

# Intermolecular $\beta$ -Sheet Results from Trifluoroethanol-Induced Nonnative $\alpha$ -Helical Structure in $\beta$ -Sheet Predominant Proteins: Infrared and Circular Dichroism Spectroscopic Study

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**2,2,2-Trifluoroethanol (TFE)-induced nonnative  $\alpha$ -helical structure in peptides and proteins has been extensively studied with circular dichroism (CD) spectroscopy. However, to date, complementary information from infrared (IR) spectroscopy has not been reported. Using both IR and CD spectroscopy, we demonstrate here that the TFE-induced nonnative  $\alpha$ -helical structure in two  $\beta$ -sheet-predominant proteins,  $\beta$ -lactoglobulin and  $\alpha$ -chymotrypsin, is unstable in comparison with those found in the  $\alpha$ -helix-predominant proteins myoglobin and cytochrome *c* under identical conditions. IR spectra showed that, immediately after dissolution of the  $\beta$ -sheet proteins in 50% (v/v) TFE, a strong amide I band component appears at  $1654\text{ cm}^{-1}$  in  $\text{H}_2\text{O}$  and at  $1650\text{ cm}^{-1}$  in  $\text{D}_2\text{O}$ , which is ascribed to  $\alpha$ -helical structure. However, the intensities of the  $\alpha$ -helical bands decrease as a function of time, concomitant with the appearance of two new band components near  $1620$  and  $1695\text{ cm}^{-1}$  in  $\text{H}_2\text{O}$  and  $1612$  and  $1684\text{ cm}^{-1}$  in  $\text{D}_2\text{O}$ , a typical IR spectral pattern for an intermolecular  $\beta$ -sheet aggregate. Clear gels begin to develop within 30 min. No similar spectral changes were observed for the  $\alpha$ -helical proteins. CD spectra suggested initially that the TFE-induced  $\alpha$ -helix was retained in the gelled state. However, further analysis of the spectra, and Gaussian function modeling with basic spectra, indicated that the apparent  $\alpha$ -helix signal was actually due to a combination of signals from intermolecular  $\beta$ -sheet and residual  $\alpha$ -helix. These results indicate that the TFE-induced nonnative  $\alpha$ -helix structure in predominantly  $\beta$ -sheet proteins**

**is unstable and readily converts to an intermolecular  $\beta$ -sheet aggregate.** © 1998 Academic Press

**Key Words:** trifluoroethanol; Fourier transform infrared; circular dichroism;  $\alpha$ -chymotrypsin;  $\beta$ -lactoglobulin.

2,2,2-Trifluoroethanol (TFE)<sup>2</sup> has been widely used in studies probing the propensity of polypeptides and proteins to form  $\alpha$ -helices (1–3), but only recently has the mechanism of helix induction by TFE come to be understood (4–6). In particular, the ability of TFE to alter the preference of extended  $\beta$ -sheet structure toward  $\alpha$ -helices has been the subject of intense investigation. Several CD spectroscopic studies have shown that predominantly  $\beta$ -sheet proteins, such as  $\beta$ -lactoglobulin, concanavalin A (7, 8), cardiotoxin analog II (9), tendimistat (10), and cellular retinoic acid binding protein (11), exhibit some type of transition from nonhelical structures to a predominantly  $\alpha$ -helical conformation in the presence of relatively high (>20% v/v) concentrations of TFE. However, the relative stability of the TFE-induced  $\alpha$ -helical structure has been largely ignored. One purpose of the current study was to determine if this structure, once formed, would be retained during prolonged exposure of the protein to TFE.

Furthermore, most studies of TFE-induced structural transitions have relied on evidence obtained with CD spectroscopy. In general, it is expected that such results should be corroborated by IR spectra, which also are sensitive to secondary structural changes in proteins and peptides (12). However, to date, no com-

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<sup>2</sup> Abbreviations used: TFE, 2,2,2-trifluoroethanol; FT-IR, Fourier transform infrared.

parison of TFE-induced structural transition in peptides and proteins has been made between CD and IR spectroscopies.

Using both Fourier transform infrared (FT-IR) and CD spectroscopy, we have chosen  $\alpha$ -chymotrypsin and  $\beta$ -lactoglobulin B as models of predominantly  $\beta$ -sheet proteins and myoglobin and cytochrome *c* as models of predominantly  $\alpha$ -helix proteins to study the TFE-induced structural transition and stability of the nonnative state. Using myoglobin and cytochrome *c*, the effects of TFE on native  $\alpha$ -helices can be examined and contrasted to those noted for TFE-induced, nonnative  $\alpha$ -helices.

## MATERIALS AND METHODS

**Materials.**  $\alpha$ -Chymotrypsin (bovine pancreas),  $\beta$ -lactoglobulin B (bovine milk), myoglobin (horse heart), cytochrome *c* (horse heart, type VI), 2,2,2-trifluoroethanol, and deuterium oxide (99.9 atom % D) were purchased from Sigma (St. Louis, MO). Isotopic 2,2,2-trifluoroethanol- $d_3$  is the product of Isotope Cambridge (Cambridge, MA).

