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Unfolding of Ervatamin C in the Presence of Organic Solvents: Sequential Transitions of the Protein in the O-state

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The folding of ervatamin C was investigated in the presence of various fluorinated and non-fluorinated organic solvents. The differences in the unfolding of the protein in the presence of various organic solvents and the stabilities of O-states were interpreted. At pH 2.0, non-fluorinated alkyl alcohols induced a switch from the native α-helix to a β-sheet, contrary to the β-sheet to α-helix conversion observed for many proteins. The magnitude of ellipticity at 215 nm, used as a measure of β-content, was found to be dependent on the concentration of the alcohol. Under similar conditions of pH, fluorinated alcohol enhanced the intrinsic a-helicity of the protein molecule, whereas the addition of acetonitrile reduced the helical content. Ervatamin C exhibited high stability towards GuHCl induced unfolding in different O-states. Whereas the thermal unfolding of O-states was non-cooperative, contrary to the cooperativity seen in the absence of the organic solvents under similar conditions. Moreover, the differential scanning calorimetry endotherms of the protein acquired at pH 2.0 were deconvoluted into two distinct peaks, suggesting two cooperative transitions. With increase in pH, the shape of the thermogram changed markedly to exhibit a major and a minor transition. The appearance of two distinct peaks in the DSC together with the non-cooperative thermal transition of the protein in O-states indicates that the molecular structure of ervatamin C consists of two domains with different stabilities.

Keywords: DSC, Ervatamin C, Intermediate, **O**-state, Physiochemical studies, Plant cysteine protease

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Introduction

The native conformation of a protein is a characteristic property of the macromolecule itself, governed by the number, arrangement, and covalent/non covalent interactions of its amino acids. Structural studies of proteins in different solvent systems can provide information about the structure of a protein molecule and of the roles of various stabilizing and destabilizing forces (Timasheff, 1993). Therefore, the effects of various fluorinated and non-fluorinated organic solvents on the structure and unfolding of ervatamin C were studied to obtain further insight of enzyme folding. Ervatamin C is a highly stable cysteine protease belonging to the papain super family, and was isolated from the latex of *Ervatamia coronaria* in our laboratory (Sundd *et al*., 1998). The enzyme (Mr 25 kDa) contains 7 tryptophans, 16 tyrosines, and 7 cysteine residues, which three disulfide bridges leaving one residual cysteine (Sundd *et al*., 1998). Preliminary crystallographic data on ervatamin C has also been reported by our group (Chakrabarti *et al*., 1999). Ervatamin C is structurally stable and does not lose proteolytic activity even after prolonged exposure to pHs in the range 2.0-12.0. Moreover, enzyme unfolding is non-cooperative at low pH (<2.0) contrary to the co-operation seen under neutral conditions (Kundu *et al*., 1999).

Organic solvents can denature proteins; moreover, the resulting products are not completely unfolded but possess a measure of conformation. In addition, the structure of a protein can be made highly ordered or disordered relative to native state by changing the solvent system employed. Generally, partially unfolded states arise due to the different contributions of hydrogen bonds, hydrophobic interactions, and electrostatic interactions of the protein molecule and the solvent system. A wide range of organic solvents, capable of denaturing proteins are known, and alcohols are most widely used as solvents due to their high miscibilities with water. Moreover, the usage of alcohols provides unique opportunities to dissect the contributions made by alkyl groups to protein conformation and to examine the changes induced by altering

alcohol chain length. Alcohols in general have three distinct effects upon proteins and polypeptides: (i) the destruction of the rigid native protein structure, (ii) the induction or enhancement of a helixes, (iii) and the dissolution of peptide aggregates. Their role in stabilizing helical structure (Nelson and Kallenbach, 1989; Lehrman *et al*., 1990), which destabilizes tertiary structure (Conio *et al*., 1970; Herskovits *et al*., 1970; Fink and Painter, 1987), is well documented. The ability of an alcohol to disrupt the internal folding of a protein increases with its chain length and hydrocarbon content, and follows the order trifluoroethanol > propanol > ethanol > methanol (Bianchi *et al*., 1970; Conio *et al*., 1970; Herskovits *et al*., 1970). Thus, the destabilization of a tertiary structure and the stabilization of a secondary structure may induce partial protein unfolding and result in intermediates, which are often referred to as **O**-states. An **O**-state is a stable intermediate on or off the normal pathway of protein folding. In some ways it appears to be similar to the "molten globule-like" state of many proteins (Ptitsyn, 1987; Kuwajima, 1989). Under conditions of moderately low pH, transition to a molten globule-like state can be induced by various means, one being the inclusion of methanol in the solvent composition (Bychkova *et al.*, 1996). The molten globule state is a commonly evoked term for partially folded protein structures with considerable secondary structure, but with few, if any, fixed tertiary structural contacts. Detailed studies of such stable intermediates can provide information useful for understanding the protein-folding problem. Concerning the nature and role of equilibrium intermediates, the following questions need to be answered: How are protein states in different organic solvents related to each other and to other known equilibrium intermediates? Do these intermediate states furnish any information regarding protein structure and folding?

In the present work extensive studies on ervatamin C were carried out in the presence of different organic solvents and solvent compositions. Different spectroscopic methods (e.g., circular dichroism, fluorescence, and absorbance) and proteolytic activity measurements were employed to detect and characterize the organic solvent induced states. In addition, differential scanning calorimetric studies were conducted to monitor the global thermal unfolding of proteins.

Material and Methods

Materials Ervatamin C was purified from the latex of *Ervatamia coronaria* as previously described (Sundd *et al.*, 1998). Sodium tetrathionate was used throughout the purification procedure to avoid any complications due to auto digestion, and the inactivated enzyme obtained was used for all of the spectroscopic studies reported here. The physical properties of the enzyme are same in the free and in the sodium tetrathionate blocked state. Guanidine hydrochloride (GuHCl), methanol, ethanol, propanol, acetonitrile and trifluoroethanol (TFE) were of spectroscopic grade and purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of the highest purity available. Samples for spectroscopic measurements were centrifuged, and filtered through 0.45 mm filters. Exact protein concentrations and pHs were determined.

Methods

Protein concentration Protein concentrations were determined spectrophotometrically using an extinction coefficient ($\varepsilon^{1\%}_{1cm}$) of 24.6 at 280 nm (Sundd *et al.*, 1998).

