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ORGANIC SOLVENT INDUCED REFOLDING OF ACID DENATURED HEYNEIN: EVIDENCE OF DOMAINS IN THE MOLECULAR STRUCTURE OF THE PROTEIN AND THEIR SEQUENTIAL UNFOLDING

N. K. Prasanna Kumari and M. V. Jagannadham

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India

Abstract: Organic solvent (methanol and trifluoroethanol) induced-refolding of the acid denatured heynein (α+β class of protein) is studied using optical spectroscopy. In the organic solvent induced state (O-state) heynein exhibits considerable amount of secondary structure and the fluorescence emission maximum shifts to lower wavelength. At a certain concentration of methanol a conformational switch occurs from α+β to β-sheet state in the protein, while the α-helicity enhances with TFE. Chemical- induced unfolding of heynein in O-state is non-cooperative due to differential stabilization of α and β regions of the protein. On the other hand thermal unfolding of heynein in **O**-state indicates an overall destabilization of the protein molecule in the case of methanol and a biphasic transition in the case TFE. These observations suggest the presence of two structural parts (may be domains) in the molecular structure of heynein with different stabilities, which unfold sequentially.

Keywords: Cysteine protease; Heynein; Molten globule; Domains; Sequential unfolding.

Introduction

Solvent plays an important role in maintaining the native structure of a protein and the conformational changes that occur in different solvents can provide information about the structure of a protein molecule as well as the role of various stabilizing and destabilizing forces that are responsible for the unique folded structure of the protein in solution. Organic solvents usually affect the native structure of a protein, thus interactions of the protein with water-organic solvent mixtures is of importance. Therefore proteins may adopt a radically different conformation with the changes in the solvent constitution and composition.

"Solvent engineering" using unconventional solvents is a potential approach to modulate the protein structure. Elucidation of the driving forces responsible for the formation of non-native states will also throw light on the protein-folding pathway. The resultant state of a protein in the organic solvent arises due to the effect of the organic solvent on hydrogen bonds, hydrophobic interactions and electrostatic interactions in the protein structure. Therefore, solvent composition modulates both the structural and functional properties of biological macromolecules (Dill, 1990).

Alcohols have distinct effects upon the structure of proteins and polypeptides like destruction of rigid native structure, induction of α-helix as well as dissolution of peptide aggregates. In general alcohols stabilize the helical structure while destabilize the tertiary structure of the protein (Fink and Painter, 1987; Nelson and Kallenbach, 1989). The stabilization of secondary structure and destabilization of tertiary structure results in partially unfolded protein, which is different from the native state and, often referred as **O-state**. Such O-state is a stable intermediate observed on or off in the protein folding pathway and similar to the "molten globule" state observed in the case of many proteins. The "molten globule state" is a partially folded structure of a protein with considerable amount of secondary structure

Corresponding Author: **Prof. M. V. Jagannadham**
E-mail: jvm@bhu.ac.in; jaganmv@satyam.net.in

but few, if any, fixed tertiary structural contacts (Kuwajima, 1989). Extensive studies were done on the folding of papain and other related cysteine proteins isolated in our laboratory as model systems. (Edwin and Jagannadham, 1998, 2000; Edwin *et al*., 2002; Kundu *et al*., 2002, Sundd *et al*., 2002; Sharma and Jagannadham, 2003; Dubey and Jagannadham, 2003; Tomar *et al*., 2009.) Studies on the folding of similar proteins from different sources and proteins within a family will complement to generalize the folding behavior of cysteine proteases.

In this direction, a conformational study of organic solvent-induced state of heynein, a new thiol protease purified in our laboratory from the latex of a medicinally important plant *Ervatamia heyneana* is initiated. The molecular weight of heynein is 23 kDa (Patel and Jagannadham, 2003). Like other thiol proteases, the molecular structure of heynein may also have two domains separated by a cleft (Turk *et al*., 1997). However, there are no reports regarding the molecular structure of heynein. The present investigation shed light on the biophysical properties of heynein at low pH in the presence of organic solvents providing an insight into the molecular structure of the protein. The current investigation, may also contribute for understanding the folding-unfolding mechanism of proteases, in the presence of organic solvents, in general and thiol proteases in specific. The conformational stabilities of the native protein as well as in the organic solvent-induced state are assessed from chemical and thermal-induced unfolding profiles and the intricacies in the unfolding are compared and discussed. Methanol and TFE was chosen as a representative of the non-fluorinated and fluorinated alkyl alcohols for a detailed study of this aspect.

Materials and Methods

Materials

Heynein was purified in our laboratory in active form from the latex of *Ervatamia heyneana* using the reported method (Patel and Jagannadham, 2003). Guanidine hydrochloride (GuHCl), TFE, methanol and were purchased from Sigma Chemical Co. (USA). All other reagents that were used were analytical grade, and solutions were prepared in doubly distilled water.

