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Alcohol and Temperature Induced Conformational Transitions in Ervatamin B: Sequential Unfolding of Domains

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The structural aspects of ervatamin B have been studied in different types of alcohol. This alcohol did not affect the structure or activity of ervatamin B under neutral conditions. At a low pH (3.0), different kinds of alcohol have different effects. Interestingly, at a certain concentration of non-fluorinated, aliphatic, monohydric alcohol, a conformational switch from the predominantly α-helical to β-sheeted state is observed with a complete loss of tertiary structure and proteolytic activity. This is contrary to the observation that alcohol induces mostly the α-helical structure in proteins. The O-state of ervatamin B in 50% methanol at pH 3.0 has enhanced the stability towards GuHCl denaturation and shows a biphasic transition. This suggests the presence of two structural parts with different stabilities that unfold in steps. The thermal unfolding of ervatamin B in the O-state is also biphasic, which confirms the presence of two domains in the enzyme structure that unfold sequentially. The differential stabilization of the structural parts may also be a reflection of the differential stabilization of local conformations in methanol. Thermal unfolding of ervatamin B in the absence of alcohol is cooperative, both at neutral and low pH, and can be fitted to a two state model. However, at pH 2.0 the calorimetric profiles show two peaks, which indicates the presence of two structural domains in the enzyme with different thermal stabilities that are denatured more or less independently. With an increase in pH to 3.0 and 4.0, the shape of the DSC profiles change, and the two peaks converge to a predominant single peak. However, the ratio of van't Hoff enthalpy to calorimetric enthalpy is approximated to 2.0, indicating non-cooperativity in thermal unfolding.

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Introduction

Denaturants (like GuHCl, urea, and temperature) are most frequently employed to probe the structural aspects of a protein in solution. Thus, equilibrium unfolding studies and the presence of intermediates/molten globule state have most often been reported at low pH or in the presence of previously mentioned denaturants (Uversky and Ptitsyn, 1994; Vanderheeren and Hanssens, 1994; Wang *et al.*, 1998; Ayed and Duckworth, 1999; Chi and Asher, 1999; Kundu *et al.*, 1999). Alcohol, often used as a protein precipitant or preservative, has other effects on proteins and polypeptides, and hence can give valuable information on the structure of proteins in solution. Alcohol disrupts the rigid tertiary structures, stabilizes, and induces helicity in protein, and dissolve peptide aggregation (Barker and Mayo, 1991; Radford *et al.*, 1992). Destabilization of the tertiary structure of a protein by alcohol (Herskovits *et al.*, 1970; Fink and Painter, 1987) and stabilization of secondary structure (Nelson and Kallenbach, 1989; Lehrman *et al.*, 1990) has often led to the induction of partially folded intermediates (Bhattacharjya and Balaram, 1997; Cort and Anderson, 1997; Gast *et al.*, 1999) in proteins, sometimes referred to as the O-state (Yang and Mayo, 1993) and is "molten-globule" like (Ptitsyn, 1987; Kuwajima, 1989). Thus, alcohol is now commonly used to induce partially folded states in proteins (Fan *et al.*, 1993; Alexandrescu *et al.*, 1994; Schonbrunner *et al.*, 1996). However, the behaviors of plant cysteine proteases of the papain superfamily and their folding aspects in alcohol have not been reported extensively, and needs consideration.

Structural and functional aspects of ervatamin B in response to acid and chemical denaturants like urea and

GuHCl were studied (preceding paper). Acid induced unfolding of ervatamin B (both in the presence and absence of salt), seems to involve an intermediate, which is predominantly β-sheeted and undergoes a cooperative transition in the presence of GuHCl. An intermediate state, similar to the acid induced state, was stabilized at a very low concentration of GuHCl at pH 2.0 due to the ionic effect of the denaturant. At higher pH (3 and 4), the GuHCl induced unfolding revealed the existence of intermediates in the unfolding pathway with strong binding to ANS at low concentrations of the denaturant. Urea that induced unfolding of ervatamin B at pH 3.0 was non-cooperative and proceeds through at least two intermediates. This suggests that there are structural parts in the molecule with different stabilities, which unfold in steps. These results can be interpreted as indirect evidence for the presence of domains in the enzyme, but they are not convincing proofs. Though acid and chemical induced denaturation of ervatamin B involves partially structured multiple intermediates, the study could not give detailed information about the molecular structure of the enzyme. An intermediate (where domains are still intact, but with conformational changes) is yet to be trapped and its unfolding characteristic studied. Hence, attempts were made to study conformational changes that are also induced by alcohol, and probe the presence or absence of other intermediates in ervatamin B that could probably provide additional information about the molecular structure. Differential scanning calorimetry (DSC), a powerful technique that provides information about the global behavior of proteins, was also used to characterize temperature-induced conformational changes in ervatamin B. Also, DSC is basically a kinetic measurement of protein denaturation (unfolding), and hence DSC is of great significance (Brewer, 1999).

Materials and Methods

Ervatamin B was purified as described (Kundu *et al.*, 2000). Spectroscopic grade methanol, ethanol, 2-propanol, TFE, acetonitrile and Molecular Biology grade glycerol were purchased from the Sigma Chemical Co. (St. Louis, USA).

Absorbance, fluorescence, spectropolarimetric measurements, enzyme assays as well as data analyses were the same as described (preceding paper).

Alcohol-induced conformational transitions Spectroscopic measurements were performed in the presence of various kinds of alcohol at desired pH. Protein samples were incubated at different alcohol concentrations at different pH for approximately 24 h at 25°C in order to attain equilibrium before all the measurements. Appropriate solutions that contained the respective alcohol in the absence of the enzyme were taken as blank. Similarly, GuHCl induced denaturation was performed as described (preceding paper). Protein samples were incubated at different denaturant concentrations in the presence and absence of alcohol for approximately 24 h at 25°C to attain equilibrium. For comparison of the results, the data that was collected as molar ellipticity were normalized and expressed as a fraction of the native protein unfolded (fraction unfolded).

Thermal unfolding Temperature induced changes in the conformation of ervatamin B (in the presence and absence of alcohol) were followed by CD (far and near UV) and fluorescence measurements. Protein samples were incubated at the desired temperature for 15 min before each measurement. The actual temperature of the sample was obtained with a thermocouple using a digital multimeter. Occasionally, samples were also checked for possible aggregations, due to the heat of light scattering measurements. Data are expressed in terms of fraction unfolded as above.