Enzyme activity assay The hydrolyzing activity of ervatamin c at various pHs and in the presence of organic solvents was determined using its denatured natural substrate azoalbumin, as previously described (Sundd *et al.*, 1998). The enzyme was incubated under the given conditions overnight in denaturant before assay.

Absorbance spectroscopy Absorbance measurements were carried out on a Beckman DU-640B spectrophotometer equipped with a constant temperature cell holder. Protein concentrations for all absorbance measurements were between 4 and 10 mM, and absorbance spectra were recorded between 260 and 320 nm.

Fluorescence spectroscopy Fluorescence measurements were carried out using a Perkin-Elmer LS-5B spectrometer equipped with a constant temperature cell holder. Protein concentrations for all the fluorescence measurements were in the range of 1-6 mM. To determine the tryptophan fluorescence of ervatamin C, excitation was performed at 292 nm and the emission spectrum was recorded from 300 to 400 nm using 10 and 5 nm slit widths for excitation and emission respectively. The temperature of the cell holder was regulated using a Julabo F-25 circulating water bath.

Circular dichroism CD measurements were made using a JASCO J-500A spectropolarimeter equipped with a 500N data processor. The instrument was calibrated with a 0.1% *d*-10-camphor sulphonic acid solution (Cassim and Yang, 1969). Conformational changes in secondary protein structure were monitored in the region between 200 and 260 nm at a protein concentration of approximately 0.1 mg/ml using a 1 mm path length cuvette, and changes in tertiary structure were observed using a 10 mm path length cuvette in the region between 260 and 320 nm at a protein concentration of approximately 0.75 mg/ml. After subtracting appropriate blanks, mean residue ellipticity was calculated, using:-

$$[\theta] = \theta_{obs} \times MRW/10cl \quad (1)$$

Where, θ_{obs} is the measured ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein in g/ml and l is the path length in cm (Balasubramanian and Kumar, 1976). A mean residue molecular weight of 110 was used, and the temperature of the cell holder was regulated using a Julabo F-25 circulating water bath.

Guanidine hydrochloride induced unfolding The chemical induced denaturation of ervatamin C, at a given pH, was performed using various denaturant concentrations. Protein samples were incubated with different concentrations of denaturant for approximately 24 h at 25°C to attain equilibrium. Final protein concentrations and denaturant were determined by

spectrophotometry and refractive index measurements respectively. Data are expressed in terms of unfolded fraction F_u calculated using the equation

$$F_u = (F_{obs} - F_n)/(F_u - F_n) \quad (2)$$

Where, F_{obs} is the observed value of the CD signal at any given denaturant concentration and F_n and F_u are the extrapolated values of native and unfolded protein respectively. Assuming a standard two-state model, GuHCl and urea transitions were fitted to the equation:

$$\Delta G_d = \Delta G_w \, m_{D\text{-}N} \Delta \quad (3)$$

Where, ΔG_w and ΔG_d are the free energy of folding in water and at a denaturant concentration of D, respectively, $m_{D\text{-}N}$ is the slope of the transition (proportional increase in solvent-accessible surface area of the transition relative to the native denatured state) and D is the denaturant concentration (Pace, 1990).

Thermal unfolding Temperature induced changes in the structure of ervatamin C were followed by CD (far and near UV) and by tryptophan fluorescence. Protein samples were incubated at the desired temperature for 15 min before each measurement. The actual temperature of the sample in the cuvette was measured using a thermocouple connected to a digital multi-meter. Occasionally, samples were also checked for possible heat induced aggregation by taking light scattering measurements.

Differential scanning calorimetry Calorimetric scans were performed using a Microcal MC-2 (Microcal, Northampton, USA) differential scanning calorimeter, in the pH range 2.0-4.0. Protein solutions of 0.6-0.8 mg/ml were extensively dialyzed against 0.02 M gly-HCl, pH 2.0, 3.0 and 0.02 M sodium acetate buffer, pH 4.0. After dialysis, the protein concentrations and pHs of the samples were checked. All solutions were degassed under vacuum before being loaded into the calorimeter cells. The calorimetric experiments were carried out at a scan rate of 60ºC/h. Buffer-buffer base lines were obtained using the same conditions and subtracted from sample curves. The DA-2 software package (Microcal) was used for automatic data collection and data analysis, which included baseline subtraction and the calculation of ΔH_{cal} and $\Delta H_{vant\ Hoff}$

Results

The effects of different organic solvents (fluorinated and non-fluorinated alcohols and acetonitrile) on the structure and function of ervatamin C were studied using various spectroscopic and activity measurements. Under neutral conditions, the activity and structural integrity of ervatamin C were retained even in the presence of high concentration (>80%) of organic solvents. But significant structural changes were seen at lower pH (<2.0), and these were dependent on solvent concentration. Ervatamin C was found to be structurally stable and biologically active even after prolonged exposure to pHs in the range 2.0-12.0 (Sundd *et al*., 1998). In lower pH range, chemically induced ervatamin C unfolding is distinctly different from that under neutral conditions,

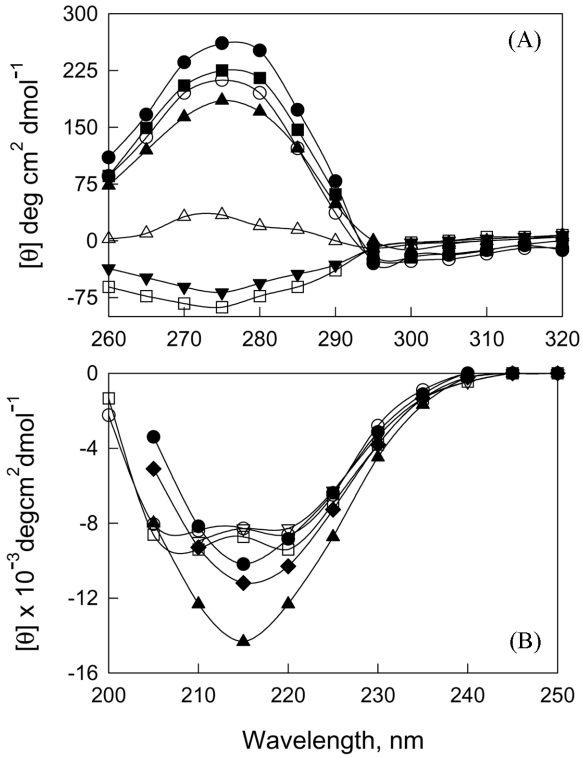

Fig. 1. Effect of methanol on CD spectra of ervatamin C. Near-UV CD spectra (A) of ervatamin C in 0.05 M gly-HCl, pH 2.0 containing (●) 0%, (■) 10%, (○) 20%, (▲) 30%, (△) 40%, (▼) 50% and (□) 90% methanol. (B) Far-UV CD spectra of ervatamin C in 0.05 M gly-HCl, pH 2.0 containing (O) 0%, (▽) 10%, (□) 30%, (●) 50%, (◆) 70% and (▲) 90% methanol. Spectra were recorded after incubating for 24 h at 25ºC. A protein concentration of 4 mM was used in each sample.