Methods

Protein Concentration

Protein concentration of heynein was determined spectrophotometrically using an extinction coefficient (ε^{280nm}, 1%) value of 21.9 (Patel and Jagannadham, 2003).

Steady-state Fluorescence Spectroscopy

The intrinsic fluorescence of the protein was measured in PerkinElmer LS-50B spectroflurometer equipped with a thermostatically controlled sample holder. The protein solution (0.04 mg/ml) was excited at 292 nm, which was determined to be suitable for measuring of both tryptophan and tyrosine fluorescence. The emission was recorded in the range of wavelengths 300–400 nm with slit widths of 5nm and 10nm for excitation and emission respectively.

Circular Dichroism

CD measurements were performed in a Jasco 500A (Jasco, Tokyo, Japan) spectropolarimeter with thermostatically controlled sample holder. Far-UVCD measurements were recorded using 0.1 mg/ml protein with a 1 mm path length cell. Near-UV measurements were made at 1 mg/ml protein concentration with a 10 mm path length cell. To study the effect of organic solvents and denaturants, the protein samples were incubated under specified conditions for 24 hrs before spectral measurements. All data were collected and the respective buffer base line was subtracted. The mean residue ellipticity was calculated, using the formula,

$$[\theta] = \theta_{obs} \times MRW/10cl,$$

Where θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, c is the concentration of protein (gm/cm^3) and l is the path length in centimeters. A mean residue molecular weight 110 was used.

Organic Solvent-induced Changes in Heynein

Organic solvent induced changes in heynein were followed by intrinsic fluorescence and far UV CD measurements. The solution containing organic solvent in the absence of the enzyme was taken as reference in the spectral measurements. All the

measurements were done at low pH as the protein is more susceptible to solvent composition. Chemical and temperature-induced unfolding of heynein in the native state as well as in the organic solvent- induced state was carried out as given below.

Chemical Induced Unfolding

Chemical induced unfolding of heynein in the absence of organic solvent and in organic solvent-induced state was performed at varying concentration of GuHCl followed by changes in the secondary structure (far-UV CD) and shift in the fluorescence emission maximum. Protein samples were incubated at different concentration of the denaturant for approximately 24 h at room temperature to attain equilibrium. Data was expressed in terms of fraction unfolded (F_u) calculated from the equation

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

where, F_{obs} is the observed value of the signal at a given denaturant concentration, F_n and F_u are the values of native and unfolded protein, respectively. Concentration of GuHCl in solution was determined from refractive index measurements. Samples for spectroscopic measurements were centrifuged, filtered through 0.45 mM filters and the exact concentration of the protein and denaturant were determined.

Thermal Unfolding

The temperature induced unfolding of heynein in the absence of organic solvent and in organic solvent-induced state was followed by changes in the secondary structure (far-UV CD) and shift in the fluorescence emission maximum. Protein samples were incubated at the desired temperature for 15 min before each measurement. The actual temperature of the sample in the cuvette was obtained with a thermocouple using a digital multimeter. Occasionally, samples were also checked for any possible aggregations due to heat by light scattering measurements. Data is expresses in terms of fraction unfolded as in the case of chemical induced unfolding.

Results

Fluorinated (methanol) and non-fluorinated (TFE) alcohol induced conformational transition of heynein and stability of the organic solvent-induced state is reported. Under neutral conditions heynein retains all the intrinsic properties as well as activity even at very high concentration of the organic solvents (Data not shown). But the changes in the properties of the protein are significant at low pH and dependent of the concentration/nature of the organic solvent. As low pH produces stress in the protein molecule in turn make the protein more susceptible and induce conformational changes easily with variations in the solvent composition.

TFE –Induced Structural Changes

Under neutral conditions, the far UV-CD spectrum of heynein exhibit strong negative ellipticities at 208 nm, 215 nm and 222 nm (Fig. 1A) suggesting that the molecule is composed of α-helix and β-sheet rich regions and belong to the α+β class of proteins (Manavalan and Johnson, 1983). Heynein looses most of the secondary structure at lower pH and the main ellipticity band is around 208 nm as shown in the same figure. The changes in the secondary structure are followed as a function of TFE concentration in the far-UV region as shown in Fig. 1B.

In the presence of 35% TFE concentration heynein exhibits enhanced ellipticity at 208 nm, 215 nm and 222 nm comparable to the native state at neutral pH (Fig. 1A) suggesting an induction of α-helicity in the molecule. At higher concentration of TFE the shape of the CD spectrum in the peptide region is similar but with higher magnitude of ellipticity at 222 nm.

Fluorescence is another useful technique for studying the conformational changes in proteins (Beechem and Brand, 1985). When a protein is excited at 292nm, the observed spectrum can be regarded as the fluorescence emission of tryptophan (Tsaprailis *et al.*, 1998). In order to get further information about structural changes, the fluorescence spectrum of heynein has been recorded, since the changes of heynein can reflect the local environment, changes around the unique tryptophan residues.