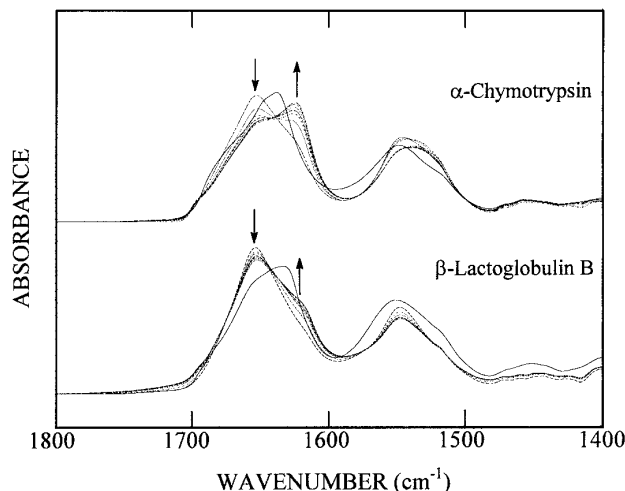
**Sample preparations.** Lyophilized proteins were dissolved directly in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4, at concentrations of 20 and 30 mg/ml. Deuterated protein samples were prepared by dissolving the lyophilized proteins in  $D_2O$  for 24 h and freeze-drying them using a Labconco 4.5 freeze drier. Then the proteins were rehydrated in 50% (v/v) TFE- $d_3$ /10 mM phosphate- $D_2O$ , pH 7.4, at concentration of 2 mg/ml.

**Infrared measurements.** Infrared spectra were measured with a Magna-IR 550 spectrometer (Nicolet) equipped with a dTGS detector. Protein samples were prepared in a liquid IR cell (P/N 20500) with  $CaF_2$  windows and a 6- $\mu m$  spacer for  $H_2O$ -based solution and a 50- $\mu m$  spacer for  $D_2O$ -based solution. For each spectrum, a 256-scan interferogram was collected in a single-beam mode with a 4- $cm^{-1}$  resolution. Reference spectra were recorded under identical scan conditions with only the corresponding solvent in the cell. Sample temperature of IR cell was maintained at 25°C using a custom-built Peltier cell temperature controller. Protein spectra were obtained according to previously established criteria and the double-subtraction procedure (13, 14). The resultant spectra were smoothed with a 7-point Savitsky-Golay smooth function to remove the white noise. Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet).

**Circular dichroism spectroscopy.** The far-UV CD spectra were measured with an Aviv 62DS spectrometer (Lakewood, NJ) equipped with a thermoelectric temperature control unit. Spectra were recorded at 25°C with 1-mm quartz cells for the 0.1 mg/ml samples and 0.01-mm path length cells for the 20 mg/ml samples. The protein concentrations were either 0.1 or 20 mg/ml in 10 mM potassium phosphate, pH 7.4, containing 50% (v/v) TFE. Background spectra were recorded under identical experiment conditions. The difference spectrum was generated by subtracting the background spectrum from the corresponding protein spectrum.

## RESULTS

**Effects of TFE on the infrared spectra of  $\beta$ -sheet proteins.** Figure 1 presents the infrared spectra of  $\alpha$ -chymotrypsin (20 mg/ml) and  $\beta$ -lactoglobulin B (20 mg/ml) in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4, at 20°C as a function of time. The spectra were recorded at 5, 20, 35, 50, 65, 80, and 95 min (ending time) after dissolution in 50% TFE solutions. The spectra

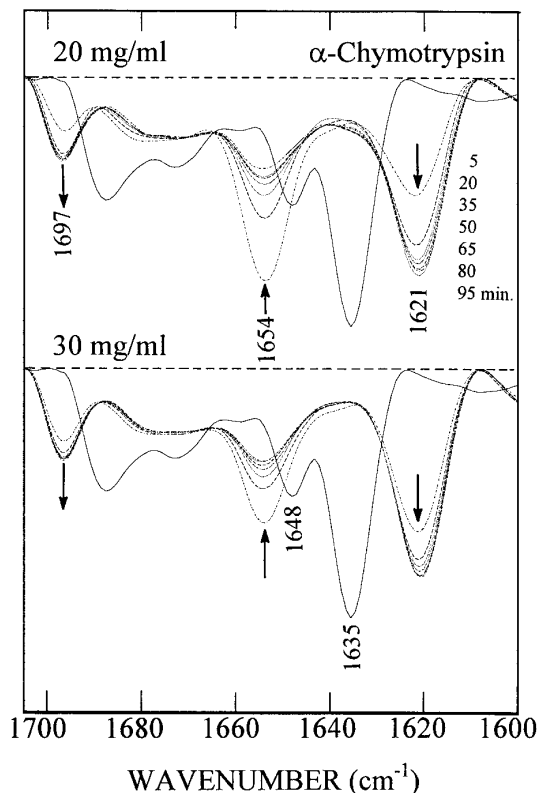


**FIG. 1.** Time-dependent infrared spectral changes of  $\alpha$ -chymotrypsin (20 mg/ml) and  $\beta$ -lactoglobulin B (20 mg/ml) in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4, at 20°C. Spectra were recorded at 5, 20, 35, 50, 65, 80, and 95 min (ending time) after the proteins dissolved in 50% (v/v) TFE. The spectra in the solid lines are the control samples in 10 mM potassium phosphate, pH 7.4. The arrows show the direction of intensity changes as a function of time.

drawn with the solid lines are for samples in 10 mM phosphate without TFE. In the absence of TFE,  $\alpha$ -chymotrypsin and  $\beta$ -lactoglobulin exhibit their amide I absorbance maxima at 1640 and 1631  $cm^{-1}$ , respectively. Both frequencies indicate a predominantly  $\beta$ -sheet structure (13–16). Immediately after the proteins are dissolved in a medium containing 50% (v/v) TFE, the amide I absorbance maxima of the two proteins shift to 1654  $cm^{-1}$ , indicative of formation of  $\alpha$ -helical structure (13–16). This spectral change suggests a massive TFE-induced conformational change from primarily  $\beta$ -sheet structure to a conformation that is predominantly  $\alpha$ -helical. These observations are consistent with numerous other reports of TFE inducing  $\alpha$ -helical structure in polypeptides and other proteins (1, 4, 11, 17–20).

