JOURNAL OF PROTEINS AND PROTEOMICS
Vol. 1, No. 2, July-December 2010, pp. 43–51

Structural Insights of the Cysteine Protease Heynein from Induction and Characterization of Non-native Intermediate States

Basant K. Patel[*#] and Medicherla V. Jagannadham[*]

Abstract: *Cysteine proteases are vital to cell physiology and many plants secrete these proteases for defense purposes. Many recent studies have reported unusually high stabilities for several plant cysteine proteases which possibly enable these proteases to function under adverse environmental conditions. Here, we have examined the conformational features of a new plant cysteine protease heynein using spectroscopic tools to understand the basis for its robust functional stability. The studies revealed structural integrity over a wide range of pH (2.5-12.0), temperature (65 °C) and urea (8M). However, at pH 2.0, the protein gets acid-unfolded (**U_A**-state) with exposed hydrophobic patches, which upon addition of more protons (pH 0.5) or anions (0.5 M KCl and 0.2 M Na_2SO_4) yields conformationally distinct refolded intermediates respectively termed: **A**-, **I_1**- and **I_2**-states. Strikingly, a high methanol level drives the **U_A**-state into a predominantly β-sheet rich conformation (**O**-state). We observed three-state unfolding kinetics of the **I_2**-state by urea, possibly suggesting presence of two domains in the heynein molecule.*

Keywords: *ANS, 8-Anilino-1-napthalene-sulphonic acid; CD, circular dichroism; GuHCl, guanidine hydrochloride; TCA, trichloroacetic acid.*

I INTRODUCTION

Partially folded protein states resembling 'molten globule'-like intermediate states are associated with chaperone action (Randall and Hardy, 1995), protein translocation across membranes (Bychkova et al., 1988) as well as amyloid fibril formation in pathological cells (Kelly, 1998). Characterization of the intermediate conformations of a protein may inform on its *in vivo* folding or misfolding (Dobson, 2003) and targeting the intermediate states may help tackle numerous highly debilitating protein misfolding diseases(Stefani and Dobson, 2003).

Here, we examine induction of intermediate protein conformations of a highly stable plant cysteine protease heynein purified from the medicinal plant *Ervatamia heyneana* (Patel and Jagannadham, 2003). As we reported previously, heynein is a 23.5 kDa molecule that contains five disulfide bridges, sixteen tyrosine and six tryptophan residues (Patel and Jagannadham, 2003). In addition to their application in food industries, the plant cysteine proteases are also vital to the plant cell physiology contributing to the host defense against pests and microbes. Thus novel plant cysteine proteases are under continuous investigation for their enzymatic and physico-chemical properties (Hernandez-Arana and Soriano-Garcia, 1988; Huet et al., 2006; Tiktopulo and Privalov, 1978). Folding studies in conjunction with X-ray structure aimed at understanding the structure-to-function relationship, have documented induction of multiple intermediate folding states of plant cysteine proteases (Edwin and Jagannadham, 2000; Sumner et al., 1993; Thakurta et al., 2004; Turk et al., 2000). Folding of several new cysteine proteases has also been investigated in our laboratory to get broader insight into the molecular design of this mechanistic class of proteins (Dubey and Jagannadham., 2003; Edwin and Jagannadham, 1998; Edwin and Jagannadham, 2000; Kundu et al., 1999; Sharma and Jagannadham, 2003; Sundd et al., 2000). Here, we carried out systematic solution studies to probe the structural features of the cysteine protease heynein and succeeded in inducing multiple intermediate protein states by modulating pH and anion contents of the solvent. These intermediate protein states exhibited different stabilities and were further characterized to gain insight into the folding aspects of heynein. Unfolding trend of one of these intermediates suggests a two-domain organization of the native molecule.

