

## Two-Phase Induction of the Nonnative $\alpha$ -Helical Form of $\beta$ -Lactoglobulin in the Presence of Trifluoroethanol

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**ABSTRACT** The trifluoroethanol-dependent induction of the nonnative  $\alpha$ -helical form of  $\beta$ -lactoglobulin has been studied by circular dichroism spectroscopy. Data analysis is performed by factor analysis and multivariate curve resolution. An intermediate form in the induction of the  $\alpha$ -helical form of the  $\beta$ -lactoglobulin has been identified at low TFE concentration. By application of an alternating least-squares algorithm, the CD spectrum corresponding to the intermediate form has been resolved. The deconvolution of this CD spectrum shows a secondary structure content more in agreement with the one predicted from the amino acid sequence than the secondary structure of the helical form obtained at higher TFE concentrations. The additional  $\alpha$ -helical content of the form present at higher TFE concentrations could be due to nonspecific interaction of TFE with the polypeptide chain.

### INTRODUCTION

The folding of a polypeptide chain *in vitro* into a native, biologically active conformation is apparently a self-assembly process (Anfinsen, 1973). The short period of time in which folding occurs implies that this process is not a random search of all possible conformations but occurs along a defined pathway with structured intermediates (Creighton, 1985). In the last years, several of these intermediate states have been characterized (Baldwin, 1993; Jennings and Wright, 1993; Dobson et al., 1994; Katoaka et al., 1993; Nishii et al., 1994). These studies have indicated that a compact denatured state keeping the greater part of the secondary structure, but without ordered tertiary interactions, is a common intermediate for a important number of protein folding pathways. This intermediate has been termed the *molten globule state* because of the preservation of the globular shape and the large flexibility of the side chains (Dolgikh et al., 1981; Ohgushi and Wada, 1983; Kuwajima, 1989; Ptitsyn, 1992).

In light of the existence of an intermediate with these characteristics, a hierarchical model has been proposed (Ptitsyn, 1987; Kim and Baldwin, 1990). In this model, the protein acquires significant native-like secondary structure before the formation of the native tertiary structure.

The hierarchical model is supported by an important body of experimental works (Peng and Kim, 1994; Nelson and Kallenbach, 1986; Segawa et al., 1991; Dyson and Wright, 1993). Nevertheless, most of these folding studies have been performed on all- $\alpha$  or  $\alpha/\beta$  proteins. Folding studies of predominantly  $\beta$ -structured proteins are not so abundant (Carlsson and Jonsson, 1995). Some of these studies confirm the hierarchical model for  $\beta$ -sheet proteins (Blanco et

al., 1994; Dill et al., 1993). However, other studies point out the possibility that nonnative  $\alpha$ -helix intermediates could be accumulated in the folding pathway of predominantly  $\beta$ -sheet proteins, such as  $\beta$ -lactoglobulin (Shiraki et al., 1995; Hamada et al., 1996), the cellular retinoic acid binding protein (Liu et al., 1994), or the FK506 binding protein (Logan et al., 1994). The secondary structure predictions indicate that  $\beta$ -lactoglobulin has a surprisingly high  $\alpha$ -helical preference (Nishikawa and Noguchi, 1991). Moreover, in the cases of cellular retinoic acid binding protein and  $\beta$ -lactoglobulin, trifluoroethanol (TFE) induces a transformation of the predominantly  $\beta$ -structure to a stable form with a high content of  $\alpha$ -helix (Shiraki et al., 1995; Liu et al., 1994). From correlation between the  $\alpha$ -helical form in the presence of TFE and the helical content predicted by the amino acid sequence, some authors have suggested that an  $\alpha$ -helical intermediate can be accumulated during the folding process of  $\beta$ -lactoglobulin and that the hierarchical model is not necessarily true for some  $\beta$ -sheet protein (Shiraki et al., 1995).