Under neutral conditions, heynein exhibits a fluorescence emission maximum of 349 nm indicating hydrophobic ambience of the

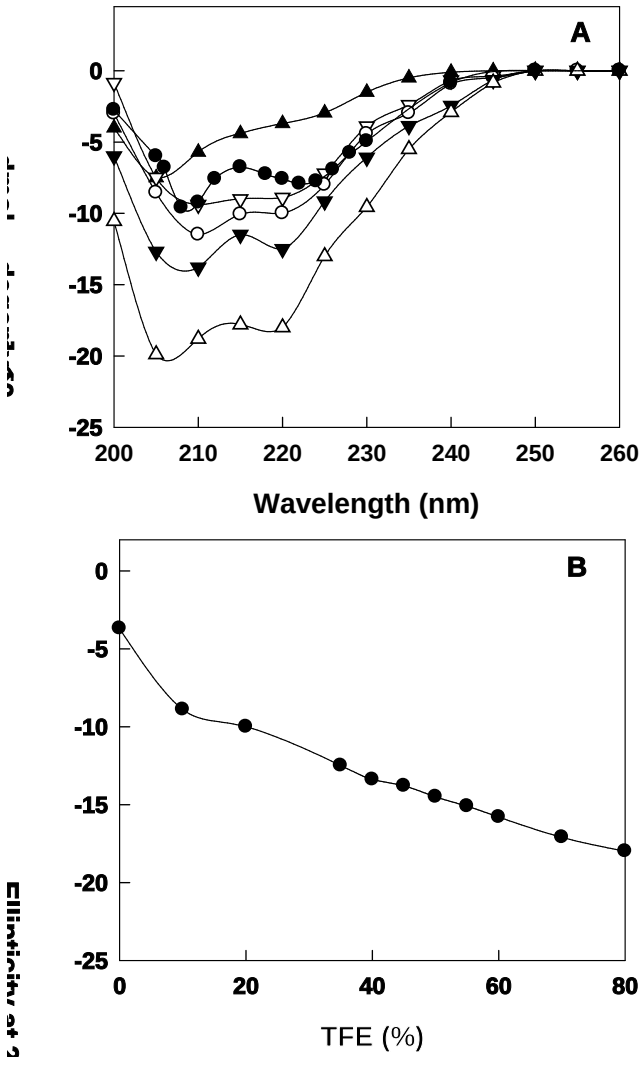

Figure 1: Effect of increasing TFE concentration on the secondary structure of heynein at pH 2.0

(A) Far UV CD spectra of heynein at different concentration of TFE at 0% (▲), 10% (●), 20% (∇), 35% (O), and 80 %(Δ). The native spectra of heynein at pH 7.0 (▼) is given for reference. (B) Change in the secondary structure (ellipticity at 220nm) with increasing TFE concentration. The protein concentration was 0.16 mg/ml in 0.05 M glycine-HCl buffer, pH 2.0 and the samples were incubated for 24h at 25°C.

tryptophan residues. Under denatured conditions, the intrinsic fluorescence spectrum of heynein showed an emission maximum at 358 nm showing a red shift of 9 nm (Data not shown). At lower pH 2.0, heynein exists in acid unfolded state with fluorescence emission maximum of 356 nm and also show good amount of ANS binding. Trifluoroethanol induced changes in fluorescence intrinsic properties of heynein are shown in Figure 2. With increasing concentration of TFE the fluorescence emission maximum suffers a blue shift of 19 nm from 356 to 337 nm by 80% of TFE concentration and the fluorescence intensity gradually decreases and attains a plateau value by 35% TFE and remains constant further. A blue shift in fluorescence emission maximum occurs when the exposed tryptophans buried in the protein molecule resulting some structure formation. The spectral parameters of tryptophan (Trp) fluorescence such as position, shape and intensity are dependent on the dynamic and electronic properties of the chromophore environment; hence study states that Trp fluorescence has been extensively used to obtain information on the structural and dynamic properties of the proteins.

The resultant protein structure in the presence of 35% TFE suggests that heynein exists in a partially unfolded state with burial of tryptophan residues in to the interior of the protein molecule along with induced secondary structure. Therefore, it can be concluded that the protein exists in a state similar to the molten globule state observed under variety of conditions in the case of other proteins. TFE-induced state is designated as O_{TFE}-state of heynein.