[*] Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh-221005, India
[*#] Corresponding author: Department of Biotechnology, Indian Institute of Technology Hyderabad, Ordnance Factory Campus, Yeddumailaram, Andhra Pradesh-502205, India, Email: basantkpatel@gmail.com, Phone: 91-40-23016008

II RESULTS

Structural Features of Heynein

In the native state at pH 7.0, the near UV circular dichroism spectrum of heynein shows positive peaks around 278 nm and 285 nm, with mean residue ellipticities of 250 and 220 deg cm^2 $dmol^{-1}$ at 278 and 285 nm respectively (**Figure 1A**). These positive peaks are suggestive of dominant contribution of the tryptophan residues to the spectrum and masking of the tyrosine's contribution. In the far UV region, the CD spectrum of heynein comprises of strong negative ellipticities at 208, 215 and 222 nm (**Figure 1B**). The ellipticity at 208 nm is more negative than that at the 222 nm, suggesting that the molecule may consists of α-helix and β-sheet rich regions and possibly belongs to the α + β class of proteins (Manavalan and Johnson, 1983). The mean residue ellipticity at 222 nm is –7920 degree cm^2 $dmol^{-1}$ in the native protein with an estimated α-helicity of 20%. At the same pH, the near and far UV spectral features of heynein are completely lost upon exposure to the denaturant 6M GuHCl indicating a thorough loss of its tertiary as well as secondary structural organizations (**Figure 1A and 1B**).

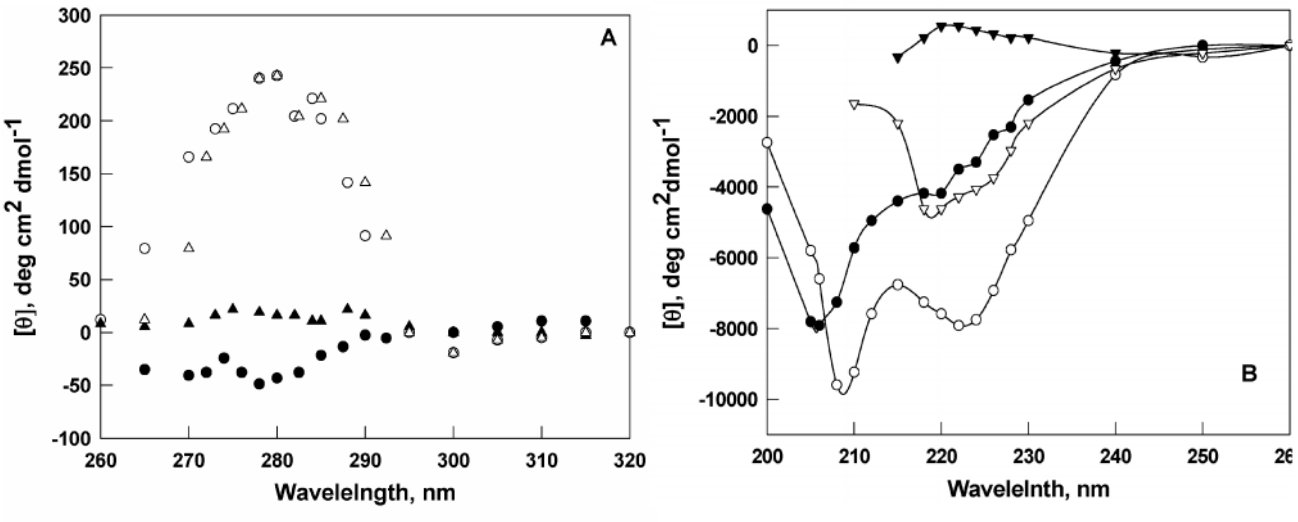

Figure 1: CD Spectra of Heynein: (a) Near UV CD Spectra of Native Heynein at pH 7.0 (O) as Compared to the 6M GuHCl pH 7.0 Denatured (●), pH 3.0 (Δ) and pH 2.0 (▼) States; (b) Far UV CD Spectra of Heynein Under Native State at pH 7.0 (O), 6M GuHCl at pH 7.0 Denatured State (▼), Acid Unfolded State at pH 2.0 (●) and 4.5 M GuHCl pH 7.0 State (▽).

The intrinsic fluorescence spectrum of heynein in the native state has an emission maximum at 349 nm indicating a relatively hydrophilic environment of its tryptophan residues (**Figure 2D**). The fluorescence spectrum of the denatured heynein showed an emission maximum at 358 nm, showing a red shift of about 9 nm (data not shown). Concurrent upon denaturation with 6M GuHCl, the tryptophan fluorescence intensity is reduced to half, indicating an increased accessibility of the quenchers to the tryptophan residues (data not shown).