The mechanism of the TFE-induced stabilization of the  $\alpha$ -helix form (Rajan and Balaram, 1996) is crucial to discriminating whether the acquisition of the native structure of  $\beta$ -lactoglobulin from the denatured state can be explained within the framework of the hierarchical model. On the basis of the studies using a two-dimensional lattice model (Thomas and Dill, 1993), the effects of TFE can be explained mainly as weakening of the nonlocal hydrophobic interaction and slight enhancement of local helical interactions. Nonetheless, recently the nonspecific induction of  $\alpha$ -helix by TFE in the case of an all- $\beta$ -sheet protein, for which secondary structure predictions methods show no propensity to exist in a helical conformation, has been reported (Jayaraman et al., 1996). This result questions the validity of the nonhierarchical model of protein folding exposed above. The understanding of the structural changes that occurs in the TFE-induced transition from the native  $\beta$ -sheet form to the  $\alpha$ -helical form may be useful in dis-

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criminating whether this putative intermediate is the more stable conformation of the protein when the nonlocal interactions are not predominant or, on the contrary, is a denatured form due to the nonspecific interaction of the TFE with polypeptide chain.

The characterization of the  $\alpha$ -helical form of  $\beta$ -lactoglobulin has been performed mainly by circular dichroism (CD) spectroscopy. However, despite the amount of information that the CD spectra contain, structural changes are monitored by using the intensity of the band at 222 nm. These approaches involve an a priori assumption that the helical content is the only structural change during the process. In this work, the transition from the native  $\beta$ -sheet form to the  $\alpha$ -helical form is monitored using the complete far-UV CD spectra obtained at different proportions of TFE. This study is performed by an approach based on the use of a family of computational and statistical techniques related to the detection of variance sources in an experimental data set, without any a priori assumption about the contribution of the different components. Among these, factor analysis (FA) techniques play a key role (Malinowski, 1991). Within FA, a recently developed multivariate curve resolution (MCR) method (Tauler et al., 1995) has been shown to be a powerful method for the study of the conformational changes in synthetic polynucleotides induced by the pH and/or ion complexation using spectrophotometric techniques (De Juan et al., 1997). Recently we have studied the possibilities of this approach in the monitoring of protein structural changes from a theoretical point of view (Mendieta et al., 1998). This work presents the application of the method to an experimental system.

## EXPERIMENTAL PROCEDURES

### Chemicals

Bovine  $\beta$ -globulin A was purchased from Sigma Chemical Co. and was used without further purification. 2,2,2-Trifluoroethanol was obtained from Aldrich.

### Circular dichroism

CD spectra were obtained using a Jasco spectropolarimeter (model 720) controlled by a personal computer implemented with the spectra acquisition software j700. Solutions of 0.1 mg/ml of protein in 20 mM HCl (pH 2) with different proportions of TFE were scanned at room temperature. A cell of 2-mm lightpath was used. Each CD spectrum was the mean of five accumulations for each run. The results were expressed as mean residue ellipticity  $[\theta]$ .

### Data treatment

CD spectra were smoothed by the Savitsky-Golay method and arranged in a data matrix of ellipticities  $\mathbf{D}$  ( $\mathbf{nR}$ ,  $\mathbf{nC}$ ), with as many  $\mathbf{nR}$  rows as number of CD spectra that were recorded at each TFE proportion, and as many  $\mathbf{nC}$  columns as wavelengths that were scanned during each spectrum.

The more probable number of components is investigated simply by inspection of the magnitude of singular values of matrix  $\mathbf{D}$  (Singular Values Decomposition, Malinowski, 1991; Henry and Hofrichter, 1992). An initial estimation of the evolution of the components is obtained by

evolving factor analysis (EFA) (Gampp et al., 1986). From this estimation, a constrained alternating least-squares (ALS) multivariate curve resolution optimization algorithm (Tauler et al., 1995) is started to try to recover the correct set of concentration profiles and individual spectroscopic responses. This recovery is based on the assumption that the instrumental responses of the chemical contributions are bilinear, i.e., they can be expressed in a matrix equation like

$$\mathbf{D} = \mathbf{CS}^T + \mathbf{E}$$

where  $\mathbf{C}$  is a matrix describing change in the chemical contributions (e.g., species distribution) with the number of rows equal to the number of experimental measured CD spectra, and the number of columns equal to the number of proposed chemical contributions.  $\mathbf{S}^T$  is the matrix of the pure individual spectroscopic contributions, with the number of rows equal to the number of proposed chemical contributions and with the number of columns equal to the number of scanned wavelengths, and  $\mathbf{E}$  is a residual matrix containing the variance not explained by  $\mathbf{C}$  and  $\mathbf{S}^T$ . The matrix decomposition equation described above is not unique. There are an infinite number of possible decompositions that are equivalent from a mathematical point of view. To overcome this difficulty, an appropriate set of constraints might be used. Conditions under which the decomposition is unique have been described previously. In particular, the so-called rotational ambiguities of curve resolution can be totally solved under especial local rank and/or selectivity conditions (Tauler et al., 1995; Manne 1995).