signifying that its molecular state differs slightly from the native state, although most of its spectral features are similar. Moreover, the enzyme has been reported to unfold to an intermediate state at pH 2.0, which is absent under neutral conditions (Kundu *et al*., 1999). Therefore, we selected pH 2.0 to investigate the folding of ervatamin C in the presence of organic solvents.

Effect of non-fluorinated alcohols on ervatamin C In the aromatic region, the CD spectra of native ervatamin C at pH 2.0 exhibits a positive peak centered at 276-278 nm and a negative band centered at 295-298 nm. Moreover, the magnitudes of the peak intensities of ervatamin C reduced on increasing the methanol concentration (Fig. 1A), for example, increasing the methanol concentration from 30% to 40% caused a sharp decline in ellipticity at 278 nm. All tertiary structural features were absent at a methanol concentration of 50%, in addition band intensities were much reduced and the spectra resembled that of the protein unfolded in 6 M GuHCl.

Similarly, changes in the secondary structure of ervatamin C were also followed using far-UV CD as a function of increasing methanol concentration, as shown in Fig. 1B. At

methanol concentrations of up to 30%, the protein was α-helical and showed ellipticities equivalent to those of the native state with two negative bands at 220 nm and at 208 nm. The further addition of methanol, to a final concentration of 50%, resulted in the sudden appearance of a negative peak at 215 nm, which is characteristic of a β-sheet conformation. These results contradict the notion that all alcohols are strictly helix inducers. Moreover, a loss of proteolytic activity (data not shown) also occurred concomitantly with the loss of tertiary structure, and these were followed by a loss of secondary structure. In the presence of 50% of methanol ervatamin C lost all rigid tertiary structure and activity, but substantial secondary structure was retained with an a-helical to b-sheet switch in the secondary structure. Thus, it is concluded that at pH 2.0 in presence of 50% methanol the enzyme exists in an intermediate state with molten globule like characteristics. We have assigned this methanol induced intermediate state as the $\mathbf{O}_{met}$-state of ervatamin C. Similar conformational switches from a-helical to b-sheet were found to occur in the presence of ethanol and propanol, but at lower alcohol concentrations, 45% and 40% respectively (Data not shown).

Effect of fluorinated alcohol on ervatamin C TFE induced conformational changes in ervatamin C at pH 2.0 as monitored by circular dichroism (in both the near- and far-UV), tryptophan fluorescence, and by proteolytic activity measurements. Increases in TFE concentrations reduced the amplitude of ellipticity at 278 nm and the near-UV CD spectrum was substantially attenuated at a TFE concentration of 25% indicating the complete loss of the rigid native tertiary structure. CD spectra at higher TFE concentrations closely resembled the spectrum of ervatamin C in 6 M GuHCl (Fig. 2A).

TFE induced changes in the far-UV region, which reflect overall changes in the secondary structure of ervatamin C, are presented in Fig. 2B. On increasing the concentration of TFE to 90%, ellipticities at 208 nm and 220 nm, characteristic of a-helicity in the protein molecule, were enhanced, implying an overall increase in the proteins helical content.

At pH 2.0, TFE concentration increases induced changes in ellipticity occurred in two stages. In the concentration range 0-25% (the first stage) $[\theta]_{222}$ remained close to the native state whereas $[\theta]_{278}$ decreased. The breakdown of the native tertiary structure and the formation of a non-native secondary structure with enhanced CD amplitude occurred during this stage. The second stage, took place in the TFE concentration range 30-80%, and involved changes in secondary structure (ellipticity at 222 nm) following a sigmoidal transition, thus indicating an increase in helicity.

The loss of proteolytic activity (data not shown) and changes in the tertiary structure (fluorescence and monitored by θ_{278} of ervatamin C preceded changes in the secondary structure (monitored at θ_{222}). At a TFE concentration of 25%, ervatamin C lost all of its proteolytic activity and rigid tertiary structure in concert with the formation of an enhanced secondary structure. Hence, ervatamin C in 25% TFE had characteristics of the molten globule state. To avoid complications in the presentation of different organic solvent induced states, we define this intermediate state as the $\mathbf{O}_{TFE}$ state of ervatamin C.

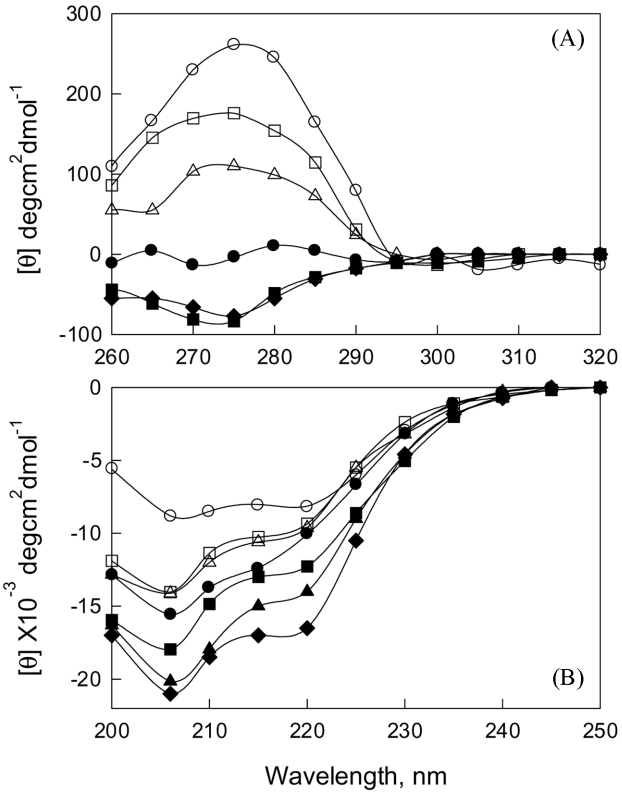

Fig. 2. Effect of TFE on the CD spectra of ervatamin C. Effect of TFE on the (A) Near- and (B) far-UV CD spectra of ervatamin C in 0.05 M gly-HCl, pH 2.0 containing (○) 0%, (□) 10%, (△) 20%, (●) 25%, (■) 40%, (▲) 60% and (◆) 90% TFE. Spectra were recorded after incubating the samples for 24 h at room temperature.