Differential scanning calorimetry Calorimetric scans were collected using a Microcal MC-2 (Microcal, Northampton, USA) differential scanning calorimeter, in the pH ranges 2.0-4.0. Protein solutions of 0.7-1.1 mg/ml were extensively dialyzed against a 0.02 M glycine buffer, pH 2.0 and pH 3.0, and a 0.02 M sodium acetate buffer, pH 4.0. After dialysis, the protein concentration and pH of the samples were checked. All of the solutions were degassed under vacuum before being loaded into the calorimeter cells. The calorimetric experiments were conducted at a scan rate of 60°C/h. Buffer-buffer base lines were obtained under the same conditions and subtracted from sample curves. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board for automatic data collection and analysis that included baseline subtraction and calculation of ΔH_{cal} and $\Delta H_{van't\ Hoff}$. In the calculations of molar quantities a molecular weight of 26,000 was used.

Results

Conformational states of ervatamin B in alcohol at neutral pH Three types of alcohol have been used to induce conformational changes in ervatamin B-non-fluorinated, aliphatic, monohydric alcohol (like methanol, ethanol and 2-propanol) fluorinated alcohol like TFE, and polyhydric alcohol like glycerol. A typical far-UV CD spectrum of ervatamin B in 50% alcohol under neutral conditions is shown in Fig. 1. The native spectra remains unchanged in the presence of all of the alcohol with some increase in ellipticity. Thus, it seems ervatamin B is stable in all of the alcohol studied. Near-UV CD spectra could not be recorded under similar conditions of pH, because the enzyme tends to precipitate at the higher protein concentration that is required for such measurements. The activity persists in the presence of various kinds of alcohol with a slight increase in the values (data not shown). Proteolytic activity of ervatamin B in the presence of 50% 2-propanol could not be done, due to the low solubility at neutral pH. Acetonitrile was used as an arbitrary organic solvent.

Conformational states of ervatamin B in 50% alcohol at low pH Ervatamin B retains the native structure and activity

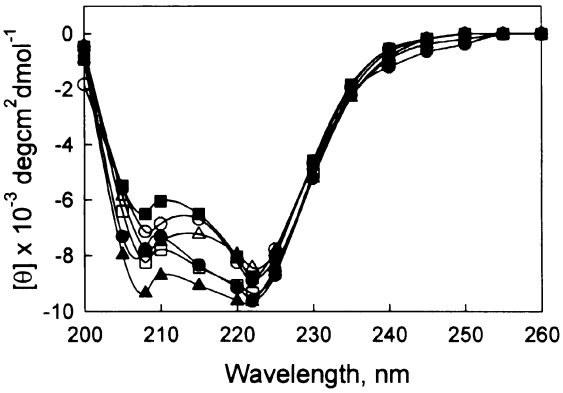

Fig. 1. Effect of various kinds of alcohol on the secondary structure of ervatamin B at neutral pH. The enzyme was incubated in various kinds of alcohol under neutral conditions at a concentration of 0.1 mg/ml for 24 h at 25ºC, and the far-UV CD spectra was collected. Alcohol concentration was 50% (v/v). Alcohol used was (●) methanol, (△) ethanol, (▲) TFE, and (□) glycerol. (○), in the absence of any alcohol. Acetonitrile (■) was used as an arbitrary organic solvent.

in the pH range 3.0-10.5. As low pH produces stress in the molecule, it can therefore be easily subjected to conformational changes. Therefore, the effect of various kinds of alcohol on ervatamin B was studied at pH 3.0 using near-UV CD, far-UV CD, and proteolytic activity measurements. Disruption of tertiary structure, loss of proteolytic activity, decrease in secondary structural content, or induction of α-helical structure are seen as expected. Interestingly, different kinds of alcohol induced different effect s on the enzyme, especially at the level of the secondary structure. Fifty-percent glycerol had no significant effect on the tertiary structure (Fig. 2A) or proteolytic activity (data not shown) of the enzyme. However, molar ellipticity in the far-UV region increased slightly, indicating some additional helical structure. (Fig. 2B). The presence of 50% TFE or non-fluorinated alcohol disrupted the tertiary structure (Fig. 2A) along with loss of proteolytic activity. TFE induced α-helicity in the enzyme with a marked increase in ellipticity in the far-UV range (Fig. 2B). Interestingly, the shape of the far-UV CD spectra also changed, the negative Cotton effect at 208nm was more pronounced than that at 222 nm. The reverse was true in the absence of TFE. The unique observation was the conformational switch, from α-helix to β-sheet (Fig. 2B) seen in ervatamin B, in the presence of 50% methanol, ethanol or 2-propanol with the appearance of a typical negative peak around 215-216 nm.

Conformational states of ervatamin B in 50% methanol at various pH In view of the conformational transition from α-helix to β-sheet in 50% methanol, ethanol, or 2-propanol at pH 3.0, further studies were focused or the presence of these kinds of alcohol as a function of pH also. As methanol being the simplest alcohol in the series, it was chosen as a

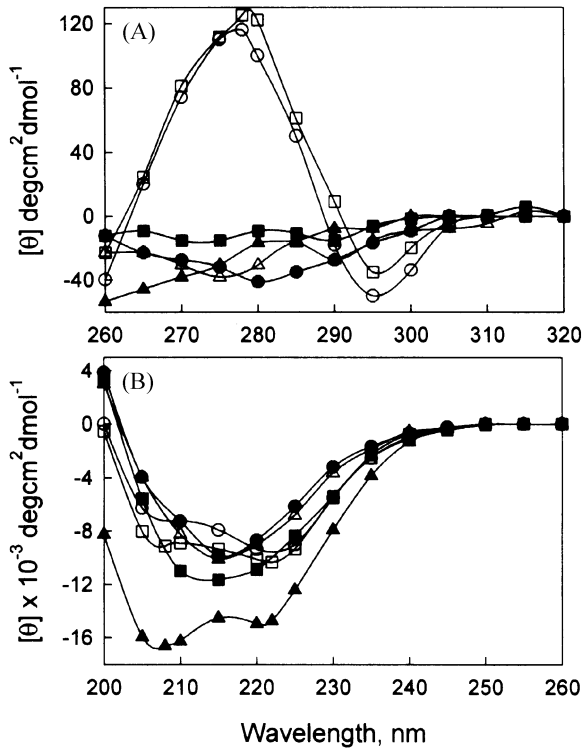

Fig. 2. Effect of alcohol on the (A) tertiary and (B) secondary structure of ervatamin B at pH 3.0. The enzyme was incubated in various kinds of alcohol for 24 h at 25°C. Alcohol concentration was 50% (v/v). Alcohol used was (○) none, (●) methanol, (△) ethanol, (■) 2-propanol, (▲) TFE, and (□) glycerol. The enzyme concentration was 0.7 mg/ml and 0.1 mg/ml for near-UV and far-UV CD measurements, respectively.