The ALS optimization procedure is started by solving iteratively the equation previously given from an initial estimation of the concentration profiles derived from evolving factor analysis (Gampp et al., 1986) and constraining at each stage of the iterative optimization the concentration profiles to be nonnegative. Other constraints implemented during the ALS optimization (Tauler et al., 1995) are the closure (sum of the concentration of all forms at different TFE proportions is equal to the total amount of protein) and the selectivity (at some TFE proportions only one form prevails). The selectivity constraint is very useful in this case, because the native conformation is, by definition, the form present at physiological conditions. Moreover, the requirement of no modifications in the CD spectra beyond the 30% of TFE (even at TFE concentrations of 80%) allows us to suppose that at high TFE proportions the only structure present in solution is the nonnative helical form. Additional confirmation about the presence of only one form in this region of the experimental data is obtained from the evolving factor analysis (see Results). This iterative procedure is carried out until the recovered profiles ( $\mathbf{C}$  and  $\mathbf{S}^T$ ) and the experimental data fitting do not improve. Details about the implementation of this method have been described elsewhere, and it has been applied to different types of chemical data (De Juan et al., 1997; Mendieta et al., 1998).

The content of secondary structure was evaluated by least-squares deconvolution, using as a basis spectra corresponding to  $\alpha$ -helix,  $\beta$ -sheet, and random coil, described by Chen et al. (1974). These spectra have been deduced from the analysis of five representative proteins. Even if  $\beta$ -turns are not taken into account and the contributions of aromatics and other groups are ignored, a reasonable estimation of the  $\alpha$ -helix, random coil, and, in lower extension,  $\beta$ -structures is achieved (Greenfield, 1996).

## RESULTS

### Circular dichroism spectroscopy

Fig. 1 shows the far-UV CD spectra of  $\beta$ -lactoglobulin A in the presence of different concentrations of TFE at pH 2. In the absence of TFE the CD spectrum presents a minimum close to 213 nm, which is consistent with the high  $\beta$ -sheet content of the  $\beta$ -lactoglobulin. The addition of TFE does not change the spectra until the concentration reaches 12% (v/v). Then the spectra change dramatically to a typical  $\alpha$ -helix spectrum, with two minima at 208 nm and 222 nm.

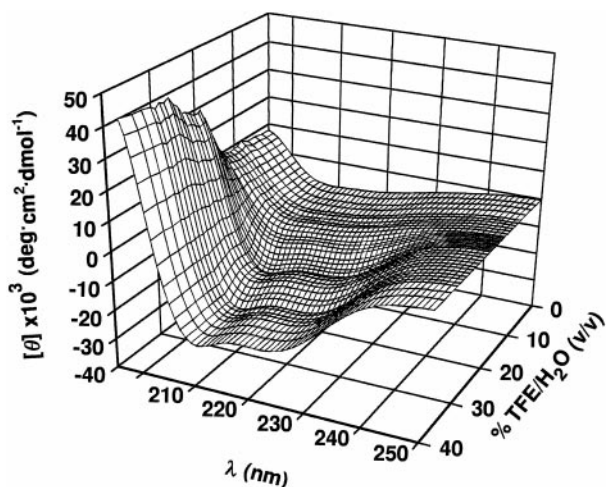


FIGURE 1 Evolution of the far-UV CD spectra of  $\beta$ -lactoglobulin A in the presence of various concentrations of TFE at pH 2. (The mesh was plotted from the experimental data matrix after smoothing by Savitsky-Golay methods.)

Fig. 2 shows the evolution of the ellipticity at 222 nm versus the concentration of TFE. The more important change in  $[\theta]_{222}$  occurs between 12% and 18% TFE (v/v), but between 20% and 28% TFE, an increase in the ellipticity at 222 nm can be observed. An additional increase in TFE concentration up to 80% does not cause changes in the CD spectra (data not shown). It is possible to observe a similar behavior in the data presented by Hamada et al. (1996), but in our data these additional changes are clearer because of the larger number of spectra recorded in our case at these TFE concentrations. This result suggests a two-phase process in the acquisition of the  $\alpha$ -helical content of the  $\beta$ -lactoglobulin in the presence of TFE. However, the use of a single wavelength to monitor the structural change (univariate analysis) does not allow us to obtain information about changes in other structures different from the  $\alpha$ -helix. For example, it is not possible to discriminate whether the  $\beta$ -sheet-to- $\alpha$ -helix transition occurs in two phases, or on the