Low pH Induces an Acid-unfolded (U_A) State of Heynein

As our previous studies on heynein (Patel and Jagannadham, 2003) revealed its functional stability over a broad range of pH (2.5-12.0), we probed its protein conformation under different pH conditions. Furthermore, when the CD ellipticities of heynein at 278 nm and 222 nm were analyzed as a function of pH to respectively estimate the tertiary and secondary structural contents, both levels of structure were retained from pH 2.5-12.0 (**Figure 2**). Thus, the previously observed functional stability of heynein over this pH range is also reflected in its conformational stability. Although, the tertiary structure of heynein is lost below pH 2.5 (**Figure 2B**), the secondary structural content however, showed an interesting trend (**Figure 2C**) and the CD ellipticities at 222 nm were comparable at pH 1.0 and 2.0 but decrease to pH 0.5 caused induction of some secondary structure as seen by decrease in the CD ellipticity at pH 0.5 (**Figure 2C**). Thus, the protein is maximally acid unfolded at pH 2.0, which we designated as the 'U_A-state' of heynein. However, as probed by the hydrophobic dye ANS, despite the substantial loss of its secondary structure, detectable hydrophobic clusters were remnant in the U_A-state, as indicated by a large increase in the ANS fluorescence intensity accompanied by blue shift in its wavelength of the emission maximum (**Figure 3**). Furthermore, unlike in the pH range 3.0 - 7.0 where the temperature induced unfolding of heynein is cooperative with very little

differences in the unfolding trend (**Figure 4**), the structure of heynein is lost at much lower temperature at pH 2.0 (Tm- 43.3 ± 0.5 ^{o}C) and the conformational stability of the protein is decreased Δ(ΔG)) by about 6 kcal/mol relative to the native protein (**Table 1**). Thus the pH 2.0 state is an acid unfolded $\mathbf{U_A}$-state of heynein.

U_A-state can be Refolded into Multiple Distinct Intermediate Protein States

Anions such as chloride and sulfate induced refolding in the acid unfolded state of heynein driving the molecule into states similar to the **A**-state previously obtained at pH 0.5. For the same concentration of the salt, the divalent sulfate anions imparted greater degree of refolding compared to the monovalent chloride anions as monitored by decrease in CD ellipticity at 222 nm (**Figure 5**). The maximal refolding of the $\mathbf{U_A}$-state was observed at respective concentrations of 0.5M and 0.2 M of the chloride and sulfate anions and the respective refolded states were termed as $\mathbf{I_1}$- and $\mathbf{I_2}$-states. Above these salt concentrations, substantial protein aggregation was observed as monitored by turbidimetry (data not shown).