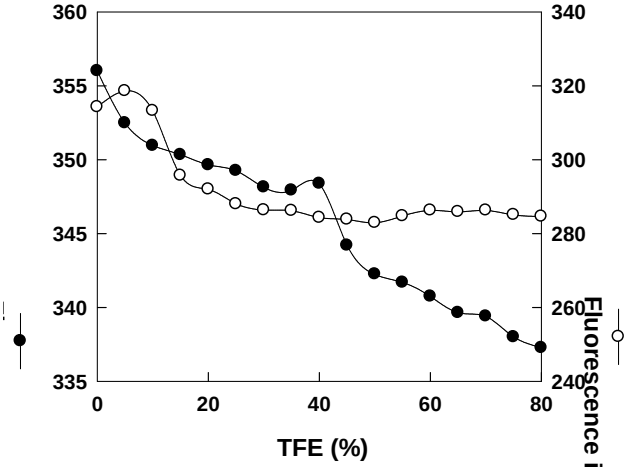

Figure 2: Effect of TFE on intrinsic fluorescence of heynein at pH 2.0

The fluorescence spectra of heynein change in wavelength emission maximum (●) and fluorescence intensity (○) was recorded with increasing TFE concentration in 0.05 M glycine-HCl buffer, pH 2.0 at 25°C. The protein sample at a concentration of 0.016 mg/ml was incubated under specified conditions for 24h at 25°C before measurements. The excitation wavelength was 292 nm with slit widths of 10nm and 5nm for excitation and emission respectively.

Methanol Induced Structural Changes of Heynein

Changes in the secondary structure of heynein in the presence of methanol were followed by CD measurements. CD spectrum of heynein at different concentrations of methanol is shown in Figure 3. A gradual increase in the ellipticity at 222 nm took place up to 35% methanol concentration indicating an induction of α-helicity in the protein molecule. Further increase in methanol concentration to 40% resulted in a sudden appearance of a negative peak at 215 nm, which is the characteristic of predominantly β-sheet in the conformation of the protein. At higher concentrations of methanol the shape of the CD spectra remains same but with higher magnitude of ellipticity at 215nm. Similar conformational switch from α-helix to β-sheet is observed with ethanol, propanol and butanol but at lower concentration of the organic solvent (Data not shown). Besides, light scattering measurements reveal that no protein aggregation took place after induction of β-sheet upon addition of alcohol.

Fluorescence emission maximum of heynein suffers a blue shift of 15 nm from 356 nm to 341 nm upon addition of methanol, in the solvent composition (Fig. 4) and the transition can be fit to a two state transition. The change in fluorescence intensity with increasing methanol concentration also followed a sigmoidal curve. Such change in fluorescence emission maximum reveals that the tryptophan residues are buried inside the protein molecule in the presence of the organic solvent. Thus, it is concluded that at pH 2.0 and in the presence of 40% methanol the enzyme exists in an intermediate state with molten globule like characteristics. Methanol induced intermediate state is designated as $\mathbf{O_{met}}$-state of heynein.

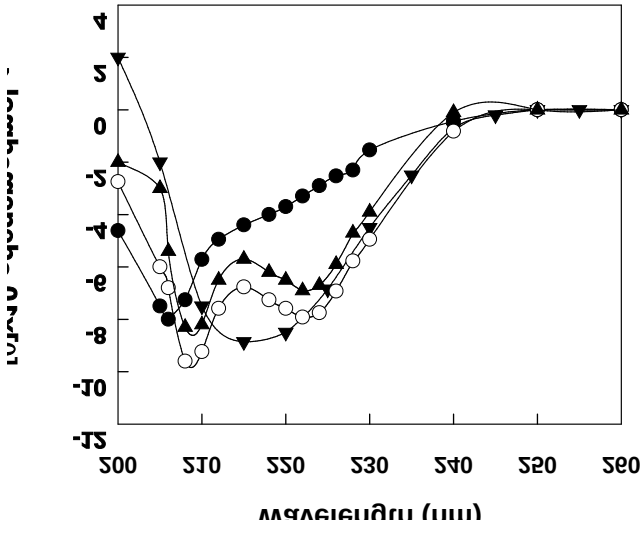

Figure 3: Effect of increasing methanol concentration on the secondary structure of heynein at pH 2.0

Far UV CD spectra were recorded at different concentration of methanol in 0.05 M glycine-HCl. pH 2.0. The protein concentration was 0.16 mg/ml and the samples were incubated for 24h at 25 °C. The methanol concentrations were 0 % (●) 35%, (▲), 40 %(▼) and Far UV CD spectra of heynein at pH 7.0 (O) included for reference.

