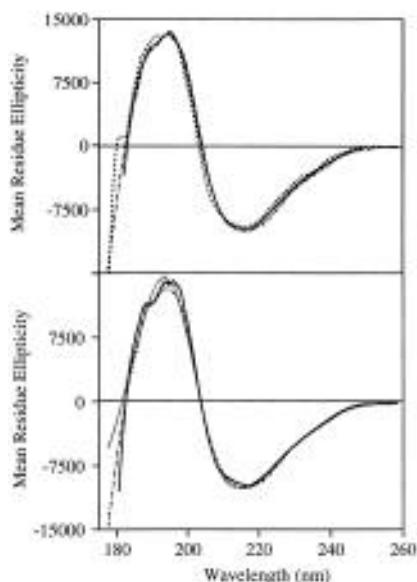

FIGURE 8: Far-UV CD spectra of BLG A and B in 10 mM phosphate/H_2O or D_2O buffers. (Top) Spectra of variants A. (Bottom) Spectra of variant B. Proteins in H_2O (—), D_2O after 24 h exchange (- - -), and D_2O after two H−D exchange and lyophilization cycles (· - ·).

best indicator of the progression of H−D exchange is the increase in the ratio between intensities for the two low-wavenumber β-sheet bands (i.e., 1621/1632 cm^{-1}). For both proteins, this ratio increases more rapidly, and to a greater extent, when they are dissolved directly in D_2O-based buffer. More importantly, for any given time after exchange and with any one of the H−D exchange methods, the 1621/1632 cm^{-1} ratio is greater for variant A than for variant B. Therefore, the spectral differences detected in D_2O solutions are due to the greater relative structural mobility of variant A.

The secondary structures of two BLG variants were also examined by CD spectroscopy. For both variants, the position of the intense negative band at 216 nm indicates the proteins possess predominantly β-sheet structure (Figure 8). With either variant, the spectra recorded in H_2O and D_2O solutions were essentially identical, indicating that the secondary structural compositions of the proteins were not

altered by H−D exchange. Also, the CD spectra of the two proteins recorded in D_2O solution, before and after heat treatment, are identical (data not shown), again indicating that the secondary structure of the proteins is the same and is not irreversibly altered by heat treatment under conditions employed in this study.

DISCUSSION

Hydrogen−Deuterium Exchange Behavior of Proteins. H−D exchange has been used for decades in infrared studies of structural dynamics and secondary structure of proteins, for the purpose of monitoring exchange-dependent changes in the amide II region (Nielsen, 1960; Blout et al., 1961) or for avoiding strong interference of water absorbance in the amide I region (Timasheff & Susi, 1966; Susi & Byler, 1986; Surewicz & Mantsch, 1988). Since the pioneering work of Hvidt and Linderstrøm-Lang (1954), the H−D exchange behavior of proteins has been extensively investigated (Woodward & Hilton, 1979; Hvidt & Nielsen, 1966; Englander & Kallenbach, 1984), employing a variety of means to monitor the exchange. It has generally been accepted that the rate of H−D exchange and the extent of exchange at a given time period depend on the structural mobility of the protein. The more mobile (less rigid) the protein structure, the faster the rate of H−D exchange becomes and the greater the final equilibrium level of exchange. The peptide NH protons of random (unfolded) polypeptides exchange readily with aqueous solvent deuteron, whereas those of native (folded) proteins are retarded by many orders of magnitude. Nevertheless, most of the interior backbone protons of native proteins have measurable accessibility to solvent (Woodward & Hilton, 1979). Earlier studies have also shown that, by exchange of the protons of polypeptide and protein backbones with deuterons, the hydrogen-bonding strength in the macromolecules was increased, which, in turn, increased significantly their conformational stability (Calvin et al., 1959; Hermans et al., 1959; Scheraga, 1960).

Two different H−D exchange models have been put forward to explain the structural motion involved: solvent penetration and local unfolding/breathing [for review, see Englander and Kallenbach, (1984)]. In the former model, solvent molecules and ion catalysts (OH^- and H_3O^+) are thought to enter the protein due to structural fluctuation and to catalyze the rate-limiting exchange event within the protein matrix. In the latter model, the H−D exchange of proteins is explained by a transient open state of local structure in which hydrogen-bonded groups are separated and made available for H−D exchange catalysis. In either case, even if two proteins do not have significant differences in structure, but do have differences in conformational mobility, the degree of H−D exchange will be different.