Acetonitrile induced ervatamin C structural changes Acetonitrile is widely used for reverse phase chromatography, for the purification of proteins, and sometimes to preserve proteins. The effect of acetonitrile on the tertiary structure of ervatamin C as a function of increasing acetonitrile concentrations at constant pH is shown in Fig. 3A. With increased acetonitrile concentration, the amplitude of the ellipticity at 278 nm reduced drastically, and at a concentration of 25% all spectral features were lost suggesting that the rigid native tertiary structure of the protein had been reduced to a form similar to unfolded protein in 6 M GuHCl.

Although increasing concentrations of acetonitrile caused only a slight reduction in the ellipticity at 220 nm with some enhancement of CD amplitude at 208 nm, indicating a breakdown of the intact native secondary structure (Fig. 3B),

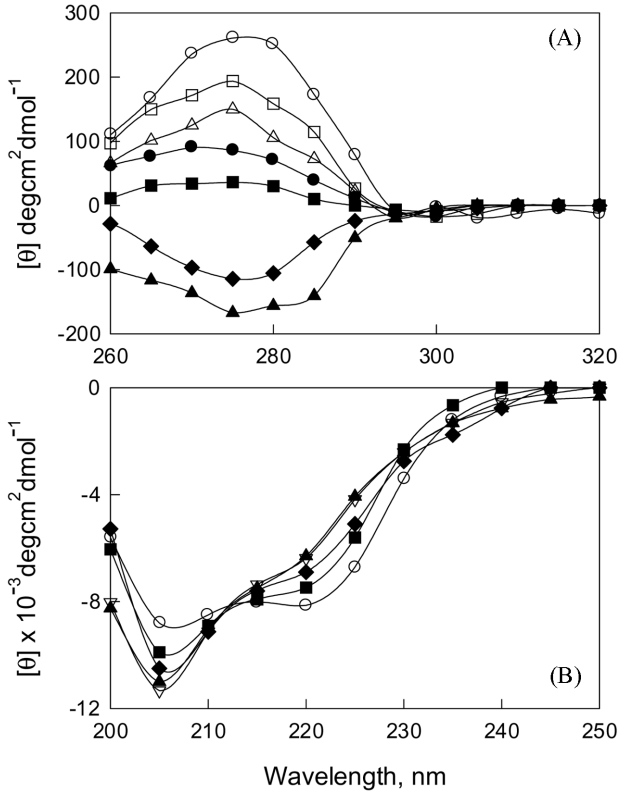

Fig. 3. Effect of acetonitrile on the CD spectra of ervatamin C. Circular dichroism of ervatamin C 0.05 M gly-HCl, pH 2.0. (A) Near- and (B) far-UV CD spectra of ervatamin C in (○) 0% (■) 5%, (△) 10%, (●) 15%, (■) 20%, (◆) 25%, (▽) 50%, (▲) 70% acetonitrile. Protein concentrations were 0.1mg/ml in the far-UV and 0.75 mg/ml in the near-UV. Spectra were recorded after incubating samples for 24 h at room temperature.

an isodichroic point was observed at 212-213 nm. A single cross-over point like this during transition suggests the presence of a two-state transition for the unfolding of ervatamin C, i.e., from the a-helix to the unfolded state without the intervention of a non-native (b-sheet) state, as was observed in presence of methanol. CD data could not be collected beyond an acetonitrile concentration of 70%, as the samples showed signs of aggregation even at low protein concentrations. The loss of the native rigid tertiary structure and activity (data not shown) occurred before any significant changes in the secondary structure of ervatamin C. All protein changes were complete at an acetonitrile concentration of 25%, when the enzyme had no tertiary structure or activity but an enhanced secondary structure. We have defined this molten globule-like intermediate state as the $\mathbf{O}_{ace}$-state of ervatamin C.

GuHCl induced unfolding As described above, it appears that ervatamin C is driven into different types of **O**-state depending on the type and concentration of the organic solvent used (50% methanol, 25% TFE, or 25% acetonitrile). Moreover, ervatamin C exhibited different structural features

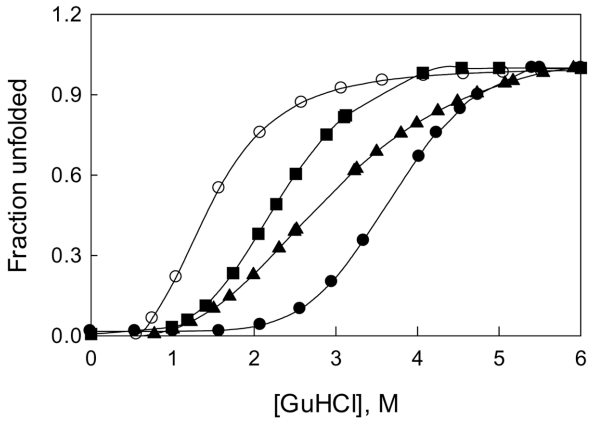

Fig. 4. Stability of the organic solvent induced states of ervatamin C. GuHCl induced denaturation of ervatamin C in (○) buffer and in the presence of (▲) 25% TFE, (●) 50% methanol, or (■) 25% acetonitrile as determined by far-UV CD. The denaturation of ervatamin C was followed by monitoring ellipticity at 220 nm for TFE and acetonitrile, or at 215 nm for methanol. The buffer used was 50mM gly-HCl, pH 2.0 at 25ºC.

in different organic solvent induced states (**O**-states). Therefore, we followed the unfolding of the molecule to gain an insight of the its structural changes and other subtle differences. GuHCl-induced unfolding of the **O**-state relative to that of the protein in the absence of organic solvent at pH 2.0 is shown in Fig. 4. Changes in its secondary structure upon unfolding were followed by taking far UV CD measurements. The data obtained was normalized and then analyzed using equations (2) and (3) in *Methods*. Respective thermodynamic parameters along with corresponding transition midpoints are summarized in Table 1. The transition midpoints of the unfolding of ervatamin C and $DG_{U\text{-}N}$ in the **O**-states were higher in the presence than that in the absence of organic solvents. The extent of stabilization followed the order $\mathbf{O}_{met}$-state > $\mathbf{O}_{TFE}$-state > $\mathbf{O}_{ace}$-state. Differences in the stability, secondary structural features, and the fact that the **O**-states have no tertiary structure, confirm that the organic solvent induced states of ervatamin C differ from each other and from the native state.