However, the  $\alpha$ -helical structures induced by TFE in these two proteins are apparently unstable, as they convert readily to structures resembling an intermolecular  $\beta$ -sheet aggregate, as evidenced by appearance of bands near 1623 and 1697  $cm^{-1}$ . These bands appear as the band at 1654  $cm^{-1}$  diminishes as a function of time. The appearance of a more intense feature band near 1625  $cm^{-1}$  and a higher energy band near 1690  $cm^{-1}$  is the typical spectral pattern associated with intermolecular antiparallel  $\beta$ -sheet aggregates (21–25). Such spectra are frequently observed in the thermally denatured proteins (21–25).

**Second-derivative spectra.** The TFE-induced conformational transitions in  $\beta$ -sheet proteins can be seen more clearly in the resolution-enhanced second-derivative spectra (13, 14). The progression from the native

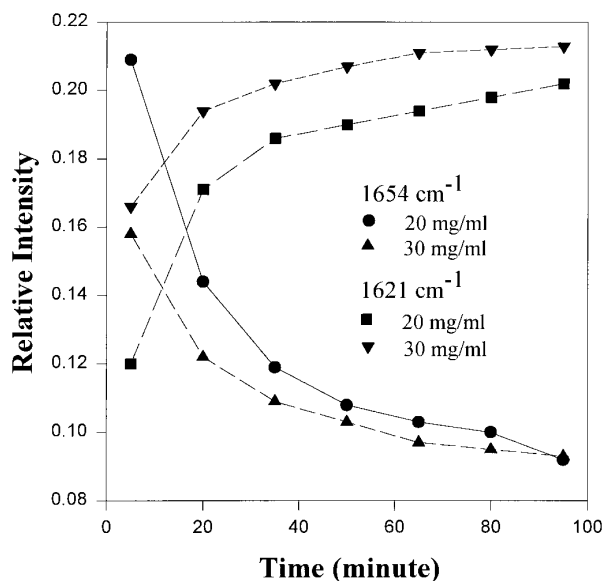


**FIG. 2.** Second-derivative amide I spectra of  $\alpha$ -chymotrypsin in 50% (v/v) TFE/10 mM potassium phosphate at concentrations of 20 and 30 mg/ml. The spectra in the solid lines are the control samples in 10 mM potassium phosphate. Spectra were normalized to equal areas for easier presentation. The arrows represent directions of intensity changes as a function of time.

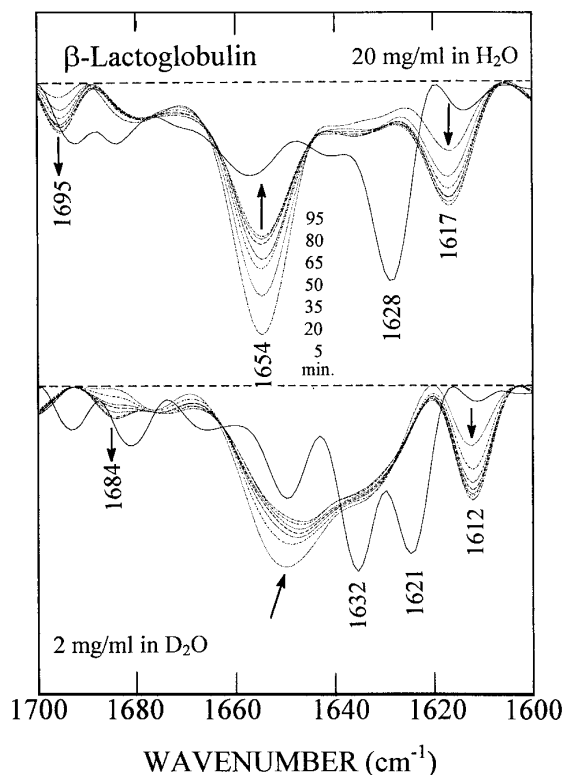
conformation, which is high in intramolecular  $\beta$ -sheet, to a nonnative  $\alpha$ -helical structure, and subsequently to intermolecular  $\beta$ -sheet aggregates, produces many vibrational components that overlap. Use of second-derivative spectroscopy allows these components to be resolved. Figure 2 presents the second derivative of the amide I spectra of  $\alpha$ -chymotrypsin at 20 and 30 mg/ml concentrations in 50% (v/v) TFE as a function of time. All spectra were normalized to equal areas for easier comparison. Again, the spectra in the solid lines are the control samples in 10 mM phosphate buffer without any TFE added. The second-derivative spectrum of  $\alpha$ -chymotrypsin in the absence of TFE exhibits a strong band at 1635  $\text{cm}^{-1}$ , assignable to the native intramolecular  $\beta$ -sheet structure, and a relatively weaker band at 1648  $\text{cm}^{-1}$ , assignable to unordered structure (13, 14, 25). Immediately after dissolution of the protein in 50% (v/v) TFE solution, the spectrum of  $\alpha$ -chymotrypsin displays a strong band at 1654  $\text{cm}^{-1}$ , assignable to  $\alpha$ -helical structure (13–16). In addition, there are two relatively weaker bands at 1621 and 1697  $\text{cm}^{-1}$  assignable to intermolecular antiparallel  $\beta$ -sheet aggregate (21–25). The intensity of the 1654- $\text{cm}^{-1}$  band decreases as a function of time, accompanied by an increase in