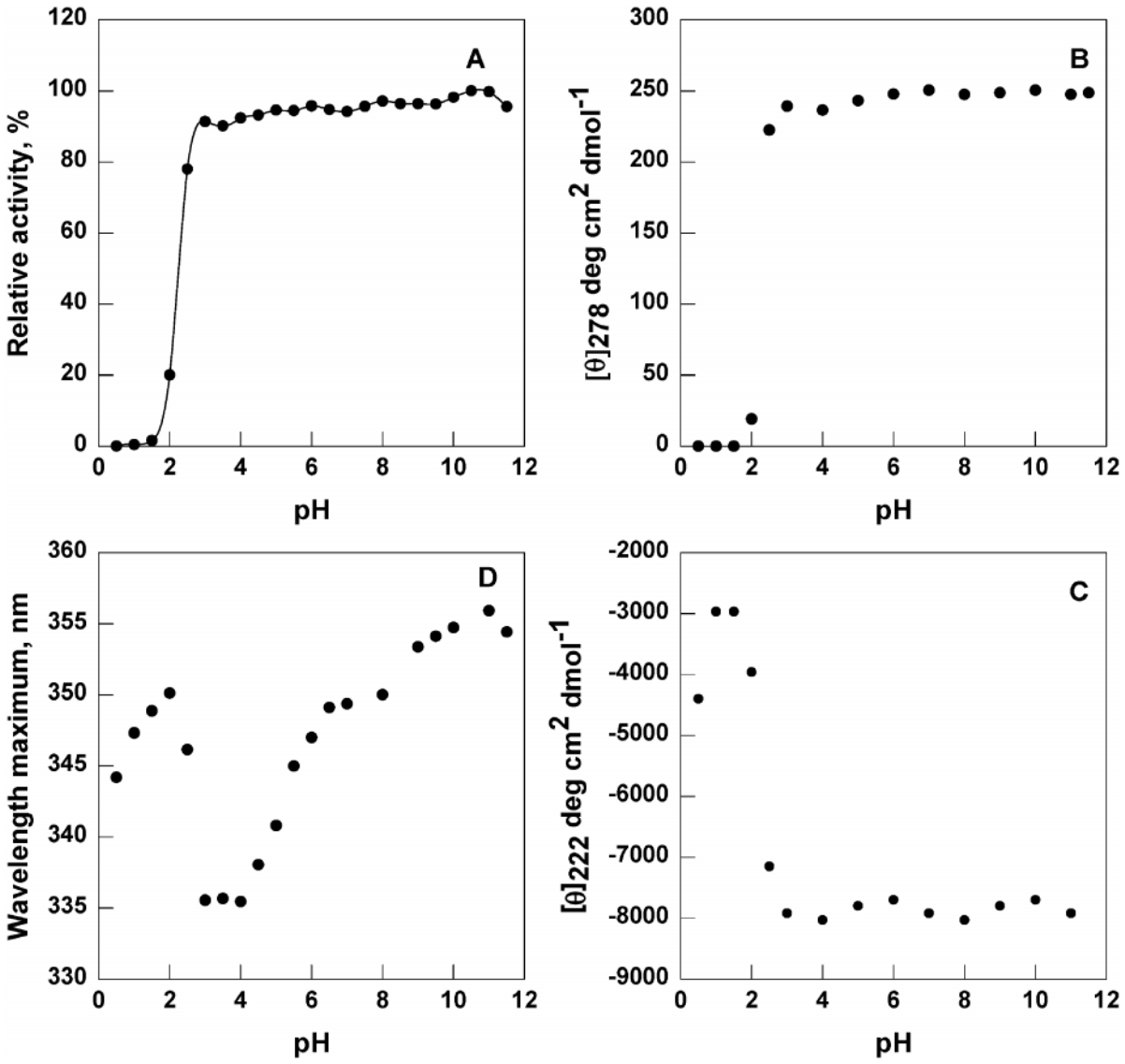

Figure 2: pH Induced Conformational Changes in Heynein: (a) Effect of pH on Proteolysis by Heynein (b) Effect of pH on Tertiary Structure of Heynein as Monitored by near UV CD Spectra and Represented as CD Ellipticity at 278 nm (θ_{278}) (c) pH Induced Changes in Secondary Structure of Heynein as a Function of pH Monitored by Far UV CD and Represented as CD Ellipticity at 222 nm (θ_{222}) (d) Effect of pH on Wavelength of Fluorescence Emission Maximum for Tryptophan Residues of Heynein. The Protein Concentrations for Near UV CD, Far UV CD and Tryptophan Fluorescence were Respectively 1 mg/mL, 0.1 mg/mL, and 0.025 mg/mL and 10 μg of the Protein was Equilibrated at a Particular pH and its Proteinase Activity Assayed at pH 8.0 to Assess its pH Stability.

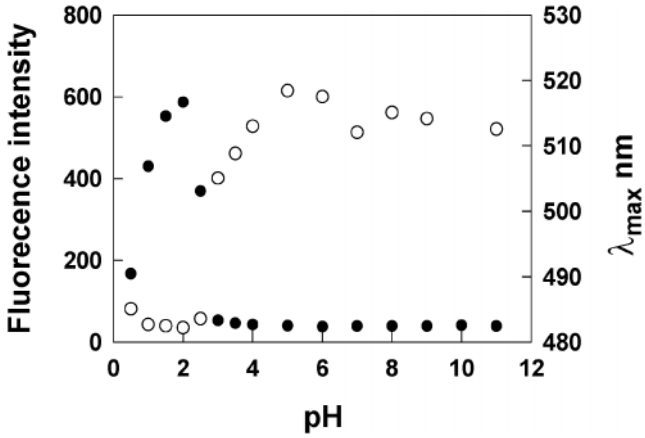

Figure 3: Binding of ANS Dye to Heynein: The Exposure of Hydrophobic Surfaces in Heynein at Different pH, were Studied by ANS Binding Assay at a Molar ratio of 1:100 of Protein to ANS Monitored by its Extrinsic Fluorescence intensity (●) and the Wavelength of Emission Maximum (O).