representative for further studies. Moreover, with an increase in alkyl chain length, the solubility decreases. Therefore, higher alcohol was not used, leaving methanol the best choice. The order of alcohol induced destabilization of tertiary structure and stabilization of secondary structure is as follows: TFE>propanol>ethanol>methanol (Bianchi *et al.*, 1970; Herskovits *et al.*, 1970). Thus, the transition that is induced by methanol is expected to occur in a wider range of alcohol concentration than that induced by other kinds of alcohol, which would enable one to investigate the transition in detail. However, although the present study is focused on the effect of methanol, other kinds of alcohol, such as ethanol or propanol induce similar effects, but at different effective concentrations. Fig. 3A shows the near-UV CD spectra of ervatamin B in 50% methanol as a function of pH. At pH 5.0 and 6.0 no significant changes in the tertiary structure were seen, but by pH 4.0 a large decrease in the molar ellipticity was observed. This was followed by a complete loss of tertiary structure, which lowered the pH to 3.0 and below. The loss of proteolytic activity also follows a similar trend with a change in pH (data not shown). The far-UV CD spectra of ervatamin B at various pH are shown in Fig. 3B. Up to pH 4.0, the spectra are typical of α + β class of protein with two

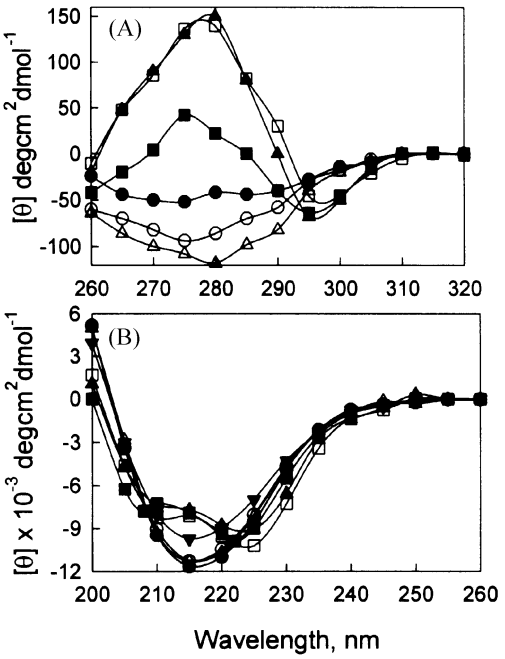

Fig. 3. Effect of 50% methanol on (A) tertiary and (B) secondary structure of ervatamin B at pH (□) 6.0, (▲) 5.0, (●) 4.0, (■) 3.0, (△) 2.0, and (○) 1.0. The enzyme concentrations are the same as in Fig. 2. The enzyme was incubated in 50% methanol at different pHs for 24 h at 25°C.

negative peaks at 222 and 208 nm. At and below pH 3.0, a conformational switch to a β-sheet was observed in presence of 50% methanol. Since all of the structural properties are intact at pH 3.0 in the absence of alcohol, further studies on the conformational transitions that are due to the presence of alcohol are done at pH 3.0. At very low pH (1.0 and 2.0), in the absence of alcohol, the enzyme exists in an acid-unfolded state with no typical CD spectra (accompanying paper). Thus, the β-sheet that is seen in the protein structure is due to the presence of 50% methanol only.

Conformational states in increasing concentrations of methanol at pH 3.0 The effect of 50% methanol has been reported so far. It would be interesting to see the effect of increasing methanol concentration, as well on the structure of ervatamin B. Fig. 4A shows the near-UV CD spectra of ervatamin B with increasing concentrations of methanol. Initially, there was some increase in the ellipticity, up to 30% methanol, followed by a gradual decrease. There was a further increase in methanol concentration and all the tertiary structure was lost by 45-50% methanol. The loss of proteolytic activity as a function of methanol concentration was also followed a similar trend (Fig. 4B). The far-UV CD spectra of ervatamin B under similar conditions are shown in Fig. 4C. The spectra are typical of native state up to 40% methanol. Beyond this concentration there was some perturbation in the secondary structure. Also, by 48% methanol, α to β switchover was observed. In 50% methanol the spectra was distinctly typical of

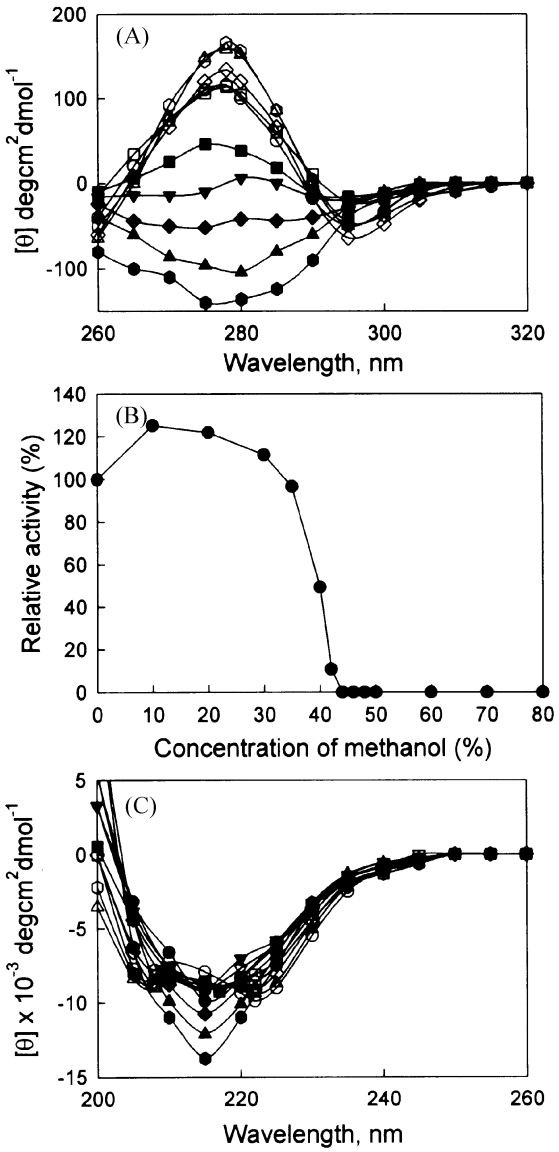

Fig. 4. Effect of increasing concentration of methanol on (A) tertiary structure (B) activity, and (C) secondary structure of ervatamin B at pH 3.0. The enzyme was incubated at different concentrations of methanol for 24 h at 25°C before CD and activity measurements. The concentration of methanol at (○) 0%, (◇) 10%, (△) 20%, (◈) 30%, (□) 35%, (■) 40%, (▽) 42%, (▼) 45%, (●) 48%, (◆) 50%, (▲) 60%, (⧫) 70%. The enzyme concentrations were 0.7 mg/ml and 0.1 mg/ml for near-UV and far-UV CD, respectively. Proper aliquot that contained 3 μg of the enzyme was assayed for proteolytic activity, as described in Methods. The activity of the enzyme in absence of methanol is taken as 100%.