the band intensities at 1621 and 1697  $\text{cm}^{-1}$ . The overall pattern of spectral changes suggests a rapid conformational transition induced by TFE from a native intramolecular  $\beta$ -sheet to a largely  $\alpha$ -helical structure. Subsequently, spectra indicative of the formation of intermolecular  $\beta$ -sheet aggregates appear. It should be noted that no attempt was made to measure spectral change less than 5 min after the addition of TFE, due to the time requirements of sample preparation and data collection. Therefore, the exact rates of appearance of the  $\alpha$ -helical band at 1654  $\text{cm}^{-1}$  and the time frame for the first appearance of the intermolecular  $\beta$ -sheet aggregate bands at 1621 and 1697  $\text{cm}^{-1}$  are unknown.

Figure 3 shows a plot of a relative intensity change at 1654 ( $\alpha$ -helix) and 1621  $\text{cm}^{-1}$  (intermolecular  $\beta$ -sheet aggregate) as a function of time. The conformational transition from a native intramolecular  $\beta$ -sheet to an  $\alpha$ -helix occurs immediately after dissolving the protein in 50% TFE solution, whereas the transition from a nonnative  $\alpha$ -helix to an intermolecular antiparallel  $\beta$ -sheet aggregate takes about 90 min to complete. The small amount of intermolecular  $\beta$ -sheet aggregate in the first spectral measurement (5 min after dissolving in TFE) is likely due to rapid conversion of  $\alpha$ -helices to intermolecular antiparallel  $\beta$ -sheet aggregate within the sample manipulation time of the experiment. As expected for a process involving intermolecular interactions, within the 90-min time frame of the experiments, the extent of intermolecular  $\beta$ -sheet formation is greater at higher protein concentrations.



**FIG. 3.** Relative intensity change at 1654 ( $\alpha$ -helix) and 1621  $\text{cm}^{-1}$  (intermolecular  $\beta$ -sheet) as a function of time for the  $\alpha$ -chymotrypsin in 50% (v/v) TFE as shown in Fig. 2.

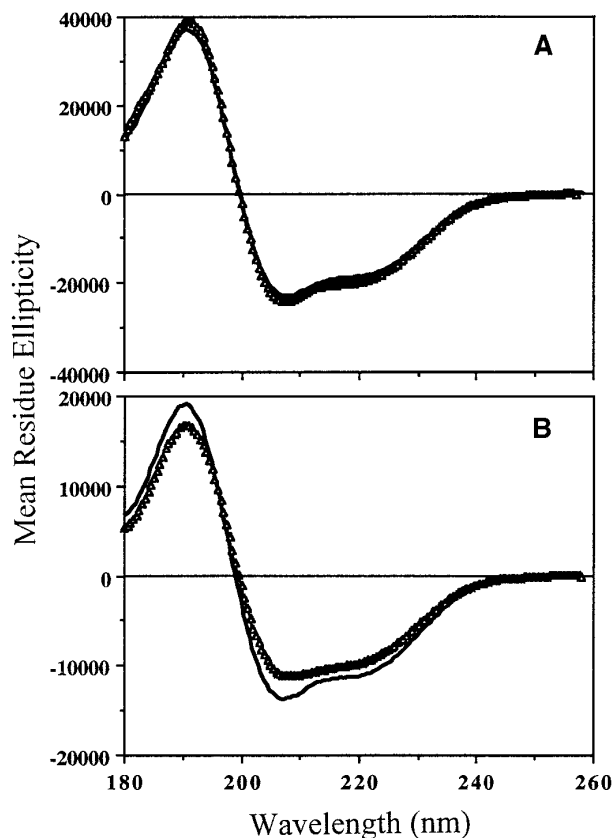


**FIG. 4.** Second-derivative amide I spectra of  $\beta$ -lactoglobulin B in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4. (Upper spectra) 20 mg/ml protein in  $\text{H}_2\text{O}$ -based solution. (Lower spectra) 2 mg/ml protein in  $\text{D}_2\text{O}$ -based solution. The spectra in the solid lines are the control samples in 10 mM potassium phosphate. Spectra were normalized to equal areas for easier presentation. The arrows represent directions of intensity changes as a function of time.