Table 1: Temperature Induced Unfolding of Heynein at Different pH[a]

pH	ΔS_m *cal/mol/deg*	ΔH_m *kcal/mol*	T_m *°C*	ΔT_m *°C*	ΔG (25°C) *kcal/mol*	$\Delta(\Delta G)$ *kcal/mol*
7.0	206.2	70.6	69.2 ± 0.5		9.1 ± 0.2	
4.0	205.0	70.0	68.0 ± 0.5	-1.2	8.8 ± 0.2	-0.3
3.0	188.2	62.5	58.9 ± 0.5	-10.3	6.3 ± 0.2	-2.8
2.0	176.8	55.9	43.3 ± 0.5	-25.9	3.2 ± 0.2	-5.9

[a]The conformational stabilities have been calculated using the unfolding transitions in Figure 4 using the method of Becktel and Schellman (Becktel and Schellman, 1987).

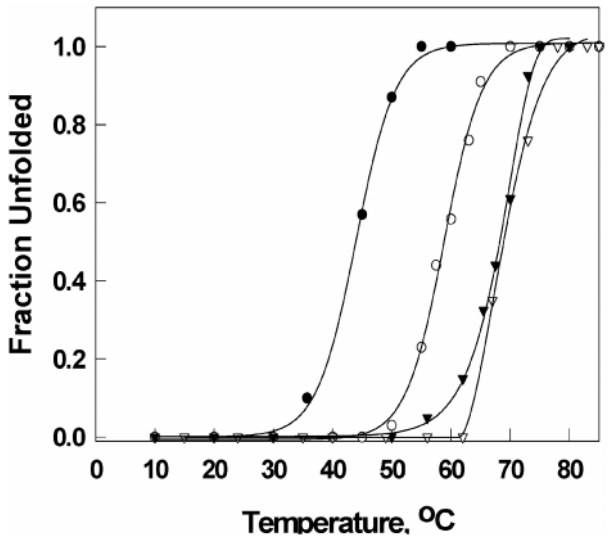

Figure 4: Thermal Unfolding of Heynein at Different pH: Effect of Temperature on the Secondary Structure of Heynein was Studied by far UV CD. The Unfolding Behavior at pH 7.0 (▽), pH 4.0 (▼), pH 3.0 (O) and pH 2.0 (●) was found to be Cooperative.

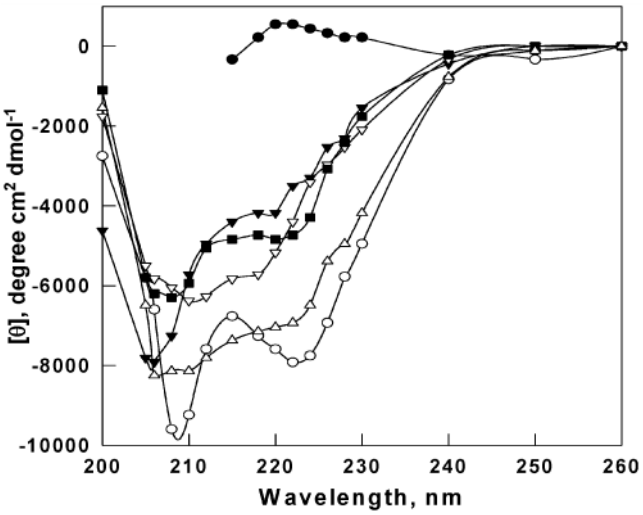

Figure 5: Effect of Anions on the Far UV CD Spectrum of Acid Unfolded U_A-state: The Far UV CD Spectrum of Native Protein (O) and 6M GuHCl Denatured Protein (●) have been Compared with Acid Unfolded Protein at pH 2.0 (▼), Acid Refolded State at pH 0.5 (▽), 0.5 M KCl Refolded State (■), and 0.2 M Na_2SO_4 Refolded State (▽).