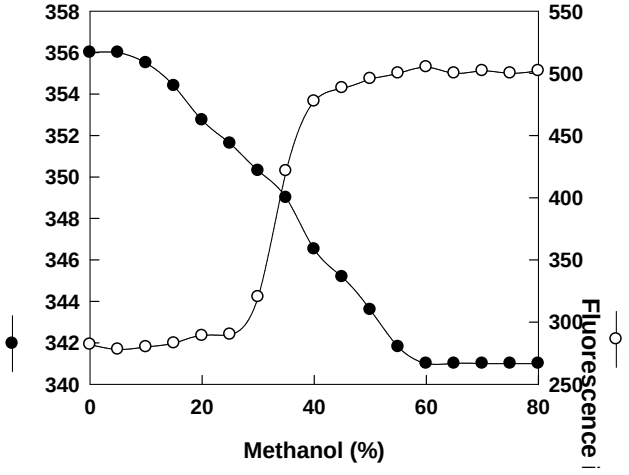

Figure 4: Effect of methanol on intrinsic fluorescence of heynein at pH 2.0

Change in wavelength emission maximum (●) and fluorescence intensity (○). Spectra were recorded with increasing methanol concentration in 0.05 M glycine-HCl buffer, pH 2.0 at 25°C. The protein sample at a concentration of 0.016 mg/ml was incubated under specified conditions for 24h at 25°C before measurements. The excitation wavelength was 292 nm with slit widths of 10nm and 5nm for excitation and emission respectively.

Stability and Unfolding of Organic Solvent- induced State of Heynein

In the presence of TFE and methanol, heynein exhibits the characteristics of molten globule state observed in the case of many proteins. Understanding the behavior of organic solvents-protein complex is of vital interest and allows gaining insight into the binding mechanism between the two components as well as the effect of solvent binding on the protein structure in the complex. One approach in this direction is to see the equilibrium unfolding of the protein as well as the protein-solvent complex, where stability

and unfolding behavior indeed could be different. Such differences will shed light on the subtle changes induced in the protein structure and stability due to interaction with the solvent. GuHCl and temperature induced unfolding of heynein and heynein-solvent complex were carried out by following far UV CD and intrinsic fluorescence measurements.

GuHCl Induced Unfolding

The conformational stability of proteins can be measured by equilibrium unfolding studies using GuHCl, a commonly employed protein denaturant. Analysis of solvent denaturant curves using the denaturants can provide a measure of the conformational stability of the proteins (Pace, 1990). Structural characterization of the partially folded intermediates, which are stabilized/ destabilized under variety of conditions, provided significant inputs on the forces responsible for such stabilization/destabilization. Therefore, characterization of these intermediates during denaturant induced folding/unfolding of the protein is necessary. GuHCl induced unfolding of heynein in the absence of organic solvent, when monitored by changes in secondary structure and fluorescence, showed a cooperative and concurrent transition with out any detectable intermediates in the unfolding pathway (Figure 5A and 5B). The loss of fluorescence and secondary (CD) structure reflects a good correlation of structural integrity of the molecule. This observation suggests that the molecule unfolds as a single entity. Chemical-induced loss in the secondary structure as well as in tertiary structure followed a single transition with transition midpoints of 2.9±0.05 and 2.68±0.05 M GuHCl respectively. Similarly the GuHCl-induced unfolding of heynein in the presence of 40% methanol and 35% TFE are also shown in the Figure 5A and Figure 5B. The unfolding transitions are non-cooperative and biphasic in nature with the two solvents. The transition midpoints of the two transitions by CD are 1.8±0.08M and 3.8±0.09M GuHCl in the case of methanol and 2.8±0.10M and 4.6±0.10M GuHCl with TFE. While the transition midpoints of the two transitions by fluorescence are 1.8±0.08M and 3.5±0.09M GuHCl with methanol and 2.7±0.10M and 4.3±0.10M GuHCl with TFE. Stability and other unfolding parameters of heynein in the presence and absence of organic solvents are shown in Table 1.