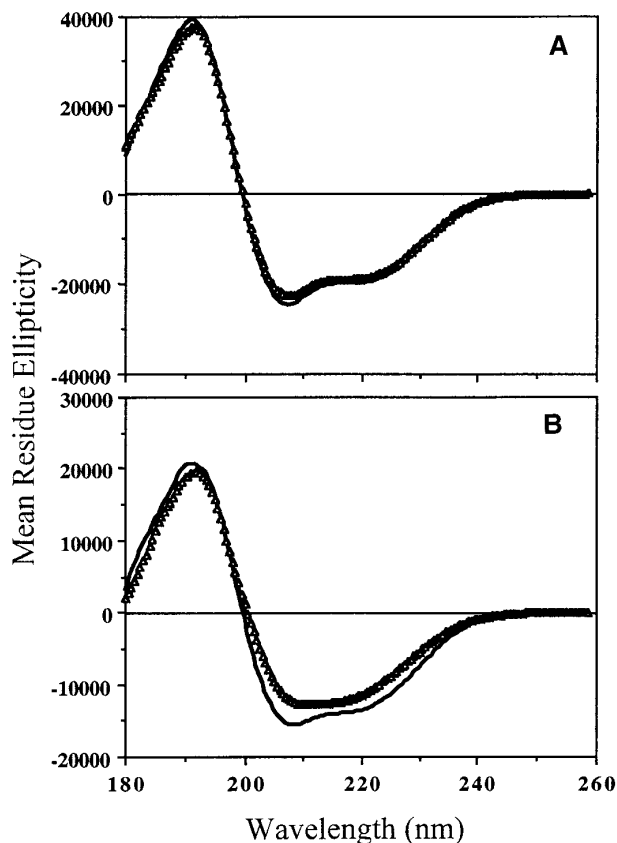
Figure 4 presents the second-derivative amide I spectra of  $\beta$ -lactoglobulin in 50% TFE/ $\text{H}_2\text{O}$  buffer at 20 mg/ml concentration and in 50% TFE- $\text{d}_3/\text{D}_2\text{O}$  buffer at 2 mg/ml concentration. All spectra were normalized to equal areas for easier comparison. Only one major band component ascribed to the native intramolecular  $\beta$ -sheet was observed at  $1628\text{ cm}^{-1}$  in the spectrum of the control sample in  $\text{H}_2\text{O}$  solution, whereas two major band components ascribed to the native intramolecular  $\beta$ -sheet were observed at  $1632$  and  $1621\text{ cm}^{-1}$  in  $\text{D}_2\text{O}$  solution. Upon dissolution of protein in 50% TFE, strong band components ascribed to  $\alpha$ -helix at  $1654\text{ cm}^{-1}$  in  $\text{H}_2\text{O}$  and at  $1650\text{ cm}^{-1}$  in  $\text{D}_2\text{O}$  were observed immediately. Again, the TFE-induced  $\alpha$ -helical band decreased as a function of time, accompanied by an increase in the intensities of bands at  $1617$  and  $1695\text{ cm}^{-1}$  in  $\text{H}_2\text{O}$  and at  $1612$  and  $1684\text{ cm}^{-1}$  in  $\text{D}_2\text{O}$ . Both sets of signals are indicative of the formation of intermolecular  $\beta$ -sheet aggregates in that particular solvent. At a concentration of 20 mg/ml, clear gels developed in both protein samples. However, no visible gelation was observed at the concentration of 2 mg/ml.

**Far-UV CD spectra.** Upon addition of TFE, clear indication of  $\alpha$ -helix formation was observed in the far-UV (175–250 nm) CD spectrum of both  $\alpha$ -chymotrypsin and  $\beta$ -lactoglobulin, either at 0.1 (Fig. 5) or at 20 mg/ml (Fig. 6). This wavelength region is known to reflect the secondary structure composition of the proteins (26). The presence of negative bands at 222 and 208 nm and a positive band below 200 nm is characteristic of  $\alpha$ -helical structures. Upon setting for 3 h, the high-concentration solutions turn viscous and then stiff, indicating gel formation has occurred. At the lower concentration, the  $\alpha$ -chymotrypsin sample becomes significantly more viscous as well, while the  $\beta$ -lactoglobulin sample did not appear to gel, at least within the 3-h time frame.

For both proteins, the CD spectral changes were slight, especially for  $\beta$ -lactoglobulin. Casual inspection would suggest little change had occurred in the secondary structure composition as a function of time, i.e., the  $\alpha$ -helical structure induced by TFE is relatively stable. However, examination of the difference spectra (before and after the 3-h incubation period) provides a clearer indication of the time-dependent structural changes (Fig. 7). At low concentration (0.1 mg/ml),  $\beta$ -lactoglob-



**FIG. 5.** The far-UV CD spectra of  $\beta$ -lactoglobulin B (A) and  $\alpha$ -chymotrypsin (B) in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4, at  $25^\circ\text{C}$  before (—) and after ( $\Delta$ ) a 3-h incubation period. The protein concentration is 0.1 mg/ml and the path length is 1 mm.



**FIG. 6.** The far-UV CD spectra of  $\beta$ -lactoglobulin B (A) and  $\alpha$ -chymotrypsin (B) in 50% (v/v) TFE/10 mM potassium phosphate, pH 7.4, at 25°C before (—) and after ( $\Delta$ ) a 3-h incubation period. The protein concentration is 20 mg/ml and the path length is 0.01 mm.

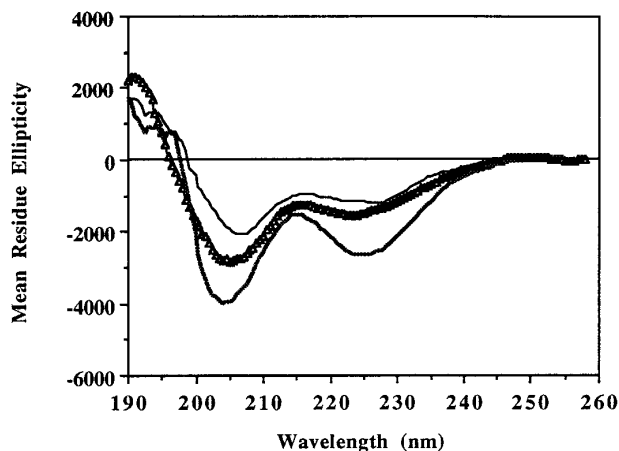
ulin displays very little change in the far-UV CD (Fig. 5A). Even at 20 mg/ml, the changes appear to be insignificant (Fig. 6A). However, the difference spectrum exhibits a broad negative band near 223 nm, a stronger negative band at 207 nm, a crossover wavelength of 199 nm, and positive ellipticity below 199 nm (Fig. 7). Similar CD difference spectra were observed for  $\alpha$ -chymotrypsin at both 0.1 and 20 mg/ml (Fig. 7). The magnitudes were larger for  $\alpha$ -chymotrypsin, resulting from an even greater level of conversion from  $\alpha$ -helix to intermolecular  $\beta$ -sheet. In fact, the TFE-induced  $\alpha$ -helical structure appears to be unstable, even at low  $\alpha$ -chymotrypsin concentration (Fig. 7).