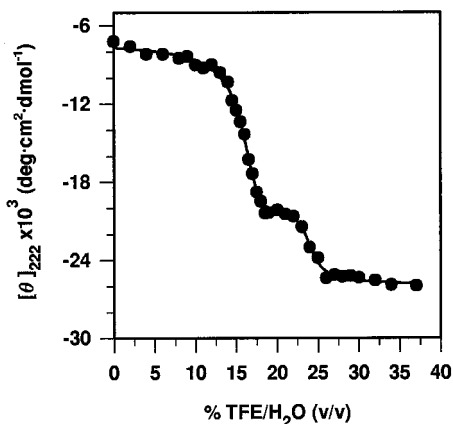


FIGURE 2 Dependence on TFE concentration of the ellipticity at 222 nm of  $\beta$ -lactoglobulin A at pH 2.

contrary, a part of the  $\beta$ -sheet structure is previously disorganized to random coil during the first phase and a random coil-to- $\alpha$ -helix transition occurs during the second phase of the process.

### Factor analysis of the experimental data

With the purpose of obtaining all of the structural information that the changes in the CD spectra contain, factor analysis techniques were applied to the study of the process. CD data were arranged in a data matrix form (see Experimental Procedures). The study of the mathematical structure of the data matrix yields a dynamic picture of the changes in the CD spectra with increasing concentration of TFE (Malinowski, 1991; Henry and Hofrichter, 1992). Singular values decomposition allowed us to discriminate whether the data can be explained by a linear combination of two CD spectra (corresponding to the native-like acid form and the nonnative  $\alpha$ -helical form) or if more components are necessary to explain the process. Fig. 3 a shows the singular values of the data matrix as a function of the number of considered components. Three singular values are higher than the singular values associated with the background noise level. No clear conclusions about the numbers of significant factors (2 or 3) can be inferred from this plot. However, the logarithmic plot (Fig. 3 b) shows that this third singular value is a significant component of the data matrix that is larger than those associated with noise at the bottom.

To rule out the possibility that the third singular value could be due to the presence of only one very divergent spectrum, the experimental data matrix was also studied by evolving factor analysis techniques (Gampp et al., 1986). This approach provides an estimation of the regions where the concentrations of the different components are changing. The method is based on the evaluation of the magnitude of the singular values associated with all of the submatrices built up by successively adding one by one all of the rows

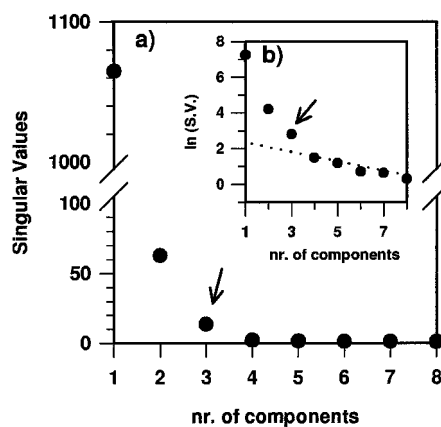


FIGURE 3 Singular values of the experimental CD data matrix obtained for  $\beta$ -lactoglobulin A in the presence of various concentrations of TFE at pH 2. (a) Linear plot. (b) Logarithmic plot

of the original data matrix (i.e., each spectrum was obtained at a different TFE concentration). The calculations are performed in two directions, forward (in the same direction of the experiment) and backward (in the opposite direction of the experiment). Singular values related with significant components become larger and were clearly distinguished from the singular values associated with noise. Fig. 4 shows the forward and backward EFA corresponding to the experimental data matrix. Singular values related to the noise are at the bottom of the plot. When the analysis is performed in the forward direction, three lines emerge (*lines 1, 2, and 3* in Fig. 4). The first component (*line 1*) is present from the beginning of the experiment, but components 2 and 3 appear at just the same TFE concentration at which the changes in the  $[\theta]_{222}$  are detected (see Fig. 2). A similar picture is obtained when the disappearance of the components is studied (backward analysis). In this case, it is more evident that there is a gradual disappearance of the component 2' in relation to the component 3'. The backward analysis also provides a picture of the mathematical structure of the data matrix region corresponding to values of TFE concentration between 30% and 40%. The submatrix built with the spectra obtained in this range of concentrations can be explained with only one component. This result is an additional support for the application of the selectivity constraint in this region, which makes the multivariate resolution of the whole experimental data matrix easier.