β-sheet. With a further increase in methanol concentration, this state was stabilized. Another conformational transition that resulted in enhanced ellipticity was observed. A similar result, α-helix to β-sheet switchover, was observed with the addition of ethanol or propanol to ervatamin B, but at much lower concentrations as 44% ethanol and 40% propanol. It appears

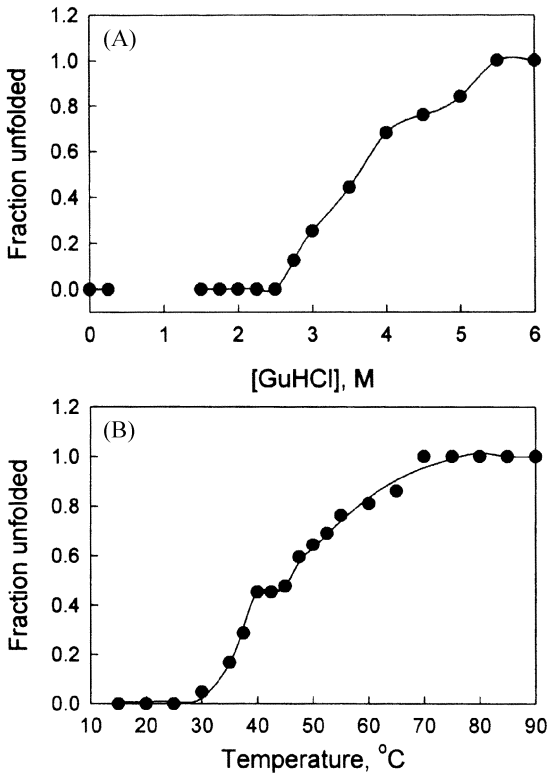

Fig. 5. Equilibrium unfolding of ervatamin B in 50% methanol at pH 3.0 by (A) GuHCl (B) temperature. (A) Enzyme at a concentration of 0.1 mg/ml in 50% methanol, pH 3.0 was incubated at different concentrations of GuHCl for 24 h at 25°C. The far-UV CD signal between 0.5 and 1.25 M GuHCl could not be measured accurately due to aggregation. (B) Enzyme, at a concentration of 0.1 mg/ml, in 50% methanol, pH 3.0 was incubated at different temperatures for 15 min. Transition was monitored by ellipticity at 215 nm.

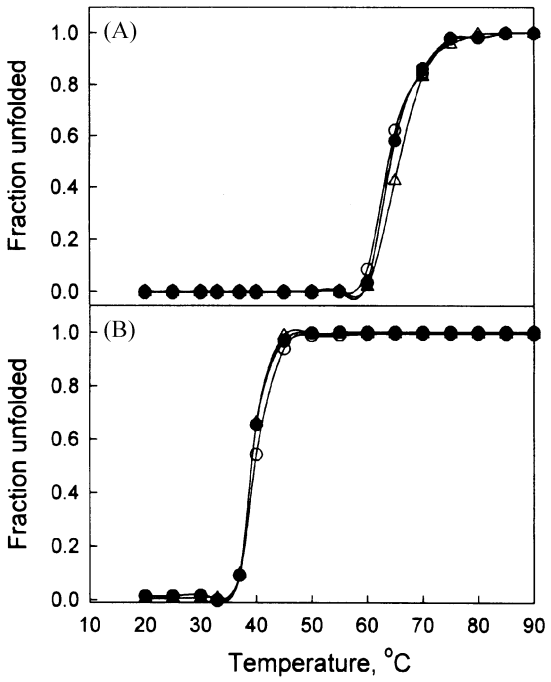

Fig. 6. Equilibrium thermal denaturation of ervatamin B at (A) pH 7.0 (B) pH 3.0. Denaturation of ervatamin B was monitored by change in ellipticity at 278 nm (●), ellipticity at 222 nm (△), and proteolytic activity (○). Protein concentration was 0.7 mg/ml for near-UV CD; 0.1 mg/ml for far-UV CD and 3 μg of the enzyme was used for proteolytic activity. The protein samples were incubated at the specified temperatures for 15 min before measurement.

that the conformational switch is dependent of hydrocarbon chain length.

Effect of enzyme concentration on conformational states in 50% methanol, pH 3.0 The formation of protein aggregates in the presence of methanol can result in a CD spectrum typical of β-sheet, or the promotion of β-structures can result in aggregation (Waterhous and Johnson, 1994). To rule out this possibility, light scattering experiments were performed with increasing concentrations of enzyme. No aggregation was found. Moreover, since aggregation is concentration dependent, far-UV CD spectra of ervatamin B were collected in 50% methanol in the concentration range of 0.05-1.0 mg/ml at suitable sensitivities. All of the spectra were found to be overlapping (data not shown). Any aggregation would have resulted in some variations in the CD spectra. Hence, the formation of β-sheet, due to any aggregation of the protein molecules, is ruled out. The methanol induced state of ervatamin B with predominant β-structure is referred to as the O-state.

Stability of the O-state and its unfolding Intermediate states in protein folding/unfolding are usually stable. The stability of ervatamin B in the O-state was determined by following GuHCl and temperature induced unfolding. Unfolding of the protein was monitored by ellipticity at 215 nm. As shown in Fig. 5A, the GuHCl induced transition curve for the O-state of ervatamin B is biphasic with transition midpoints of 3.3 M and 4.9 M GuHCl. In the absence of methanol, however, the chemically induced unfolding transition midpoint is 1.16 M GuHCl. (preceding paper). Thus, ervatamin B in the O-state is more stable to GuHCl denaturation compared to the native state. It is important to note that between 0.5 to 1.25 M GuHCl some visible aggregation of the protein was seen. Therefore, the far-UV CD signal in this concentration range of GuHCl could not be measured accurately. However, at higher concentration of GuHCl no aggregation of protein was detected.