**Infrared analysis of  $\alpha$ -helical proteins.** To see whether the observed TFE-induced conformational transition from an  $\alpha$ -helix to an intermolecular anti-parallel  $\beta$ -sheet aggregate is specifically associated with nonhelical proteins, we examined the infrared spectra of myoglobin and cytochrome *c*, two predominantly  $\alpha$ -helical proteins (>70% of the residues are involved in  $\alpha$ -helices in the native structure of myoglobin) in the presence of 50% TFE. In the absence of TFE, the spectra of myoglobin and cytochrome *c* exhibit strong band components ascribed to  $\alpha$ -helix at 1654

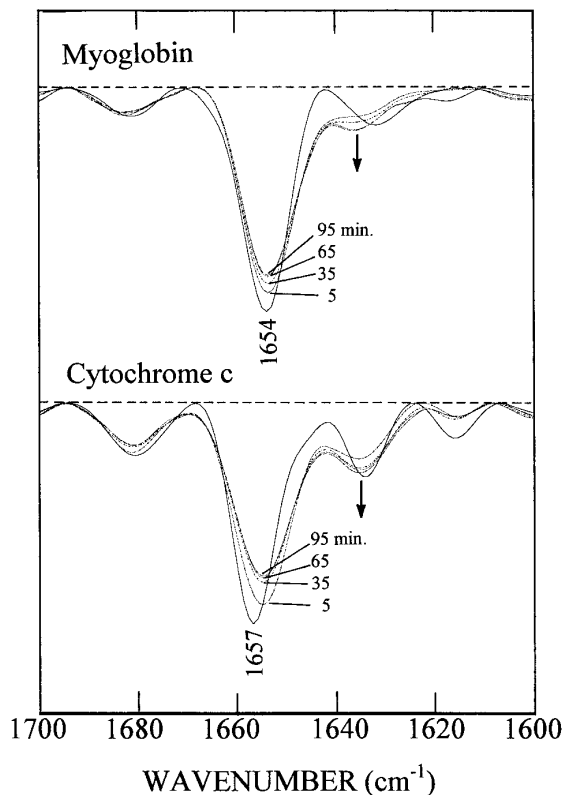
and 1657  $\text{cm}^{-1}$ , respectively (Fig. 8). In the presence of TFE, the frequency of the  $\alpha$ -helical band remains unchanged at 1654  $\text{cm}^{-1}$  for myoglobin, while it shifts about 3  $\text{cm}^{-1}$  from 1657 to 1654  $\text{cm}^{-1}$  for cytochrome *c*. In the spectrum for myoglobin, a relatively small intensity decrease at the 1654  $\text{cm}^{-1}$  band was observed as a function of time, accompanied by a small intensity increase near 1637  $\text{cm}^{-1}$ . This band seems to correspond to the band at 1635  $\text{cm}^{-1}$  observed in the control sample without TFE. No amide I band components associated with intermolecular  $\beta$ -sheet aggregates were detected, and no visible gelation occurred in the solutions of the two  $\alpha$ -helical proteins, even though the protein concentrations were 20 mg/ml.

## DISCUSSION

**CD spectra of the conversion of  $\alpha$ -helices to intermolecular  $\beta$ -sheet structures.** Despite numerous studies on TFE-induced conformational changes in the literature, the structural stability of the nonnative  $\alpha$ -helical structure in peptides and proteins has rarely been reported, even for all  $\beta$ -sheet proteins (7, 8, 27). At least in part, this may be due to the difficulties in recognizing the structural transition from an  $\alpha$ -helix to an intermolecular  $\beta$ -sheet structure by CD spectroscopy, as the spectral changes are not dramatic, especially at low protein concentrations. This may be surprising, as it is usually believed that the signal from an  $\alpha$ -helix is much more intense than that from a  $\beta$ -sheet (10, 26). However, it has been shown that the CD spectra of thermally induced protein gels, composed almost exclusively of intermolecular  $\beta$ -sheets, are relatively intense ( $[\theta]_{217} \sim -15,000^\circ \text{ cm}^2 \text{ dmol}^{-1}$ ) (28). Furthermore, the intensity of a  $\beta$ -sheet CD spectrum can increase dramatically, particularly if the sheet is highly twisted (29). Finally, there may be partial conversion of



**FIG. 7.** The far-UV CD difference spectra for  $\alpha$ -chymotrypsin (0.1 mg/ml,  $\Delta$ ; 20 mg/ml,  $\cdots$ ) and  $\beta$ -lactoglobulin B (20 mg/ml, —) for spectra taken at 0 and 3 h.