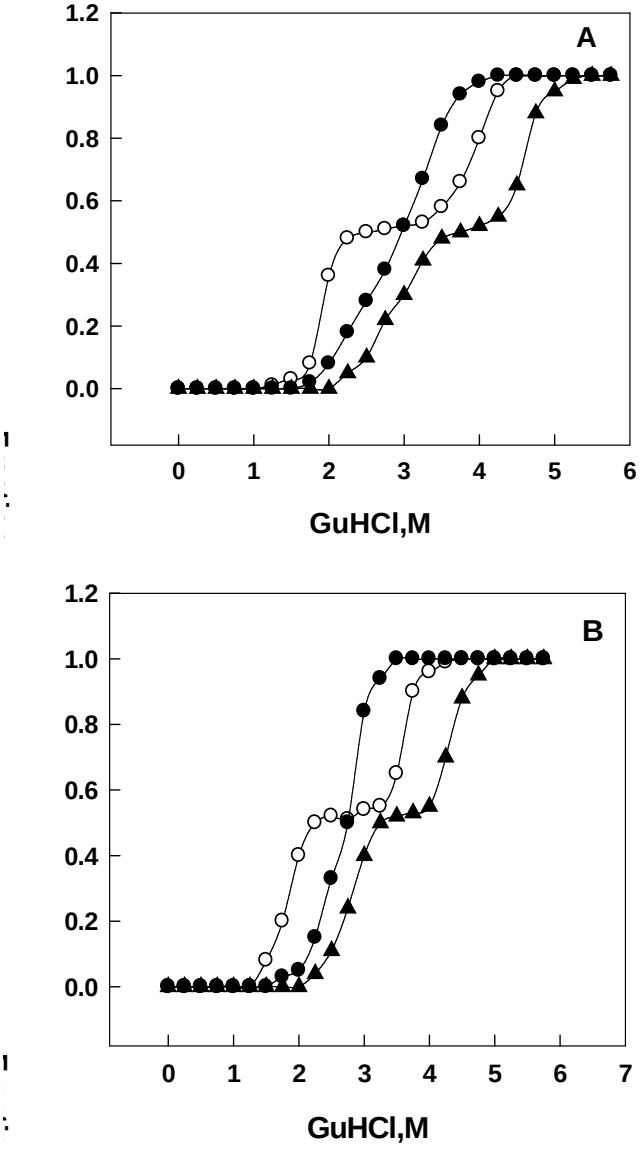

Figure 5: GuHCl induced unfolding of heynein in presence and absence of organic solvent

Unfolding transition monitored by (A) ellipticity and (B) fluorescence emission maximum. Enzyme was incubated at different concentrations of GuHCl for 24h at 25 °C in presence of (O) 40% Methanol and (▲) 35% TFE and (●) in the absence of organic solvent. The ellipticity at 222nm was monitored in the case of TFE while ellipticity at 215 was used with methanol. Fraction unfolded was calculated as described in materials and methods.

Temperature Induced Unfolding

As already pointed out, at a critical concentration of the TFE and methanol, heynein exists in a state

Table 1
Stability and Unfolding Parameters of Heynein in the Absence and Presence of Organic Solvent

Condition	*Denaturant*	*Method*	*Transition midpoint (C_m) or(T_m)*	
pH 2.0	GuHCl	CD $[\theta]_{222}$	2.9± 0.05M	
		Fluorescence	2.6± 0.06M	
35% TFE	GuHCl	CD $[\theta]_{222}$	2.8± 0.10M	4.6±0.10M
		Fluorescence	2.7±0.10M	4.3±0.10M
40% Methanol	GuHCl	CD $[\theta]_{215}$	1.8±0.08M	3.8±0.09M
		Fluorescence	1.8±0.08M	3.5±0.08M
pH 2.0	Temperature	CD $[\theta]_{222}$	47.8±0.5°C	
		Fluorescence	50.9±0.5°C	
35%TFE	Temperature	CD $[\theta]_{222}$	37.5±0.5°C	58.5±0.5°C
		Fluorescence	38.7±0.5°C	61.1±0.5°C
35%Methanol	Temperature	CD $[\theta]_{215}$	39.8±0.5°C	
		Fluorescence	39.9±0.5°C	

Chemical-induced unfolding was done at 25°C.

different from the native protein in terms of secondary and tertiary structural content. Temperature-induced structural changes in a protein will provide ample information about the molecular structure of the protein molecule. The temperature-induced unfolding transition of heynein in the presence and absence of organic solvents is also followed by far UV CD (ellipticity at 222 nm in the case of TFE, ellipticity at 215 nm in methanol) and changes in fluorescence emission maximum are shown. Temperature induced unfolding of heynein in presence of two organic solvents (methanol and TFE) was carried out using circular dichroism as a measure of secondary structure and fluorescence as a measure of tertiary structure. Thermal denaturation of heynein at pH 2.0 in the absence of any organic solvent is sigmoidal following a single cooperative transition and the transition midpoints are 47.8 ± 0.5 °C by CD and 50.9 ± 0.5 °C by fluorescence respectively (Figure 6). While, in the presence of TFE unfolding of heynein was non-cooperative as the transitions are biphasic. The transition curve intersects the transition curve obtained in the absence of organic solvent at 47.8 ± 0.5 °C and the fraction unfolded is 0.4. In presence of TFE, thermal unfolding of heynein is non-cooperative and the transition curve is biphasic with transition mid points 37.5 ± 0.5 °C and 58.59 ± 0.5 °C (followed by CD 222nm,) 38.73 ± 0.5 °C and 61.16 ± 0.5 °C (followed by fluorescence), respectively.

On the other hand, thermal denaturation of methanol induced-state of heynein is cooperative with a lower transition midpoint 37.5 ± 0.5 °C (followed by CD) and 39.9 ± 0.5 °C (followed by fluorescence) reflecting the overall destabilization of the protein.. Stability and other temperature-induced unfolding parameters of heynein in the presence and absence of organic solvents are shown in Table 1.