Similarly, thermal denaturation of ervatamin B in the O-state that was monitored by changes in ellipticity at 215 nm resulted in a biphasic transition curve with transition midpoints of 36.3°C and 58.5°C (Fig. 5B).

Conformational transition from denatured state Additions of 50% methanol at pH 3.0 to completely unfolded ervatamin

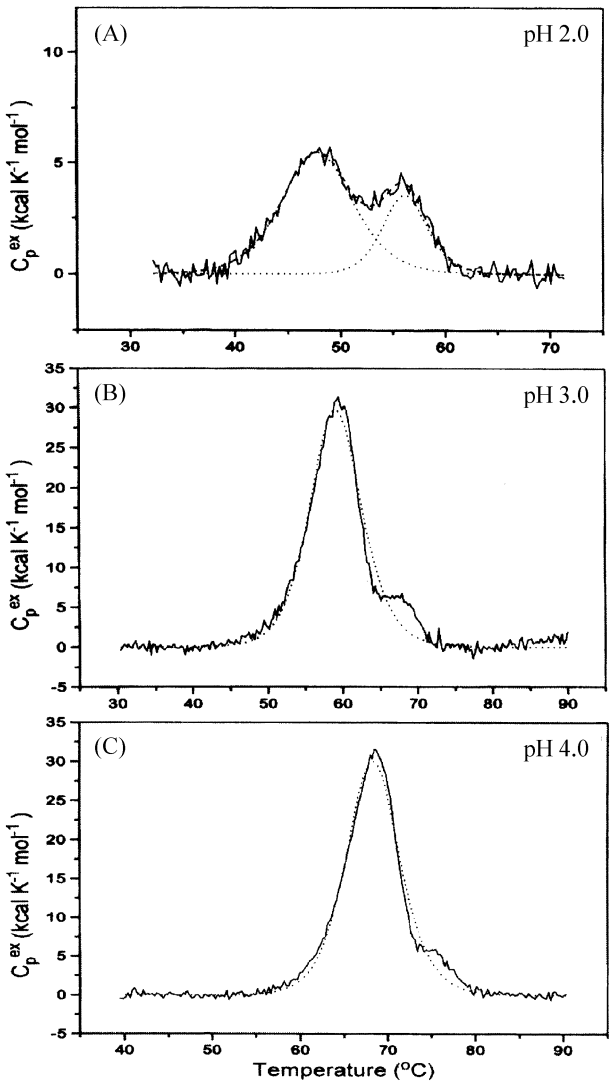

Fig. 7. Excess heat capacity function versus temperature for ervatamin B at pH (A) 2.0 (B) 3.0 and (C) 4.0. (A) The enzyme was extensively dialyzed against 0.02 M glycine-HCl, pH 2.0 before the scan. The protein concentration was 0.77 mg/ml. The deconvolution result is consistent with two distinct two-state transitions. A second scan of the sample indicated irreversibility. The scan rate was 60°C/h. The dotted line represents the fitted curves. (B) and (C) The calorimetric scans were performed with a protein concentration of 0.731 mg/ml and 1.043 mg/ml at a scanning rate of 60°C/h in 0.02 M glycine-HCl, pH 3.0, and 0.02 M sodium acetate, pH 4.0, respectively. The excess heat capacity function was obtained after subtracting the baseline from the heat capacity function. Reheating of the samples indicated irreversibility of thermal denaturation. The dotted line represents the fitted curve.

B (in 6 M GuHCl) also induced the formation of β-sheet. The resultant far UV CD spectrum was identical to the spectra seen in the case of native protein under similar conditions (data not shown).

Table 1. Thermodynamic characteristics of ervatamin B denaturation

pH	t_m (°C)	ΔH_{cal} (kcal/mol)	$\Delta_{Hvan't\ Hoff}$ (kcal/mol)	$\Delta H_{cal}/\Delta H_{van't\ Hoff}$
3.0	59.1	274	95.3	2.8
4.0	68.3	245	114	2.1

Thermal unfolding by spectroscopy The effect of temperature on the secondary structure of ervatamin B at neutral pH was monitored by changes in the mean residue ellipticity at 222 nm (Fig. 6A). The enzyme maintains its native structure up to approximately 60°C without any major change in ellipticity value. Beyond this temperature, a gradual loss of secondary structure is observed and the transition is cooperative with a transition mid-point (t_m) of 65.9°C. Similarly, the change in tertiary structure, monitored by ellipticity at 278nm, followed a cooperative transition with a t_m of 64.6°C (Fig. 6A). The loss in proteolytic activity and temperature of the enzyme followed a single transition with a transition midpoint of 64.3°C (Fig. 6A). The enzyme retained its full activity up to 60°C. The fluorescence intensity declined monotonously and showed no regular transitions (data not shown). Thus, the transition curves that are followed by different probes are cooperative and coincidental with an average t_m of 65.0 ± 0.1°C. At pH 3.0, the temperature-induced loss in tertiary structure, proteolytic activity, and secondary structure followed a single transition with a t_m around 39.3 ± 0.5°C (Fig. 6B). Thus, at lower pH the enzyme was largely in the native state with no significant loss in structure or activity up to about 30°C, though the transition temperature shifts to a lower value.

Differential scanning calorimetry At pH 2.0 the thermogram showed two peaks with low ΔC_p values with transition midpoints of 47.9 and 56.2°C (Fig. 7A). The ΔH_{cal} values for the two transitions were 49.5 and 20 kcal/mole.

The DSC curves of ervatamin B, corrected for buffer-buffer tracings, that were obtained at pH 3.0 and 4.0 and with a rate of heating at 60°C/h are shown in Figures 7B and 7C. The endotherm showed a single predominant peak. At these pH values, the thermal unfolding of ervatamin B was apparently irreversible. This is indicated by the fact that no endotherm was observed by rescanning of the samples after being cooled from the first scan. In order to determine the enthalpy of unfolding, the calorimetric curves were corrected for the difference in permanent heat capacity, ΔC_p^{ex}, by subtraction of a linear base line (buffer-buffer) that connected the initial and final temperatures of the transitions. An excess heat capacity function, C_p^{ex}, was calculated and from it ΔH_{cal} and $\Delta H_{vant\ Hoff}$ were determined. The thermodynamic characteristics of ervatamin B denaturation are shown in Table 1.

At higher temperatures, a small shoulder was observed in both cases, which could not be deconvoluted as another peak.

Discussion

Solvent plays an important role in maintaining the native structure of a protein. Structural studies of proteins in the presence of different solvent systems can provide information about the role of various stabilizing and destabilizing forces that are responsible for their unique folded structure in solution. With this view, studies of ervatamin B in the presence of alcohol was performed.