X-ray crystallographic studies have shown that the amino acid residues 64 and 118 of bovine BLG are located at a turn and a β-sheet structure, respectively (Figure 1). Comparison of the crystal structures determined at 2.5 Å resolution has revealed that no significant conformational differences between the A and B variants resulted from substitutions at positions 64 and 118 (Monaco et al., 1987). In contrast, infrared data for the proteins in H_2O solution show small but readily detectable differences between the two variants. The small differences at the band ascribed to

a high-wavenumber β-sheet component near 1693 cm^{-1} and the band ascribed to a turn at 1684 cm^{-1} are consistent with the locations the substituted residues. The two most likely explanations for the apparent discrepancy between X-ray crystallography and infrared studies are as follows. (1) The structural differences between two variants are subtle and, thus, cannot be detected by X-ray crystallography, and (2) the relatively low resolution of X-ray diffraction data (2.5 Å) makes detection of small structural differences difficult. We should point out that the structural differences between the two variants were also not detected by CD, which serves as a reference for us to interpret the spectral changes that were observed by FT-IR and that were a result of differences in protein conformational mobility.

The infrared spectral differences between variants A and B become much more apparent in D$_2$O solutions. These spectral differences are not due to a different global structure being induced by H$-$D exchange, as there was no alteration of the far-UV CD spectra. It is interesting that, with additional heat treatment, the infrared spectral differences between two variants remain, especially in a region ascribed to β-sheet structure. This result suggests that the substitutions at residues 64 and 118 alter not only the conformational mobility but also the hydrogen-bonding strength of the core structure as well.

As noted in the Results, on the basis of the greater degree to which H$-$D exchange altered its infrared spectra, the A variant has greater structural mobility than the B variant. This finding is consistent with the observation of different gelation properties of the A and B variants (Huang et al., 1994). The A variant has a lower gelation temperature (72.3 °C) and a higher initial, gelling rate (15.4 Pa/min) than the B variant (80 °C and 2.9 Pa/min, respectively). These differences were ascribed to the greater conformational mobility of variant A, which may increase the "chance" at any given instant that there will be enough protein molecules in the right conformation to lead to gelation.

Amide I Spectral Changes Induced by H$-$D *Exchange.* It has been shown in earlier infrared studies (Byler & Susi, 1986; Susi & Byler, 1986) that amide I bands of proteins in D$_2$O solution are generally shifted to a lower wavenumber (red-shifted) relative to that seen in H$_2$O solution. This shift is the result of H$-$D exchange at the N$-$H groups of backbone structures. However, it is apparent from the spectra shown in Figure 2 that the amide I band absorbance maxima of BLG shifted about 2 cm^{-1} to a higher wavenumber (blue-shifted) during H$-$D exchange. Results of quantitative analysis of variant B (Table 2) indicate that the observed blue shifts in the amide I bands result from absorbance redistribution between two major β-sheet-related bands, which are located at 1639 and 1628 cm^{-1} in H$_2$O and at 1634 and 1621 cm^{-1} in D$_2$O, respectively. For example, with variant B in H$_2$O solution, the area under the 1628 cm^{-1} band accounts for 28.1% of the total amide I band area, while the area under the 1639 cm^{-1} band accounts for only 18.7%. In D$_2$O solutions, however, the area under 1634 cm^{-1} is greater than the area under 1621 cm^{-1}. Even though both bands were red-shifted about 6 cm^{-1} upon H$-$D exchange, the absorbance redistribution between them has resulted in an overall blue shifting in the overall amide I composite band [cf., Hübner et al. (1990)]. Similar results are seen with variant A (Table 1). Since the amount of β-sheet structure remains unchanged (Table 3) during H$-$D

exchange, the absorbance redistribution is most likely due to a splitting of the 1628 cm^{-1} band in H$_2$O to both the 1632 and 1622 cm^{-1} bands in D$_2$O. The exact reason for this band splitting is not clear. It may result from a partial hydrogen exchange in the antiparallel β-sheet barrel, which in turn causes some topographical changes in the core structure.

Conclusions. Our results demonstrate that H$-$D exchange may not alter the secondary structural composition of protein genetic variants, as documented with CD spectroscopy. However, such exchange can greatly alter the infrared spectra of the proteins in the amide I region. The relative degree of this alteration is dictated by the relative conformational mobility of the variants. Thus, H$-$D exchange can be used to "magnify" protein structural differences that are not readily detectable by simple measures of secondary structural composition. This finding indicates that the combination of infrared spectroscopy and H$-$D exchange has great potential for use in the characterization of conformational changes of proteins induced by naturally occurring point mutations or site-directed mutagenesis.

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