Discussion

Organic solvents usually affect the native structure of a protein. Upon placing an enzyme in a non-aqueous medium, the protein is subjected to a number of factors that can alter its native, aqueous-based structure and function (Singer, 1962). The native secondary and tertiary structure of the enzyme is maintained by the interactions of the several non-covalent forces, including hydrogen bonding, ionic, hydrophobic and Van der Walls interactions (Schulz and Schimer 1979). Disruption of such forces due to addition of organic solvent to enzyme in aqueous solution can lead to diminished substrate binding and catalytic turnover. Addition of organic solvent can also cause varying effects by opening up the protein molecule, depending on the nature of the organic solvent. Thus structural studies of a protein in different organic solvents can provide information about the structure of the protein molecule and the role of various stabilizing and destabilizing forces responsible for the unique

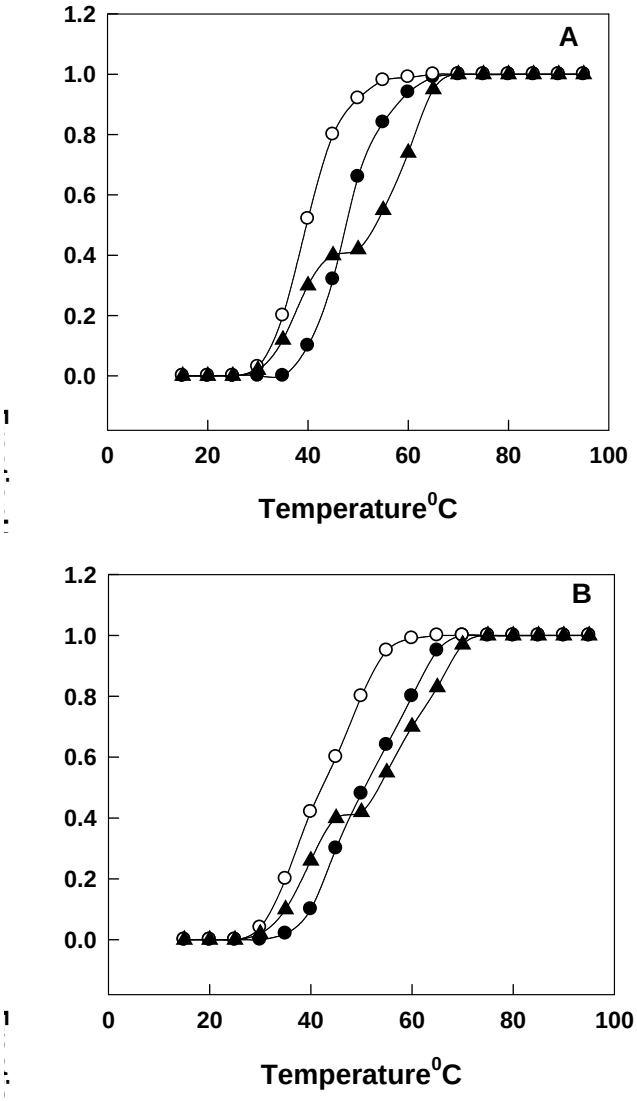

Figure 6: Thermal induced unfolding of heynein in presence and absence of organic solvent

Transitions were monitored by (A) ellipticity and (B) fluorescence intensity. Enzyme was incubated at different temperature for 15 min. (O) 40% Methanol (▲) 35% TFE and (●) in the absence of organic solvent. The ellipticity at 222nm was monitored in the case of TFE while ellipticity at 215nm was used with methanol. Fraction unfolded was calculated as described in materials and methods.

folded structure of the protein in solution. With this view, studies of heynein in the presence of different organic solvents were performed. Folding pattern of a polypeptide may differ significantly with changes in solvent environment. A group of solvents, including alcohols and ketones was found to stabilize the proteins at low concentration, but denature the proteins at higher concentration. The stabilizing effect of an alcohol increases with increase in chain length. Organic solvent alters the native structure of the protein by disrupting hydrophobic interactions between the nonpolar side chains of amino acids. Relatively high concentration of the solvent is required to unfold the ordered structure of a polypeptide chain (Herskovits and Jaillet, 1969).

The action of organic solvent on the protein is a function of the proton donor or proton acceptor character. The energy of hydrogen bonds depends on the solvent and the competition between peptide hydrogen bonds with solvent molecule. When solvent molecules are linked by strong hydrogen bonds the equilibrium is shifted toward the right i.e. the stabilization of hydrogen bonds. At low pH the enzyme is vulnerable to structural changes and hence any slight perturbation causes loss in tertiary structure and proteolytic activity. Subsequent hydrogen bond formation with peptide carbonyl groups, which leave many of the intermolecular interactions unaffected, promotes secondary structure formation.