At neutral pH, the addition of different kinds of alcohol to ervatamin B had no effect on the enzyme, as all the tertiary and secondary structural features were intact with complete retention of proteolytic activity. At lower pH the protein molecule is under stress, because of the electrostatic repulsions that are due to the increased positive charge. Thus, the addition of different kinds of alcohol tend to cause varying effects by opening up the molecule, depending on the nature of alcohol. The effect of TFE and glycerol on ervatamin B is consistent with the effect of alcohol on other proteins. TFE tends to give an increase in helical content at neutral pH. This tendency enhances at low pH where the protein is becoming more flexible. Glycerol has little effect on ervatamin B at neutral pH or at pH 3.0, whereas other alcohol that was studied has pronounced effects on the conformation of ervatamin B. The most striking observation (in the presence of methanol/ethanol/2-propanol) is the conformational switch from α-helix to β-sheet that occurred at low pH (at a critical concentration of alcohol followed by a further increase in ellipticity with an increase in alcohol concentration). The induction of a helical structure, a conformational switch from β-sheet to α-helix, and random coil to β-sheet in the conformation of proteins in the presence of different kinds of alcohol are reported for proteins of different structure types (Jackson and Mantsch, 1992; Alexandrescu *et al.*, 1994; Liu *et al.*, 1994; Jayaraman *et al.*, 1996; Ranjan and Balaram, 1996; Kumaran and Roy, 1999). An induction of β-sheet by methanol was recently reported in muscle acylphosphatase as a step in the formation of the native β-sheet structure (Chiti *et al.*, 1998). The conformational switch, α-helix to β-sheet, that was observed in ervatamin B in the presence of methanol is quite unique. The conformational switch in ervatamin B occurred in two steps. (1) The formation of β-sheet at a critical concentration of methanol was accompanied by loss of tertiary structure and proteolytic activity. (2) This was followed by a gain in ellipticity with increasing concentration of the organic solvent. The additional β-sheet content that is present at higher concentrations of methanol could be due to the non-specific interaction of methanol with the polypeptide chain. Such a methanol induced two-step transition is also seen in cytochrome c (Bychkova *et al.*, 1996) and ubiquitin (Wilkinson and Mayer, 1986).

A methanol induced state in ervatamin B is predominantly of β-sheet and non-native. There have been few reports of non-native intermediates in proteins (Liu *et al.*, 1994; Logan *et al.*, 1994; Hamada *et al.*, 1996; Kuwajima *et al.*, 1996; Mendieta *et al.*, 1999). The solvent composition must be a very important factor in determining the secondary structure of a given amino acid sequence *in vitro*. It can override the importance of only amino acid sequence in determining a secondary structure. This implies that the microenvironment, which is seen by a secondary structure that is due to non-local interactions of amino acids from the tertiary structure of a protein, determines the secondary structure of a polypeptide (Waterhous and Johnson, 1994). Thus, the formation of the non-native state in ervatamin B can be attributed to the local propensity of the amino acid sequence to form β-sheet in the presence of methanol, which is converted to native α-helical conformation under the influence of non-local (tertiary) interactions. Protein folding not only changes the local environment by removing many amino acids from the polar environment of the protein exterior, it may also introduce a micro-solvent that is due to non-local amino acids that are now nearby, due to tertiary structure. This is supported by the fact that the addition of methanol to denatured ervatamin B (in 6M GuHCl) or to A-state also induces the β-sheet. Thus, it seems that the formation of β-sheet is a pre-requisite for further folding of the enzyme into its native state. Hence, probably the folding of ervatamin B, like the folding of β-lactoglobulin (Hamada *et al.*, 1996), provides a unique example; the local secondary structure preference is very different from the final native preference, and should be useful in understanding the mechanism of protein folding. Such formation of β-sheet imparts stability to the enzyme. These intermediate structural units, which are predominantly stabilized by hydrogen bonding, flicker back and forth with the unfolded state until their interactions result in the formation of more stable, larger units of structure with the attainment of α-helices too (probably through both hydrogen-bonding and hydrophobic interactions), and undergo final conformational reorganization to attain the proper active site. The correlation between α-helices and activity is evident from the thermal unfolding characteristics of ervatamin B that is monitored by CD and proteolytic activity. The thermal transitions of ervatamin B, both at neutral and low pH, that is monitored by ellipticity at 222 nm (and hence in α-helical content) and the enzyme activity were coincidental. This indicates the involvement of the α-helical segments in maintaining the enzyme activity (Chopra *et al.*, 1983).

The switch from α-helix to β-sheet results in the formation of an intermediate state that is different than the native state at pH 3.0 or denatured state (in 6 M GuHCl). This intermediate state has no tertiary structure or proteolytic activity. However, it has substantial secondary structure that is non-native like (β-sheet), and the enzyme in this state is sticky. This intermediate state, with only a subset of native folding interactions, can be referred to as the O-state (for alcohol-induced state). It exhibits characteristics of the “molten-globule” like state.

Induction of the O-state, due to the presence of alcohol, is reported in the case of few other proteins (Timasheff, 1970; Nakano and Fink, 1990; Harding *et al.*, 1991; Yang and

Mayo, 1993). In most of the proteins studied, there was a decrease in β-sheet structure and increase in α-helix structure, as usually happens in the presence of alcohol. Contrary to such observations, β-sheet was induced in ervatamin B in the presence of alcohol.

Equilibrium unfolding of ervatamin B, in the O-state (both by GuHCl and temperature) is biphasic, whereas the native protein (at pH 3.0) exhibits a single unfolding transition. The biphasicity in the unfolding transitions suggests the presence of two structural parts in the molecular structure of the protein with different stabilities. The differential stabilization of the structural parts in the O-state may be a reflection of the differential stabilization of local conformations in methanol. Since the enzyme belongs to α + β class proteins, it can be assumed that one domain has predominant α-helices while the other predominant β-sheet. Thus, the two transitions that are seen in the presence of methanol correspond to the sequential unfolding of these domains. A similar case of uncoupling of cooperative transitions in the presence of methanol was reported in ribonuclease A (Brandts *et al.*, 1989). Thus, the domain interactions in ervatamin B, which are perturbed due to the presence of methanol, might be dominated by hydrophobic stabilization. Also, different domains have different relative hydrophobic stabilization.