These results confirm that three principal components are associated with the changes that occur during the transition from the native-like form to the  $\alpha$ -helical form of the  $\beta$ -lactoglobulin.

### Multivariate curve resolution

The analysis of the experimental data has shown that changes in the CD spectra of the  $\beta$ -lactoglobulin induced by

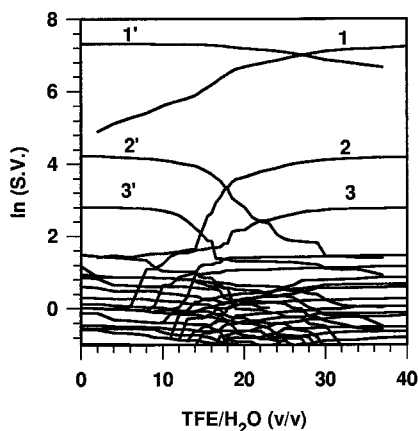


FIGURE 4 Evolving factor analysis of the experimental data matrix. Lines 1, 2, and 3 correspond to the three principal singular values obtained in the forward analysis. Lines 1', 2', and 3' correspond to the three principal singular values obtained in the backward analysis (see the text for further explanations).

TFE cannot be explained as a simple  $\beta$ -sheet-to- $\alpha$ -helix transition. The number of components detected suggests that a third component distinct from the native-like acid form (present at low TFE concentrations) and the nonnative  $\alpha$ -helical form (present at high TFE concentrations) is necessary to explain the process. Therefore, our goal is to obtain the spectrum corresponding to this intermediate form.

An abstract estimation of the concentration profiles corresponding to the three components can be obtained from EFA (Gampp et al., 1986). From this initial estimation of the concentration profiles, the best least-squares estimation of the CD spectroscopic contributions can be estimated. However, this least-squares solution is a purely mathematical solution that will not be optimum from a chemical point of view, as stated previously in the data treatment.

The following constraints have been used to achieved the resolution of the experimental data: 1) nonnegativity of the concentrations; 2) closure (the sum of the concentrations of the three components present at each TFE proportion is equal to 1); and 3) selectivity (in this case, the spectrum obtained in the absence of TFE has been considered to be the corresponding spectrum of the native-like acid form, and the spectrum obtained at 40% of TFE has been considered to be the corresponding spectrum of the nonnative  $\alpha$ -helical form).

Fig. 5 shows the spectra (Fig. 5 a) and the concentration profiles (Fig. 5 b) corresponding to the three components (I, II, III) obtained after the application of the ALS optimization algorithm with the constraints described above. The

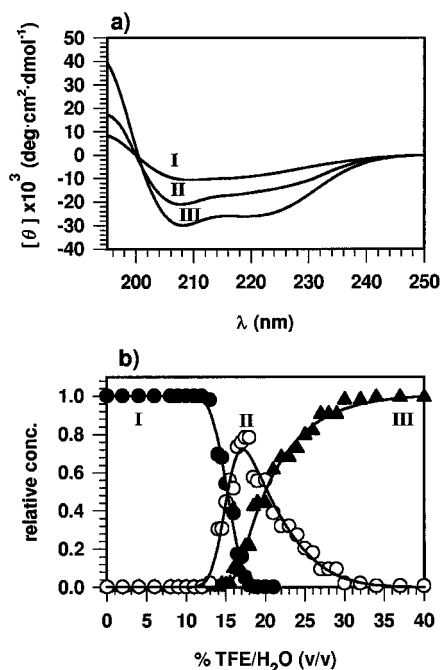


FIGURE 5 Alternating least-squares optimization of the experimental data with nonnegativity, closure, and selectivity constraints. (a and b) Individual spectra (a) and the concentration profiles (b) corresponding to the three components (I, II, and III) detected.

percentage of lack of fit (unexplained variance) corresponding to the resolution of the system in these conditions was only 3.714%. As expected, because of the selectivity constraint, spectra I and III (Fig. 5 a) are easily recognized as corresponding, respectively, to the spectra of the native-like acid form and the nonnative  $\alpha$ -helical form of the  $\beta$ -lactoglobulin. Spectrum II is the ALS-estimated spectrum corresponding to the intermediate form. The simple visual inspection of this spectrum shows a lower content of  $\alpha$ -helix than the nonnative  $\alpha$ -helical form. More information about the secondary structure of this intermediate is obtained after deconvolution (see below).