Furthermore, the urea induced unfoldings of the **U_A-, A-** and **I_1**-states were cooperative with different transition mid-points when monitored by CD ellipticity changes at 222 nm (**Figure 6**). Urea induced unfolding was employed to study the stability of the different anion induced refolded states of heynein, as urea allows the investigation of salt effects due to its non-ionic nature (Pace and Grimsley, 1988). Interestingly, the urea induced unfolding of the divalent anion induced refolded **I_2**-state followed three-state transition (**Figure 6**). Such trend was not observed under any other condition studied. Further analyses of the urea-induced unfolding transitions of heynein revealed that the conformational stabilities of the three refolded states are different (**Table 2**).This observation suggests that in the **I_2**-state, different parts of the molecule with distinct stabilities, unfold sequentially and independently.

Strikingly, the acid unfolded **U_A**-state of heynein undergoes a transition to a β-sheet rich conformation if incubated with methanol. This conversion to β-sheet, as followed by a decrease in the CD ellipticity at 215 nm, embraces a trend consistent with a cooperative process. Also, concurrent with the β-sheet induction, a systematic increase in tryptophan fluorescence intensity was also observed. The transition mid-point for the conversion to β-sheet as monitored by CD ellipticity at 215 nm was in close proximity of 35% of methanol (**Figure 7**) and the process attains completion by 40% methanol. This 40% methanol induced state at pH 2.0 is named as the '**O**-state' of the molecule. The temperature induced unfolding of the **O**-state of heynein followed a broad

unfolding transition with about 30 °C wide transition zone, possibly manifesting a non-cooperative structure melting with accumulation of multiple transient intermediates (data not shown).

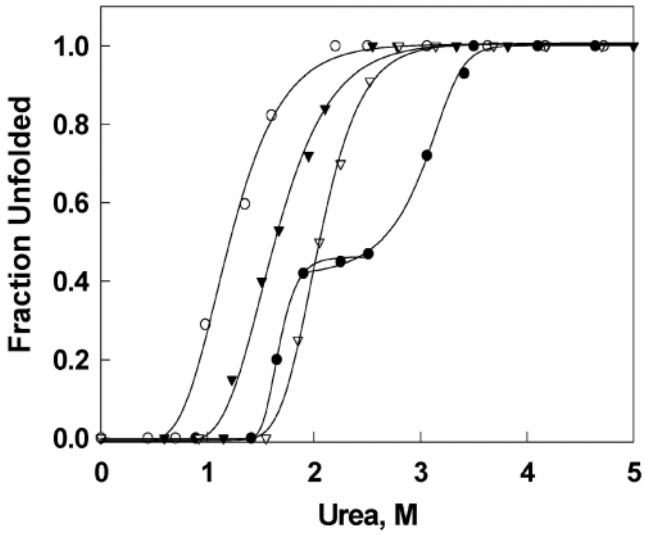

Figure 6: Urea Induced Unfolding of the U_A-, A- and the Anion-induced Refolded States: Heynein in the U_A-state (O) was Incubated for 24 hours with pH 0.5 Buffer or, 0.5 M KCl or 0.2 M Na_2SO_4 to Obtain the A- (▼), I_1- (▽) and I_2-states (●), which were Subsequently Incubated with Increasing Concentrations of Urea for 24 hours and the Far UV CD Spectra were Collected and Fraction of the Unfolded Protein was Estimated Using the Ellipticity Value at 222 nm.

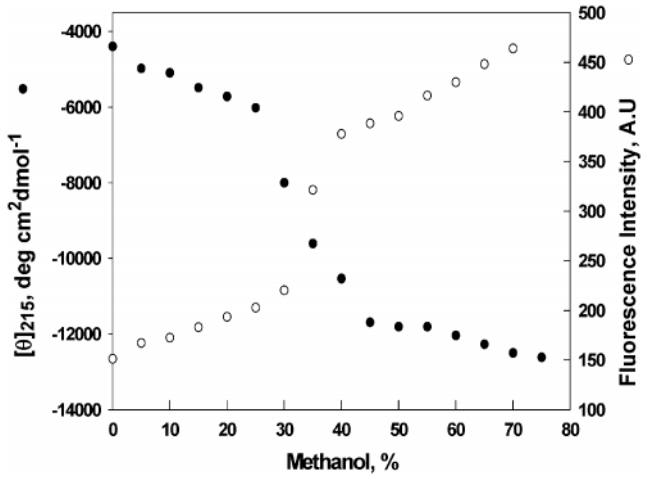

Figure 7: Effect of Methanol on the U_A-state: Acid Unfolded Heynein at pH 2.0 was Incubated in Presence of Increasing Methanol Concentrations for 24 hours, and Structure was Monitored by Far UV CD and Tryptophan Fluorescence Intensity. The Results have been Represented as Changes in CD Ellipticity at 215 nm (●) and Tryptophan Fluorescence Intensity (O) as a Function of the Methanol Concentration.