Temperature induced unfolding Temperature induced unfolding of ervatamin C in the organic solvent induced state (**O**-state), as determined by changes in secondary structure is shown in Fig. 5. The thermal unfolding of ervatamin C was followed by determining changes in mean residue ellipticity at 220 nm in the case of trifluoroethanol and acetonitrile. Whereas, for the $\mathbf{O}_{met}$-state, the unfolding of the protein was monitored by mean residual ellipticity at 215 nm, as the protein in this state exists predominantly as a β-sheet. The thermal denaturation of ervatamin C at pH 2.0 in the absence of any organic solvent was cooperative. However, in all the O-states, the unfolding of ervatamin C was non-cooperative and the transition curves were biphasic. A summary of the thermal

Table 1. Thermodynamic parameters of the GuHCl unfolding of ervatamin C in different states

Condition	Transition mid point (C_m)	ΔG_{U-N} (kcal mol^{-1})	m_{U-N} (kcal mol^{-1} M^{-1})
pH 2.0, 25°C	1.6 ± 0.1 M	-1.25 ± 0.1	-1.17 ± 0.1
$\mathbf{O}_{met}$-state	3.5 ± 0.1 M	-3.5 ± 0.2	-1.0 ± 0.1
$\mathbf{O}_{TFE}$-state	2.7 ± 0.1 M	-1.9 ± 0.2	-0.7 ± 0.1
$\mathbf{O}_{ace}$-state	2.2 ± 0.1 M	-1.5 ± 0.2	-0.9 ± 0.1
pH 7.0, 25°C (Curve not shown)	4.8 ± 0.1 M	-11.0 ± 0.2	-1.8 ± 0.1

The calculated thermodynamic parameters are approximate, as they do not take into account interactions present in native ervatamin C that are irreversibly lost on unfolding

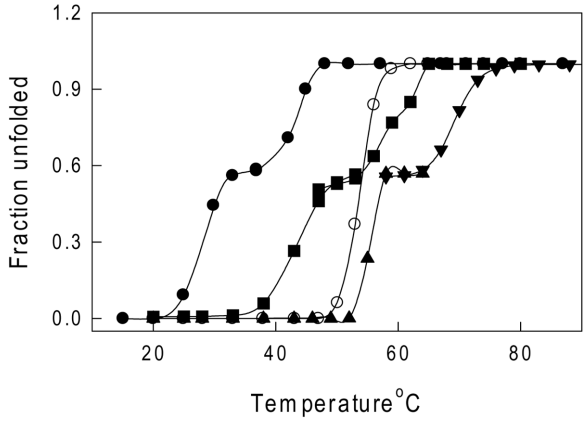

Fig. 5. Thermal stability of the organic solvent induced states of ervatamin C. Thermal unfolding of ervatamin C in (○) buffer, (■) 50% methanol, (▲) 25% TFE, and in (●) 25% acetonitrile at pH 2.0. Ervatamin C denaturation was followed by monitoring ellipticity at 220 nm for TFE and acetonitrile or 215 nm for methanol. Data have been normalized versus the fraction of unfolded protein. The buffer used was 0.05 M gly-HCl at pH 2.0.

stability of ervatamin C in the presence of organic solvent is shown in Table 2. The thermal transition of ervatamin c in the $\mathbf{O}_{met}$-state is biphasic in nature and crosses the transition curve of the protein in the absence of methanol, at the same pH at 53°C. Similarly, in the $\mathbf{O}_{TFE}$-state, the thermal transition is non-cooperative and biphasic with two transition midpoints higher than the midpoint observed in the absence of TFE. The temperature induced unfolding of ervatamin C in the $\mathbf{O}_{ace}$-state also occurred in two steps with two transition midpoints lower than the midpoint observed in the absence of acetonitrile, suggesting the uncoupling of the two domains and a resulting unfolding of the domains. It is evident that the presence of acetonitrile in the solvent composition destabilizes the whole protein molecule.

Differential scanning calorimetric studies on ervatamin C

In our continuous efforts to shed light on the molecular structure of ervatamin C, the thermal unfolding of the protein was also monitored by DSC. A differential scanning calorimetric thermogram of ervatamin C obtained at pH 2.0 and 3.0 is shown in Fig. 6. The thermograms obtained were deconvoluted into two peaks with specific transition temperatures at both pHs. Both components of the transitions were affected to a similar extent by pH increases. However, the intensity of the second transition was lower at pH 3.0. This observation is consistent with earlier results obtained in the presence of organic solvents, and suggests that the enzyme is composed of discrete domains, each of which unfold as a single entity. To determine the enthalpy of unfolding, calorimetric curves were corrected for permanent heat capacity difference, $\Delta C_{p,}$ by subtracting of a linear base line (buffer-buffer) connecting the initial and final temperatures of the transitions and the excess heat capacity function, C_p^{ex}, was calculated and from it ΔH_{cal} and $\Delta H_{vant\ Hoff}$ were then determined. The thermodynamic characteristics of ervatamin C denaturation are shown in Table. 3.