The evolution of the concentration profiles (Fig. 5 b) shows a fast substitution of the native-like acid form (profile I) by the intermediate form (profile II), suggesting a highly cooperative transition between the two structures in the 10–15% range of TFE. At higher proportions of TFE, a more gradual transition between the intermediate form and the nonnative  $\alpha$ -helical form is observed.

### Evolution of the secondary structure during the TFE-induced transitions

To obtain more information about the structural changes that occur in the presence of TFE, the content of secondary structure of the ALS-estimated spectra of the three components was estimated by least-squares deconvolution, using as basis spectra those corresponding to  $\alpha$ -helix,  $\beta$ -sheet, and random coil described by Chen et al. (1974). Results of these estimations are shown in Table 1. The secondary structural content of the native-like acid form differs of the one estimated from the x-ray structure reported by Papiz et al. (1986), but it is close to that obtained by other authors from deconvolution of CD data (Hamada et al., 1996). The spectrum corresponding to the nonnative  $\alpha$ -helical form presents a high percentage of  $\alpha$ -helix, with no  $\beta$ -sheet structure detected. More interesting are the results obtained for the intermediate form. The secondary structure content estimated for its spectrum is rather close to that reported by Shiraki et al. (1995) for  $\beta$ -lactoglobulin on the basis of a joint prediction method (48%  $\alpha$ -helix, 13%  $\beta$ -sheet and 39% random coil).

Once the secondary structural content of the spectra corresponding to the three components has been estimated, the

**TABLE 1** Secondary structure content of the spectra recovery after the ALS optimization

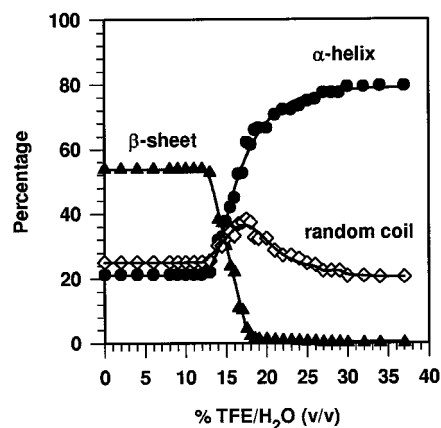
	Native-like acid form	Intermediate form	Nonnative $\alpha$ -helical form
$\alpha$ -helix	21.14	56.11	79.61
$\beta$ -sheet	53.81	2.14	0.00
Random coil	25.05	41.75	20.39

The content of the secondary structure was evaluated by least-squares deconvolution, using as basis spectra corresponding to  $\alpha$ -helix,  $\beta$ -sheet and random coil those described by Chen et al. (1974).

proportions of  $\alpha$ -helix,  $\beta$ -sheet, and random coil present at each TFE concentration can be calculated, taking into account the concentration profiles obtained by the ALS optimization method (Fig. 5 b). Fig. 6 shows the evolution of the different secondary structures versus the TFE proportion. For TFE proportions higher than 12%, the  $\beta$ -sheet structure quickly disappears, whereas the  $\alpha$ -helix increases. At the same concentration of TFE, the random coil content increases, suggesting that a fraction of the  $\beta$ -sheet structure present in the native-like acid form of the  $\beta$ -lactoglobulin is disorganized to a random coil. At 18% of TFE, the random coil content reaches a maximum and then decreases until it reaches values close to those present in the acid form. At  $\sim$ 30% TFE, the  $\alpha$ -helix content reaches a maximum, and beyond this TFE proportion no additional changes are detected. These results suggest that the first phase of the two detected phases in the TFE induction of the nonnative  $\alpha$ -helical form of the  $\beta$ -lactoglobulin is not a simple  $\beta$ -sheet-to- $\alpha$ -helix transition, but a  $\beta$ -sheet-to-random coil transition also occurs. The second phase of the process could be explained by a simple random-to- $\alpha$ -helix transition.