Organic solvent increases the compactness of the molecule which in turn results enhanced ellipticity because the charged state of the protein molecule induces favorable interactions with the organic solvent. Upon lowering the pH, protein molecule is under stress because of the electrostatic repulsions due to the increased positive charge. In the presence of methanol a conformational switch from the native α-helical to predominantly β-sheet conformation was observed in the case of heynein. When the methanol concentration was increased from 35% to 40% the resultant intermediate exhibited substantial amount of secondary structure, which is predominantly β-sheet (O_{met}-state). While, TFE enhances the native secondary structure of heynein to a greater extent, in O_{met}-state state beta sheet structure of the protein is prominent. Moreover, the conformational stabilities of the organic solvent induced state are also different (Table 1). Generally alcohols have two main effects on proteins; they stabilize helical structure of proteins while destabilizing their rigid tertiary structure. These combined effects of alcohols may induce partially folded intermediates. Knowledge

of partially structured states should provide an insight into the relative strengths and different type of interactions that can stabilize a given section of the polypeptide chain and may provide information regarding the interactions responsible for directing the folding of proteins. The existence of an intermediate in alcohol induced protein denaturation was long back supported by Timasheff (1970). All the known papain like cysteine proteinases, except cathepsin-C, are monomers whose structure consists 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 solvent is consistent with the view that heynein has two domains that unfolds sequentially. However thermal denaturation of methanol induced states of heynein is cooperative with lower transition midpoints, of 39.8±0.5°C (followed by CD) 42.3±0.5°C (followed by fluorescence) reflecting the destabilizing properties of the protein. Due to differential stabilization and destabilization of α or β-rich domains of heynein, in O-state, the differences in the stabilities of the domains become more significant. Similarly, in heynein (O_{TFE}-state) the two transition midpoints were higher to that in the absence indicate that the protein molecule as a whole is stabilized. This increase in stability can be attributed to the hydrogen bonding property of TFE.

Moreover, the GuHCl induced unfolding of heynein in presence of TFE and methanol are found to be non-cooperative while thermal unfolding was cooperative in presence of methanol and non-cooperative in TFE. These differences in unfolding may be attributed to the ionic nature of GuHCl, which mean at low pH lower than that this the GuHCl molecule is fully dissociated, i.e., in to Gu+ and Cl. More over the presence of these ions could influence the stabilization/destabilization properties of the protein (Mayr and Schimid, 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.

The interactions between domains or subdomains in proteins often involve the close apposition of hydrophobic surfaces and account for most of the structural stability of the protein (Palme *et al.*, 1997). Hydrophobic interactions have been prominently implicated in determining the native configuration of proteins in aqueous solution. These interactions are actually not of a single relatively well-defined character, as are electrostatic and hydrogen bond interactions, but are rather a set of interactions responsible for the immiscibility of non-polar substances and water. Proteins contain a substantial proportion of amino acids such as phenylalanine, valine, leucine etc with non-polar side chain residues. These non-polar groups should tend therefore, other factors permitting to cluster on the inside of the protein molecule away from the aqueous environment, as a result of these interactions. Organic solvents basically perturb the hydrophobic interactions in protein. Thus, in case of heynein it appears that the two domains have a different relative hydrophobic stabilization, and the addition of additives like organic solvents (known to weaken hydrophobic interactions), leads them to move in the uncoupling direction. The well-separated thermal transitions suggest significant difference in their intrinsic stability; possibly one of the two domains is less stable and unfolds at a lower temperature than the other, causing the denaturation process to approach a sequential mechanism. However, in presence of GuHCl, the molecule was observed to unfold as a single unit. This may be due to the reason that GuHCl being a strong denaturant unfolds the molecule as a whole and not in a sequential manner. A similar uncoupling of the domains has been reported in RNase unfolding, long considered the standard for single transition proteins. The unfolding transition of RNase can be split into two transitions by either raising the concentration of methanol in the solvent at a constant apparent pH, or by lowering the pH (Brandts *et al.*, 1979). There are several ways in which transitions might be uncoupled, thereby allowing the study of individual domains. First, is addition of a ligand that binds to one domain only can act to alter its relative intrinsic stability and there by possibly uncouple transitions. Second is a selective mutation in the body of one domain that act to change relative intrinsic stabilities and will push towards uncoupling. Third method is change in

solvent conditions may in some instances promote uncoupling. This was seen dramatically for RNase A where the addition of methanol appears to completely uncouple its thermal transitions (Brandts *et al.*, 1989). Thus hydrophobic interactions play a major role in stabilizing the native conformation of proteins. The marked reduction in hydrophobic interactions in almost any non-aqueous solvent compared to water must be critically involved in the conformational changes observed. Intramolecular hydrogen bonds are important in determining these structures, but the hydrophobic interactions make the largest single contribution to stabilizing the native conformation of these macromolecules in aqueous solution. The enhanced stability in presence of organic solvents may be due to the hydrogen bond forming tendency. Analysis of solvent denaturation suggests that heynein has probably two domains with a marked difference in intrinsic stability and on thermal denaturation unfolds sequentially.

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Abbreviations

CD, Circular dichroism; GuHCl, Guanidine hydrochloride; TFE, Trifluoroethanol; UV, Ultraviolet; F_u, Fraction Unfolded.

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