The DSC profile of ervatamin C at pH 4.0 is shown in Fig. 7. Unlike that at pH 2.0 and 3.0, the thermogram at pH 4.0 showed a predominant transition with a midpoint at 76.67°C and a second transition with much less heat capacity with a midpoint at 83.05°C. The corresponding calorimetric enthalpy values were 192 kcal/mole and 82 kcal/mole. The endotherm

Table 2. Thermal stability of ervatamin C in the presence of organic solvents

Conditions	Transition midpoints, °C		Fraction unfolded at the intersection of two transitions
	T_{m1}	T_{m2}	
pH 2.0	53.73		--
pH 2.0 + 25% TFE	55.0 ± 0.5	68.8 ± 0.5	0.50
pH 2.0 + 50% methanol	42.5 ± 0.5	59.3 ± 0.5	0.55
pH 2.0 + 25% acetonitrile	27.5 ± 0.5	42.6 ± 0.5	0.47

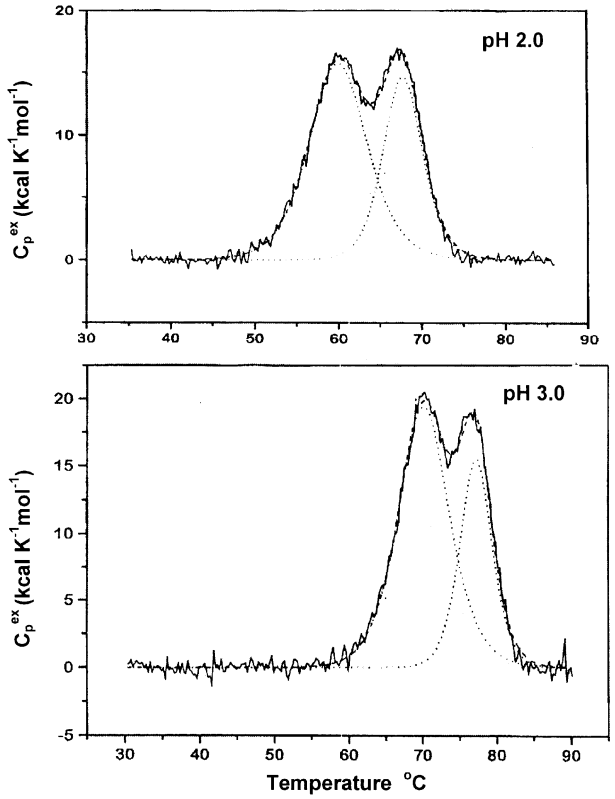

Fig. 6. DSC of ervatamin C as a function of pH. The enzyme was extensively dialyzed against 0.02 M gly-HCl at pH 2.0 or 3.0 before taking measurements. The protein concentrations were 0.677 mg/ml and 0.701 mg/ml, respectively. The de-convolution results obtained are consistent with two distinct transitions. A second scan indicated irreversibility. The scan rate used was 60°C/h, and the dotted line represents the fitted curves.

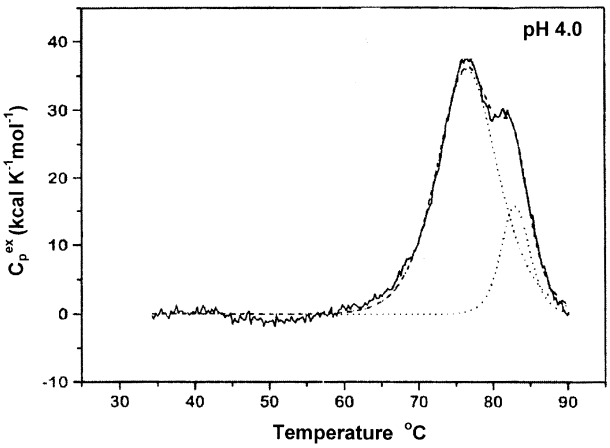

Fig. 7. Excess heat capacity function versus temperature for ervatamin C. Calorimetric scans were performed at protein concentration of 0.701 mg/ml and 0.726 mg/ml at a scanning rate of 60°C/h in 0.02 M gly-HCl, pH 3.0 or in 0.02 M sodium acetate, pH 4.0. The excess heat capacity function was obtained by subtracting baseline from the heat capacity function. Reheating of the samples indicate irreversible thermal denaturation. The dotted line represents the fitted curve.

was shifted to appreciably higher temperatures. At pH 5.0 and higher, thermal denaturation resulted in protein precipitation. This may be due to the high protein concentrations used for DSC measurements, but it was not possible to use concentrations below 0.6 mg/ml due to the sensitivity limit of the instrument used.

Discussion

The proteolytic activity of ervatamin C was preserved over a wide pH range from 2.0 to 12.0. Under neutral conditions, ervatamin C was stable even at high concentrations of organic solvent, more than 80% (Sundd *et al.*, 1998). However, the enzyme becomes susceptible to organic solvents on lowering the pH. In the presence of methanol (non-fluorinated, aliphatic, monohydric alcohol), a sudden switch from the native a-helical structure to a predominantly b-sheet conformation occurred along with a loss of tertiary structure when the methanol concentration was increased from 40% to 50%. This resultant intermediate (**O_{met}**-state) had no tertiary structure or activity, but did have a substantial secondary structure-predominantly a β-sheet structure. This α-helix to β-sheet switch may be due to the high amino acid content of the β-sheet forming amino acids, as proposed by Yang (1967). However, TFE concentration induced changes in ervatamin C occurred in two steps, the native secondary structure (ellipticity 222 nm) was preserved up to 25% TFE where as the tertiary structure was highly reduced. At above this concentration, a sharp increase in a-helical content was observed. Wilkinson and Mayer (1986) reported a similar two stage increase phenomenon in the secondary structure of ubiquitin. Thus, it could be hypothesized that in presence of TFE, ervatamin C unfolds to a non-native structure with a loss of tertiary structure followed by an increase in α-helicity, which drives the molecule to a new conformation (**O_{TFE}**-state). Moreover, the unfolding of ervatamin C, in the presence of acetonitrile at pH 2.0, revealed that it is likely that only the

Table 3. Thermodynamic characteristics of ervatamin C denaturation

	Transition Midpoint, °C		ΔH_{cal} (kcal/mole)		$\Delta H_{vant\ Hoff}$ (kcal/mole)		$\Delta H_{cal}/\Delta H_{vant\ Hoff}$	
pH	T_{m1}	T_{m2}	$\Delta H1_{cal}$	$\Delta H2_{cal}$	$\Delta H1_{vH}$	$\Delta H2_{vH}$	$\Delta H_{cal}/\Delta H_{vH}$	$\Delta H_{cal}/\Delta H_{vH}$
2.0	60.0 ± 0.5	68.0 ± 0.5	140.0 ± 2.0	96.0 ± 1.5	100.0 ± 2.0	137.0 ± 2.0	1.40	0.71
3.0	70.0 ± 0.5	77.0 ± 0.5	179.0 ± 3.0	89.0 ± 1.0	122.0 ± 2.0	127.0 ± 2.0	1.45	0.70

first stage involving the loss of tertiary structure occurs and that the reduction in a-helicity is marginal.