### DISCUSSION

The application of factor analysis and multivariate curve resolution techniques to the study of the TFE induction of the nonnative  $\alpha$ -helical form of the  $\beta$ -lactoglobulin using CD spectroscopy allowed the deduction of structural information about the conformational changes that the evolution of the CD spectra contains. The number of components detected to explain the evolution of the experimental data set showed that the changes in the secondary structure of the  $\beta$ -lactoglobulin induced by TFE cannot be explained as a simple  $\beta$ -sheet-to- $\alpha$ -helix transition. A third component different from the native-like acid form and the nonnative  $\alpha$ -helical form is necessary to explain the process. The



**FIGURE 6** Evolution of the  $\alpha$ -helix,  $\beta$ -sheet, and random coil at different TFE proportions. The secondary structure content at each TFE concentration was calculated from the deconvolution of the estimated spectra (Table 1) and the concentration profiles obtained by the ALS optimization (Fig. 5 b).

application of the alternating least-squares algorithm (Tauler et al., 1995) allowed the estimation of the CD spectrum corresponding to this intermediate form and allowed the estimation of the concentration profiles corresponding to the three forms involved in the process (Fig. 5).

The results obtained in this work suggest that the structural changes induced by TFE on the  $\beta$ -lactoglobulin are a more complicated process than was described until now, involving two differentiated phases. The evolution of the secondary structure during the TFE-induced process (Fig. 6) showed that in a highly cooperative first phase a fraction of the  $\beta$ -sheet structure corresponding to the native-like acid form is transformed to an  $\alpha$ -helix structure, whereas in another fraction of the protein, a  $\beta$ -sheet-to-random coil transition occurs. As a consequence of this first phase, an intermediate form is accumulated at low TFE concentration. In a second phase, at higher TFE proportions, a more gradual random coil-to- $\alpha$ -helix transition takes place.

The secondary structural content of the ALS-estimated intermediate (Table 1) is rather close to that reported by Shiraki et al. (1995) on the basis of a joint prediction method for the  $\beta$ -lactoglobulin. This result is consistent with the generally accepted mechanism of the TFE stabilization of the  $\alpha$ -helix. According to this mechanism, the effect of TFE can be explained mainly as weakening of the nonlocal hydrophobic interactions and slight enhancement of local helical interactions (Thomas and Dill, 1993).

The more gradual random coil-to- $\alpha$ -helix transition that occurs during the transformation of the intermediate form to the nonnative  $\alpha$ -helical form, at high TFE concentrations, can be due to the nonspecific interactions of the TFE with the polypeptide chain. The induction of  $\alpha$ -helix structures by high TFE concentrations in proteins with no propensity to exist in a helical conformation has been reported (Jayaraman et al., 1996).

The existence of a form of the  $\beta$ -lactoglobulin with a secondary structure content more in agreement with the predictions based in its amino acid sequence at low TFE concentration (when nonlocal interactions are weakened) is of interest in relation to the mechanism of protein folding. The presence of an  $\alpha$ -helical intermediate in the folding pathway of the  $\beta$ -lactoglobulin was proposed for the first time by Kuwajima et al. (1987). These authors found that in the refolding kinetics of this protein monitored by stopped-flow CD, the ellipticity at 222 nm exceeded that of the native state within the dead time of mixing and then slowly returned to the level of the native state (the overshoot phenomenon). More recently, using the same technique at various wavelengths, a partial spectrum for the intermediate form was built by Hamada et al. (1996). The estimation of the secondary structure of this spectrum is rather close to that obtained in this work. They suggest that the folding reaction of  $\beta$ -lactoglobulin follows a nonhierarchical mechanism, in which nonnative  $\alpha$ -helical intermediates formed at an early stage of the process, when local interactions are dominant, play important roles.

From our point of view, even if the  $\alpha$ -helical intermediate is present in the refolding pathway of the  $\beta$ -lactoglobulin, some kind of hierarchy remains in the process. A folding pathway like that proposed for this protein is hierarchical, not in relation to the similarities between the structure of the intermediates and the native form, but in relation to the order in which the different interactions take place. The structure acquired by the intermediate forms (native-like or not, depending on the primary structure) is driven by the local interactions that occur between nearby amino acid residues (the typical forces involved in the stabilization of the secondary structure). In a second stage, the final native structure is acquired, driven by the nonlocal interactions (the typical forces involved in the stabilization of the tertiary structure).

In summary, the proposed mathematical data analysis allowed the detection of an intermediate form in the TFE-induced transition of  $\beta$ -lactoglobulin and the resolution of the CD spectrum associated with this intermediate. The spectral results, combined with the resolved concentration profiles, also provide information concerning the nature of the changes that TFE induces in the protein.

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