Thermal unfolding of the enzyme at neutral and low pH that is monitored by different measures (CD, fluorescence and activity) is cooperative. The transition curves are also coincidental. This indicates that the molecule unfolds as a single entity. It is apparent from DSC studies that thermal unfolding of ervatamin B is irreversible. The values of real (calorimetric) and effective (van't Hoff) enthalpies of ervatamin B denaturation (determined at pH 3.0 and 4.0) differ by a factor of approximately two or higher, indicating that the transition is not a two-state transition. Thus, there must be a thermodynamically stable intermediate state. The transition into this state, as well as further transition from this state, is rather similar and independent. If, such transitions were completely independent, the ratio $\Delta H_{cal}/\Delta H_{vant\ Hoff}$ would equal exactly two, whereas for the one-step process (i.e., when both stages are strongly dependent), the ratio would approach the value unit. Thus, in the case of ervatamin B, quasi-independent transitions correspond to the transition of separate parts in the enzyme. Thus, the ervatamin B parts represent quasi-independent cooperative systems. This substantiates the hypothesis that folding of domains in ervatamin B proceeds independently. The cooperativity could be due to side-chain interactions at the domain interface. The small shoulder that is seen in the DSC profiles at pH 3.0 and 4.0 is probably due to the association of the denatured enzyme, which tends to get sticky at higher temperatures. Besides, the shoulder certainly does not represent another transition, as the profile could not be deconvoluted into two peaks as at pH 2.0. The presence of two structural parts, likely to be two domains, is clearly evident from the two distinct peaks that are seen in the DSC profile at pH 2.0. This confirms the conclusion that ervatamin B consists of two domains in its molecular structure. However, although it is tempting to assign the two transition temperatures to the melting of the two domains of the molecule, further experimental studies are needed to elucidate these assignments. Thus, it can be safely concluded that the two distinct melting transitions originate from the sequential unfolding of the unique domains in the ervatamin B. It is interesting to compare the transition midpoints of thermal unfolding of ervatamin B (at pH 3.0) that are obtained by CD and DSC measurements. In the O-state (pH 3.0, 50% methanol), the two transition midpoints that are obtained by monitoring far-UV CD are 36.3°C and 58.5°C. The former is close to the transition temperature 40°C (in absence of methanol) that is obtained by monitoring far-UV CD, whereas the latter is close to the transition temperature 59.1°C that is obtained by DSC. Such an observation can be probably explained as follows.

The molecule as a whole is not very stable at pH 3.0 compared to that at pH 7.0, but it still unfolds as a single unit. This indicates that the two parts in the protein are held by strong interactions, and are not differentially stabilized. In the presence of alcohol, the enzyme is non-native and perturbed with α-helices that are being lost. Such loss of α-helices might have reduced the stability of the domain with predominant α-helices, while the other domain is being stabilized. Hence, the enzyme unfolds sequentially with a lower transition temperature due to the unfolding of the less stable domain. A DSC profile shows a single peak, but with non-cooperativity, with a resultant transition temperature that is closer to the higher transition temperature that is observed in the presence of in methanol. The t_m that is due to the unfolding of the less stable domain is probably masked in the predominant peak. Perturbing the molecule further in order to destabilize the more stable domain (so that the ΔC_p values for denaturation of the two domains are not drastically different to mask one another), results in two peaks. This confirms that ervatamin B contains two domains in its molecular architecture. Moreover, the difference in the thermal unfolding behavior of ervatamin B, observed by CD and DSC, is probably due to the fact that the former is an equilibrium measurement while the latter is a kinetic one. The presence of intermediates in the kinetic unfolding pathway certainly has greater significance in understanding the folding pathway *in vivo*.

Such a wide range of variations for ervatamin B, ervatamin C (Kundu *et al.*, 1999) papain (Edwin and Jagannadham, 1998), and the individual characteristics do provide a sort of generalization for the folding behavior of plant cysteine proteases of the papain superfamily. Depending on the stability and structural integrity of the molecule, all of the proteases seem to unfold through intermediates with similar characteristics (though there are some differences in the backbone secondary structure), but contain domains that unfold sequentially.

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References

Alexandrescu, A. T., Ng, Y. L. and Dobson, C. M. (1994) Characterization of a trifluoroethanol-induced partially folded state of alpha-lactalbumin. *J. Mol. Biol*. **235**, 587-599.

Ayed, A. and Duckworth, H.W. (1999) A stable intermediate in the equilibrium unfolding of *E. coli* citrate synthase. *Protein Sci.* **8**, 1116-1126.

Barker, S. and Mayo, K. H. (1991) Trifluoroethanol drives platelet factor 4 subunit association rate toward the Smoluchowski-Stokes-Einstein dynamic diffusion limit. *J. Am. Chem. Soc.* **113**, 8201-8203.

Bhattacharjya, S. and Balaram, P. (1997) Hexafluoroacetone hydrate as a structure modifier in proteins: Characterization of a molten globule state of hen egg-white lysozymes. *Protein Sci.* **6**, 1065-1073.

Binachi, E., Rampone, R., Tealdi, A. and Ciferri, A. (1970) The role of aliphatic alcohol on the stability of collagen and tropocollegen. *J. Biol. Chem*. **245**, 3341-3345.

Brandts, J. F., Hu, C. Q., Lin, L. N. and Mos, M. T. (1989) A simple model for proteins with interacting domains. Application to scanning calorimetry data. *Biochemistry* **28**, 8588-8596.

Brewer, J. M. (1999) The use of differential scanning calorimetry (DSC) to determine the correctness of folding of cloned protein. *Biotechnol. Appl. Biochem.* **30**, 173-175.

Bychkova, V. E., Dujsekina, A. E., Klenin, S. L., Tiktopulo, E. I., Uversky, V. N. and Ptitsyn, O. B. (1996) Molten globule-like state of cytochrome c under conditions simulating those near the membrane surface. *Biochemistry* **35**, 6058-6063.

Chi, Z. and Asher, S. A. (1999). Ultraviolet resonance Raman examination of horse apomyoglobin acid unfolding intermediates. *Biochemistry* **38**, 8196-8203.

Chiti, F., Taddei, N., van Nuland, N. A. J., Magherini, F., Stefani, M., Ramponi, G. and Dobson, C. M. (1998) Structural characterization of the transition state for folding of muscle acylphosphatase. *J. Mol. Biol*. **283**, 893-903.

Chopra, R. K., Mukkamala, P. L. and Ananthanarayanan, V. S. (1983) Structural studies on prolyl hydroxylase. Conformation from circular dichroism spectroscopy. *Biochem. Int.* **7**, 415-421.

Cort, J. R. and Anderson, N. H. (1997) Formation of a molten-globule like state of myoglobin in aqueous hexafluoro2-propanol. *Biochem. Biophys. Res. Commun.* **233**, 687-691.