O_{met}-state protein had a predominantly b-sheet structure while the **O_{TFE}**-state showed enhanced α-helicity. On the other hand, the **O_{ace}**-state showed a marginal reduction in the α-helical nature of the protein. Moreover, all three organic solvents induced states with no tertiary structure or activity, and the conformational stabilities of all states differed (Table 1). Thus, it can be concluded that all the organic solvent states (**O**-states) of ervatamin C differ in terms of their secondary structure features and that they resemble a molten globule states. It appears that the prerequisite for b-sheet induction in the **O_{met}**-state is the loss of tertiary interactions and functional activity.

Alcohols in general have two main effects on proteins; they stabilize helical structures but destabilize rigid tertiary structures (Avdulov *et al.*, 1996; Bodkins and Goodfellow, 1996). Moreover, these combined effects of alcohols may induce partially folded intermediates. A knowledge of partially structured states, intermediate between two extreme states, should provide some insight of the relative strengths and of the different types of interactions that can stabilize a given section of a polypeptide chain, and thus may provide information regarding the interactions responsible for directing the folding of proteins. The existence of an intermediate in alcohol induced protein denaturation has long been supported (Timasheff, 1970). It was shown that near-UV CD spectra of lysozyme, and of some other proteins, change at lower alcohol concentrations than their far-UV CD spectra, suggesting that small conformational changes precede larger changes.

Temperature induced unfolding of ervatamin C in all three organic solvent systems, was non-cooperative in nature. All known papain like cysteine proteinases, except cathepsin-C, are monomers whose structures consist of two domains referred to as R and L domains (Turk *et al.*, 1997). The occurrence of two transition midpoints in the presence of organic solvents is consistent with the view that ervatamin C has two domains that unfold sequentially. The thermal unfolding transition curve of ervatamin C in its **O_{met}**-state crosses the transition curve obtained in the absence of methanol at the same pH at 53ºC, suggesting that one region in the protein molecule is stabilized while another is destabilized in the presence of methanol. Similarly, in the **O_{TFE}**-state, two transition midpoints were higher in the presence of TFE than in its absence indicating that the protein molecule as a whole is stabilized. This increase in stability can be attributed to the hydrogen bonding property of TFE. Similarly, temperature induced unfolding in the **O_{ace}**-state of ervatamin C also occurred in two steps suggesting the uncoupling of the two domains and the subsequent unfolding of these domains. Due to the different degrees of stabilization/ destabilization of α- or β- rich domains in different **O**-states, differences in the stabilities of these domains become more significant and this causes a biphasic transition. In addition, the presence of acetonitrile in the solvent composition destabilizes the protein molecule, which is depicted by a transition curve with midpoints shifting to a lower temperature. Surprisingly, higher concentrations of GuHCl were required to unfold the different **O**-states of ervatamin C than the native protein at pH 2.0, even though the former possesses no tertiary structure as detected by near UV CD. The order of magnitude of stability towards GuHCl-induced unfolding was **O_{met}**-state > **O_{TFE}**-state > **O_{ace}**-state, but thermal stability of **O_{TFE}**-state was greater than that of **O_{met}**-state**.** Moreover, the GuHCl induced unfolding of ervatamin C in the **O**-state was cooperative while thermal unfolding was non-cooperative. These differences in unfolding may be attributed to the ionic nature of GuHCl, which is an electrolyte with a pKa of ca. 11.0, which mean at pHs lower that this the GuHCl molecule is fully dissociated, i.e., into Gu^+ and Cl^-. Moreover, the presence of these ions could influence the stabilization/ destabilization properties of the protein (Mayr and Schmid, 1993), resulting in to different unfolding behavior. Alternatively, these differences in unfolding may be due to the fact that GuHCl is a strong denaturant and that it unfolds the molecule as a whole, not in a sequential manner. These complexities of the unfolding of the **O**-states of the enzyme make for interesting further study. A similar result, i.e., of increased the structural stability of a molten globule like intermediate state was reported in barstar (Khurana and Udgaonkar, 1994).

The interactions between the domains or subdomains of proteins often involve the close apposition of hydrophobic surfaces, and these domain-domain interactions account for most of the structural stability of a protein (Palme *et al*., 1997). Moreover, subunit interactions and domain interactions are known to be dominated by hydrophobic stabilization. Hydrophobic interactions have been prominently implicated as key determinators of the native configurations of proteins in aqueous solution. Actually, these interactions are not of a single relatively well-defined character, i.e., as electrostatic or hydrogen bond interactions, but are rather associated with the interactions responsible for the immiscibility of nonpolar substances and water. Proteins contain a substantial proportion of amino acids, such as phenylalanine, valine, and leucine, which contain nonpolar side chain residues that tend to cluster on the inside of the protein molecule away from the aqueous environment. Organic solvents basically perturb protein hydrophobic interactions by reducing the differential between hydrophilicity and hydrophobicity. Thus, in the case of ervatamin C it appears that the two structural domains have a different levels of hydrophobic stabilization, and that the addition of additives like organic solvents leads them in an uncoupling direction. The well-separated thermal transitions ervatamin C suggest significant differences in their intrinsic stabilities. Possibly, one of the two domains is less stable and unfolds at a lower temperature than the other, causing the denaturation process to proceed in a sequential manner. However, in presence of GuHCl, the molecule was observed

to unfold as a single unit.

The thermal denaturation of ervatamin C was also studied by high-sensitivity differential scanning calorimetry. The fact that no or very little endotherm was observed, when previously scanned protein was rescanned, indicated that the thermal denaturation of ervatamin C is irreversible under the pH conditions studied. However, thermal denaturation up to a temperature of 60-65ºC at pH 2.0 and 3.0 was reversible. This corresponded to the unfolding of only one protein domain, represented by the first endotherm peak. Thus, this intermediate conformation can refold to the native conformation on cooling (data not shown).