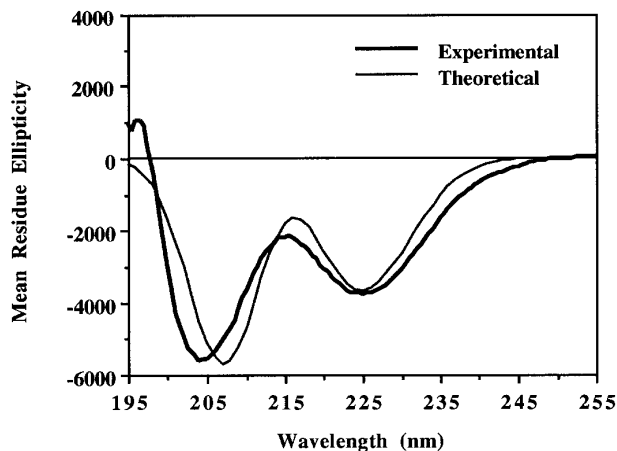
**FIG. 8.** Second-derivative amide I spectra of myoglobin (20 mg/ml) and cytochrome *c* (20 mg/ml) in 50% (v/v) TFE/10 mM potassium phosphate. (Upper spectra) Myoglobin. (Lower spectra) Cytochrome *c*. Spectra were normalized to equal areas for easier presentation. The arrows represent directions of intensity changes as a function of time.

the  $\alpha$ -helical structure to conformations other than  $\beta$ -sheet (e.g., random coil or  $\beta$ -turns), resulting in nearly equal cancellation. The conversion can be more clearly observed using difference spectra.

The difference spectra for both  $\beta$ -lactoglobulin and  $\alpha$ -chymotrypsin indicate that  $\alpha$ -helical structure is being lost upon gelation; hence, the overall pattern of negative bands at 223 and 207 nm. However, the difference in intensities of the two negative bands suggests that another structure is increasing at the same time, such as the negative band near 217 nm associated with  $\beta$ -sheet structures. This effect can be modeled using Gaussian functions to approximate the negative bands of an  $\alpha$ -helix and a  $\beta$ -sheet. Using a combination of these bands, a spectrum very similar to the experimental difference spectra can be produced (Fig. 9). These results are consistent with the  $\alpha$ -helical structure induced by TFE being lost as a function of time. Over the course of hours, the samples form a gel and the  $\alpha$ -helical signal is replaced by the strong  $\beta$ -sheet signal arising from intermolecular contacts. These two sets of signals cancel each other in large part, resulting in a CD spectrum that exhibits only minor changes in the far-UV CD region.

The spectral changes are significantly stronger for  $\alpha$ -chymotrypsin, being seen at concentrations as low as 0.1 mg/ml. For  $\beta$ -lactoglobulin, the effect can only be observed clearly at high protein concentrations, but the overall effect is the same: gel formation produces a far-UV CD signal indicative of  $\beta$ -sheet formation with loss of the TFE-induced  $\alpha$ -helix. Whether  $\beta$ -lactoglobulin does not gel at low concentrations or whether the kinetics for conversion to the more stable intermolecular  $\beta$ -sheet structure are too slow to be observed within 3 h is not known. Formation of a transparent gel coinciding with intermolecular  $\beta$ -sheet formation has been observed by CD in  $\beta$ -lactoglobulin gels formed by thermal stress (28). However, in that system, there is not  $\alpha$ -helical intermediate, as the protein undergoes a structural transition from its native conformation to a nearly complete intermolecular  $\beta$ -sheet conformation. Although structural transitions noted with TFE- and thermally induced perturbations are different, it appears that the structures of the final gels may be quite similar.

*Instability of TFE-induced  $\alpha$ -helices in predominantly  $\beta$ -sheet proteins.* At first glance, our results confirm the observations of previous studies using CD spectroscopy that nonnative  $\alpha$ -helices can be induced in predominantly  $\beta$ -sheet proteins by TFE (7–9, 11). Both FT-IR and CD spectra reveal typical spectral changes from a  $\beta$ -sheet to an  $\alpha$ -helix as  $\beta$ -lactoglobulin and  $\alpha$ -chymotrypsin dissolved in 50% TFE solution. However, our results have also shown that the nonnative  $\alpha$ -helical structure is unstable and converts readily to an intermolecular antiparallel  $\beta$ -sheet aggregate. The structural transition from  $\alpha$ -helix to intermolecular  $\beta$ -sheet is a gradual and time-dependent process. It was observed at low protein concentration (0.1–2 mg/ml) as well as at high protein concentrations



**FIG. 9.** Theoretical far-UV CD difference spectrum based on Gaussian functions for the negative CD bands of a putative  $\alpha$ -helix and  $\beta$ -sheet. The experimental difference spectrum of  $\alpha$ -chymotrypsin at 20 mg/ml (thick line) is shown for comparison.

(20 mg/ml). In contrast, native  $\alpha$ -helix in myoglobin and cytochrome *c* are not dramatically altered in the presence of TFE.

It is well known that the folding pattern of polypeptides may differ significantly with changes in solvent environments. For example, poly-L-lysine exists as a random coil at neutral pH, an  $\alpha$ -helix at alkaline pH, and an antiparallel  $\beta$ -sheet at moderately elevated temperature (30). The folding patterns of poly-L-lysine under a given condition can be further altered by the addition of short-chain alcohols. A biphasic conformational transition was observed when alcohols were added to the  $\alpha$ -helical poly-L-lysine: an  $\alpha$ -helix to  $\beta$ -sheet transformation at low alcohol concentrations and a reverse  $\beta$ -sheet to  $\alpha$ -helix transformation at higher alcohol concentrations (31). The change in folding patterns of poly-L-lysine only involves changes in the solvent environment of the polymer.