Edwin, F. and Jagannadham, M. V. (1998). Sequential unfolding of papain in molten globule state. *Biochem. Biophys. Res. Comm.* **252**, 654-660.

Fan, P., Bracken, C. and Baum, J. (1993) Structural characterization of monellin in the alcohol-denatured state by NMR: evidence for β-sheet to α-helix conversions. *Biochemistry* **32**, 1573-1582.

Fink, A. L. and Painter, B. (1987) Characterization of the unfolding of ribonuclease A in aqueous methanol solvents. *Biochemistry* **26**, 1665-1671.

Gast, K., Zirver, D., Muller-Frohne, M. and Damaschun, G. (1999) Trifluorocthanol induced conformational transitions of proteins: Insights gained from the differences between α lactalbumin and ribonuclease. *Protein Sci.* **8**, 625-634.

Hamada, D., Segawa, S-. I. and Goto, Y. (1996) Non-native α-helical intermediate in the refolding of β-lactoglobulin, a predominantly β-sheet protein. *Nature Struct. Biol.* **3**, 868-873.

Harding, M. M., Williams, D. H. and Woolfson, D. N. (1991) Characterization of a partially denatured state of a protein by two-dimensional NMR: reduction of the hydrophobic interactions in ubiquitin. *Biochemistry* **30**, 3120-3128.

Herskovits, T. T., Gadegbeku, B. and Jaillet, H. (1970) On the structural stability and solvent denaturation of proteins. I. Denaturation by alcohol and glycols. *J. Biol. Chem*. **245**, 2588-2598.

Jackson, M. and Mantsch, H. H. (1992) Halogenated alcohols as solvents for proteins: FTIR spectroscopic studies. *Biochim. Biophys. Acta* **1118**, 139-143.

Jayaraman, G., Kumar, T. K., Arunkumar, A. I. and Yu, C. (1996) 2,2,2-Trifluoroethanol induces helical conformation in an all β-sheet protein. *Biochem. Biophys. Res. Commun.* **222**, 33-37.

Kumaran S. and Roy, R. P. (1999) Helix-enhancing propensity of fluoro and alkyl alcohols: influence of pH, temperature and cosolvent concentration on the helical conformation of peptides. *J. Peptide Res.* **53**, 284-293.

Kundu, S., Sundd, M. and Jagannadham, M. V. (1999) Structural characterization of a highly stable cysteine protease ervatamin C. *Biochem. Biophys. Res. Commun*. **264**, 635-642.

Kundu, S., Sundd, M. and Jagannadham, M. V. (2000) Purification and characterization of a cysteine protease ervatamin B, with two disulfide bridges, from the latex of ervatamia coronaria. *J. Agric. Food Chem*. **48**, 171-179.

Kuwajima, K. (1989) The molten globule state as a clue for understanding the folding an cooperativity of globular-protein structure. *Proteins: Struct. Funct. Genet*. **6**, 87-103.

Kuwajima, K., Yamaya, H. and Sugai, S. (1996) The burst phase intermediate in the refolding of β-lactoglobulin studied by stopped flow circular dichroism and absorption spectroscopy. *J. Mol. Biol.* **264**, 806-822.

Lehrman, S. R., Tuls, J. L. and Lund, M. (1990) Peptide αhelicity in aqueous trifluoroethanol: Correlation with predicted helicity and the secondary structure of the corresponding regions of bovine growth hormone. *Biochemistry* **29**, 5590-5596.

Liu, Z. P., Rizo, J. and Gierasch, L. M. (1994) Equilibrium binding studies of cellular retinoic acid binding protein, a predominantly β-sheet protein. *Biochemistry* **33**, 134-142.

Logan, T. M., Theriult, Y. and Ferik, S. W. (1994) Structural characterization of the FK506 binding protein unfolded in urea and guanidine hydrochloride. *J. Mol. Biol.* **236**, 637-648.

Mendieta, J., Folque, H. and Taules, R. (1999) Two-phase induction of the nonnative α-helical form of β-lactoglobulin in the presence of trifluoroethenol. *Biophys. J*. **76**, 451-457.

Nakano, T. and Fink, A. L. (1990) The folding of staphylococcal nuclease in the presence of methanol or guanidine thiocyanate. *J. Biol. Chem.* **265**, 12356-12362.

Nelson, J. W. and Kallenbach, N. R. (1986) Stabilization of the ribonuclease S-peptide alpha helix by trifluoroethanol solution. *Biochemistry* **28**, 5256-5261.

Ptitsyn, O. B. (1987) Protein folding. Hypothesis and experiments. *J. Protein. Chem*. **6**, 273-293.

Radford, S. E., Dobson, C. M. and Evans, P. A. (1992) The folding of hen lysozyme involves partially structured intermediates and multiple pathways. *Nature* **358**, 302-307.

Ranjan, R. and Balaram, P. (1996) A model for the interaction of trifluoroethanol with peptides and proteins. *Int. J. Peptide Protein Res.* **48**, 328-336.

Schonbrunner, N., Wey, J., Engels, J., Georg, H. and Kiefhaber, T. (1996) Native-like β-structure in a trifluoroethanol-induced partially folded state of the all-β-sheet protein tendamistat. *J. Mol. Biol*. **260**, 432-445.

Timasheff, S. N. (1970) Protein-solvent interactions and protein Conformation. *Acc. Chem. Res*. **3**, 62-68.

Uversky, V. N. and Ptitsyn, O. B. (1994) "Partly folded" state, a new equilibrium state of protein molecules: four-state guanidimium chloride-induced unfolding of β-lactamase at low temperature. *Biochemistry* **33**, 2782-2791.

Vanderheeren, G. and Hanssens, I. (1994) Thermal unfolding of bovine α-lactalbumin: comparison of circular dichroism with hydrophobicity measurements. *J. Biol. Chem.* **269**, 7090-7094.

Wang, C., Lascu, I. and Giartosia, A. (1998) Bovine serum fetuin is unfolded through a molten globule state. *Biochemistry* **37**, 8457-8464.

Waterhous, D. V. and Johnson, W. C. Jr. (1994) Importance of environment in determining secondary structure in proteins. *Biochemistry* **33**, 2121-2128.

Wilkinson, K. D. and Mayer, A. N. (1986) Alcohol-induced conformational changes of ubiquitin. *Arch. Biochem. Biophys.* **250**, 390-399.

Yang, Y. and Mayo, K. H. (1993) Alcohol-induced protein folding transitions in platelet factor 4; the O-state. *Biochemistry* **32**, 8661-8671.