The propriety of applying equilibrium thermodynamics to apparently irreversible processes has been discussed by Manly *et al.* (1985) in the context of the denaturation of core protein of lac repressor, and by Edge *et al.* (1985) for the ATCase sub unit. As discussed by Edge *et al.* (1985), calculations for a model system in which protein undergoes a reversible denaturation followed by an irreversible step, show that data for such a system can be analyzed according to the vant Hoff equation and yield parameters close to those originally assigned to the reversible system. Thus, despite the irreversible nature of the thermal denaturation of ervatamin C, it is possible to analyze results in terms of equilibrium thermodynamics, as has been found for other proteins exhibiting irreversible denaturation in DSC experiments (Manly *et al.*, 1985; Edge *et al.*, 1985; Hu *et al.*, 1987; Edge *et al.*, 1988; Brandts *et al.*, 1989). Our differential scanning calorimetric results suggest that during thermal denaturation the domains unfold sequentially which results in two cooperative transitions as the thermogram progresses. However, the $\Delta H_{cal}/\Delta H_{vant\ Hoff}$ ratio was slightly higher for first transition. This may be due to strong inter-domain interactions resulting in the accumulation of intermediates that differ from the native state, or a molecular state where one domain remains intact while the other unfolds. The results of differential scanning calorimetry studies provide additional support to the view that ervatamin C is composed of two structurally distinct domains. The thermogram of ervatamin C at pH 2.0, 3.0, and 4.0 showed two distinct peaks, and a similar thermogram observed in rat brain hexokinase was been attributed to the thermal unfolding of two independent structurally and functionally distinct domains (White *et al.*, 1990). Another example is provided by yeast hexokinase B, a monomer, where calorimetric profiles characterized by the presence of two partially overlapping peaks can be ascribed to the presence of two structural domains in the native structure (Catanzano *et al.*, 1997).

The unfolding of the α and β domains is cooperative in ervatamin C, as evidenced by the ratio of calorimetric to vant Hoff enthalpies, which was approximately 1.0. It is known that many small globular proteins behave as single cooperative units on unfolding and that they show no stable intermediates during the process (Privalov, 1979; Kim, 1982). In these cases, decisive testing for the absence of stable intermediates has relied on enthalpy change comparisons as determined calorimetrically, DH_{cal} with the vant Hoff enthalpy, and $\Delta H_{vant\ Hoff}$, deduced from the temperature dependence of the equilibrium constant (Privalov and Kechinashvilli, 1974; Cantor and Schimmel, 1980; Sturtevant, 1987). The ratio $\Delta H_{vant\ Hoff}/\Delta H_{cal}$ equals unity for the simplest two state transitions where only fully folded or unfolded molecules exist. However, the unfolding of many larger proteins is more complex, and for multi-component (non two-state) processes, $\Delta H_{vant\ Hoff}$ is smaller than ΔH_{cal}.

At pH 2.0, the two peaks in the thermogram had equal ΔC_p values, though the latter peak decreased at pH 3.0-4.0 suggesting stronger interdomain interactions. Extrapolating our results to neutral pH, it appears that at a higher pH thermal unfolding may involve a single transition, indicating strong interdomain interactions within the protein molecule. Moreover, interactions between domains can be expected to influence the overall stability of a protein. Apparently, the precise behavior of a protein during unfolding depends on the delicate balance between domain-domain interactions and the intrinsic stabilities of the domains (Brandts *et al.*, 1989). Moreover, a rise in T_m at higher pH is accompanied by an increase in the area of heat absorption, representing an increase in the calorimetric enthalpy. This is possibly the result of the stabilizing effect of a reduced net positive charge on the protein as the pH increases. Interestingly, the thermal unfolding of ervatamin C at pH 2.0, as monitored by CD, is cooperative with single transition (Fig. 5). Differences between the observed transitions by CD and DSC are attributed to different heating rates (see *Methods*). The DSC experiments were carried out at a scan rate 60ºC/h, whereas to examined temperature induced protein unfolding by CD a sample was incubated at the given temperature for 15 minutes, and thus the protein is destabilized at a lower temperature, which results in a lower transition mid point. We assume in this case that the difference between the domain stabilities of ervatamin C, are small and that the protein unfolds as a single entity. Whereas, the rate of heating in the DSC experiments was more rapid, and that this resulted in a thermogram with a biphasic nature.

The present study, also demonstrates that one domain is intrinsically more stable than the other. As ervatamin C belongs to the α+β class of proteins, which have independent α-rich and β-rich regions as reported in papain, presumably the α-rich domain is intrinsically less stable than the β-rich domain (Edwin and Jagannadham, 1998; Sharma and Jagannadham 2003) and possibly its unfolding corresponds to the first DSC transition. Our DSC studies suggest that ervatamin C is a two-domain protein and that strong domain-domain interactions explain its extraordinary stability; moreover, such interactions are usually hydrophobic in nature. Organic solvents mainly weaken such hydrophobic interactions and cause sequential unfolding of the two domains. The existence of two domains in the molecular structure of ervatamin C is also supported by the temperature

transition of the **O**-state. One domain of ervatamin C is unfolded while the other is intact in the O-state, but at different temperatures (32-38°C for the $\mathbf{O}_{met}$-state; 48-57°C for the $\mathbf{O}_{ace}$-state and 57-67°C for the $\mathbf{O}_{TFE}$-state). This interpretation is based on the fact that under these conditions, the first transition corresponds to the completion of the unfolding of one domain, while the other has not yet started. Therefore, it can be safely concluded that at temperatures above those shown above protein has an unfolded á-rich domain.

Hydrophobic interactions play crucial roles in stabilizing the native conformations of proteins. Moreover, a marked reduction in hydrophobic interactions due to almost any non-aqueous water miscible solvent must be critically involved in observed conformational changes. Intra-molecular hydrogen bonds are important at determining these structures, but hydrophobic interactions make the largest single contribution to stabilizing the native conformations of these macromolecules in aqueous solution. In the absence of these interactions, the stability of a molecule reduces, as evidence by thermal transitions in the presence of acetonitrile; a similar case of thermal destabilization has been reported in lysozyme in presence of acetonitrile (Kovrigin, 2000). Several hypotheses have been proposed to account for the denaturation/ inactivation of enzymes in organic solvents (Ogino and Ishikawa, 2001); (a) water molecules in the enzyme are stripped away or replaced by solvent molecules thereby causing deformation or denaturation, (b) organic molecules bind to specific enzyme sites, (c) interfacial or surface tension of solvents destroys the tertiary structure of enzymes in two phase systems. However, enhanced stability in the $\mathbf{O}_{TFE}$- and $\mathbf{O}_{met}$-states is probably due to the hydrogen bond forming ability of alcohols.

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