The bulk dielectric constant for pure TFE is about one-third that of the value for water, and the dielectric constant for TFE-water mixture is a linear function of the mole fraction of TFE (32, 33). Nelson and Kallenbach (4) suggested that the weaker dielectric constant of TFE reduces the hydrogen bonding between amide protons and surrounding solvent molecules and promotes the intramolecular hydrogen bonding in peptides and therefore stabilizes the secondary structure of peptides. Cammers-Goodwin *et al.* (6) suggest that  $\alpha$ -helices are most often formed in the presence of TFE because they represent compact conformations that maximize intermolecular hydrogen bonding and minimize solvent exposure. Clearly, intermolecular  $\beta$ -sheet structures, as those formed in protein gels, can accomplish the same outcome.

At least for  $\beta$ -lactoglobulin and  $\alpha$ -chymotrypsin under the conditions used in the current study, the solvent-induced transitions to nonnative conformation apparently lead to structures with a high propensity for association and aggregation. Aggregates with a large content of intermolecular  $\beta$ -sheet seem to be the thermodynamically favored states for these  $\beta$ -sheet proteins in the presence of high concentrations of TFE.

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#### REFERENCES

1. Jasanoff, A., and Fersht, A. R. (1994) *Biochemistry* **33**, 2129–2135.
2. Munoz, V., and Serrano, L. (1994) *Nat. Struct. Biol.* **1**, 399–409.
3. Agou, F., Yang, Y., Gesquiere, J.-C., Waller, J.-P., and Guittet, E. (1995) *Biochemistry* **34**, 569–576.
4. Nelson, J. W., and Kallenbach, N. R. (1986) *Proteins Struct. Funct. Genet.* **1**, 211–217.
5. Rohl, C. A., Chakrabarty, A., and Baldwin, R. L. (1996) *Protein Sci.* **5**, 2623–2637.
6. Cammers-Goodwin, A., Allen, T. J., Oslick, S. L., McClure, K. F., Lee, J. H., and Kemp, D. S. (1996) *J. Am. Chem. Soc.* **118**, 3082–2090.
7. Shiraki, K., Nishikawa, K., and Goto, Y. (1995) *J. Mol. Biol.* **245**, 180–194.
8. Hamada, D., Segawa, S.-I., and Goto, Y. (1996) *Nat. Struct. Biol.* **3**, 868–873.
9. Jayaraman, G., Kumar, T. K. S., Arunkumar, A. I., and Yu, C. (1996) *Biochem. Biophys. Res. Commun.* **222**, 33–37.
10. Schönbrunner, N., Wey, J., Engels, J., Georg, H., and Kiefhaber, T. (1996) *J. Mol. Biol.* **260**, 432–445.
11. Liu, Z.-P., Rizo, J., and Gierasch, L. M. (1994) *Biochemistry* **33**, 134–142.
12. Krimm, S., and Bandekar, J. (1986) *Adv. Protein Chem.* **38**, 181–364.
13. Dong, A., and Caughey, W. S. (1994) *Methods Enzymol.* **232**, 139–175.
14. Dong, A., Huang, P., and Caughey, W. S. (1990) *Biochemistry* **29**, 3303–3308.
15. Susi, H., and Byler, D. M. (1986) *Methods Enzymol.* **130**, 290–311.
16. Byler, D. M., and Susi, H. (1986) *Biopolymers* **25**, 469–487.
17. Doty, P., Holtzer, A. M., Bradbury, J. H., and Blout, E. R. (1954) *J. Am. Chem. Soc.* **76**, 4493–4494.
18. Zimm, B. H., and Bragg, J. R. (1959) *J. Chem. Phys.* **31**, 526–535.
19. Liebes, L. F., Zand, R., and Phillips, W. D. (1975) *Biochim. Biophys. Acta* **405**, 27–39.
20. Lehrman, S. R., Tuls, J. L., and Lund, M. (1990) *Biochemistry* **29**, 5590–5596.
21. Clark, A. H., Saunderson, D. P. H., and Suggett, A. (1981) *Int. J. Pept. Protein Res.* **17**, 353–364.
22. Casal, H. L., Köhler, U., and Mantsch, H. H. (1988) *Biochim. Biophys. Acta* **957**, 11–20.
23. Byler, D. M., and Purcell, J. M. (1989) *SPIE Fourier Transform Spectrosc.* **1145**, 415–417.
24. Ismail, A. A., Mantsch, H. H., and Wong, P. T. T. (1992) *Biochim. Biophys. Acta* **1121**, 183–188.
25. Dong, A., Prestrelski, S. J., Allison, S. D., and Carpenter, J. F. (1995) *J. Pharm. Sci.* **84**, 415–424.
26. Towell, J. F., III, and Manning, M. C. (1986) in *Analytical Applications of Circular Dichroism* (Purdie, N., and Brittain, H. G., Eds.), pp. 175–205, Elsevier, Amsterdam.
27. Sivaraman, T., Kumar, T. K. S., and Yu, C. (1996) *Int. J. Biol. Macromol.* **19**, 235–239.
28. Matsuura, J. E., and Manning, M. C. (1994) *J. Agric. Food Chem.* **42**, 1650–1656.
29. Manning, M. C., Illangasekare, M., and Woody, R. W. (1988) *Biophys. Chem.* **31**, 77–86.
30. Davidson, B., Tooney, N., and Fasman, G. D. (1966) *Biochem. Biophys. Res. Commun.* **23**, 156–162.
31. Shibata, A., Yamamoto, M., Yamashita, T., Chiou, J.-S., Kamaya, H., and Ueda, I. (1992) *Biochemistry* **31**, 5728–5733.
32. Murto, J., and Heino, E.-L. (1966) *Suom. Kemistil.* **B39**, 263–266.
33. Llinás, M., and Klein, M. P. (1975) *J. Am. Chem. Soc.* **97**, 4731–4737.