Table 2: Conformational Stabilities[a] of Three Refolded States of Heynein

Heynein		*ΔG (H_2O)* kcal/mol	*Δ(ΔG)[a]* kcal/mol	*[urea]$_{1/2}$* M
pH 2.0		2.8 ± 0.2		1.2 ± 0.3
pH 0.5		4.7 ± 0.2	1.9	1.6 ± 0.3
pH 2.0 + 0.5 KCl		6.6 ± 0.2	3.8	2.1 ± 0.3
pH 2.0+ 0.2 M Na_2SO_4	transition-1	4.6 ± 0.2	1.8	1.6 ± 0.3
	transition-2	8.7 ± 0.2	5.9	3.0 ± 0.3

[a]Assessed using urea-induced unfolding curves of Figure 6 by the method of Becktel and Schellman (Becktel and Schellman, 1987) .

III DISCUSSION

Here, spectroscopic tools have been used in conjunction with denaturants to understand the structural organization of a new cysteine protease heynein. Several non-native protein intermediates with distinct conformational stabilities have been induced and characterized.

The far-UV circular dichroism spectrum of heynein is consistent with that of α + β class of proteins and possibly its molecular architecture contains distinct α and β-rich domains. Of note, two domains referred to as **R** and **L**, respectively rich in α helix and β sheet content are found in many of the papain-like cysteine proteases (Thakurta et al., 2004; Turk et al., 1997).

The retentions of its catalytic activity and tertiary as well as secondary structures from pH 2.5 to 12.0 are suggestive of a rigid native structural conformation for heynein. The native state of the protein at neutral pH has the tryptophan residues maximally exposed to the hydrophilic environment and decrease of pH to 3.0 accompanies a substantial blue shift in the tryptophan emission maximum indicating induced burial of the tryptophan residues. However, the lack of perceivable differences in the CD spectra and functional properties at same pH, indicate a local rather than a global change in the protein structure, a situation also seen for several other proteins (Kundu et al., 1999; Nandi, 1998). The observed decrease in the fluorescence emission from pH 7.0 to 3.0 can be an indication of proximity of tryptophan residues with acidic amino acid residues in the native state, and the quenching of intensity could be a consequent manifestation of protonation of the acidic residues at pH 3.0. Upon decreasing the pH from neutrality to acidic, the histidine residues of the protein are first protonated followed by the acidic aspartate and glutamate residues. The pKa of these amino acids is well above pH 2.0, therefore, at pH 2.0 the positive charges on the protonated amino groups lead to charge-charge repulsions (Tanford, 1968). In turn, the compact structure of the protein gets

perturbed driving the molecule to a much-extended conformation. At pH 2.0, heynein loses its complete tertiary fold, apart from getting functionally inactive. But some residual secondary structure is seen as monitored by its far UV CD spectrum. This state of the protein has been named as the acid-unfolded '$\mathbf{U_A}$-state'.

As observed by the gain of some secondary structure, decreasing the pH to 0.5 induces refolding of the $\mathbf{U_A}$-state and the resulting state has been termed as the acid-refolded '**A**-state'. The decreased affinity of the **A**-state to ANS indicates internalization of certain exposed hydrophobic clusters during the refolding. Upon decreasing the pH below 2.0, the positive charges (H^+) do not have effect because of the lack of potential binding sites, but the counter ions (anions) pair up with the positively charged (NH_3^+) groups thereby shielding the Columbic repulsions. This leads to the manifestation of the inherent hydrophobic forces and results in compaction of the extended polypeptide chain (Barrick et al., 1994). Despite, this general response of proteins to the decrease in pH, Fink and co-workers (Fink et al., 1994) have identified three types of intricate behavior among various proteins. The acid denaturation of heynein follows the pattern obtained for proteins, such as ribonuclease A, where an acid unfolded state precedes the formation of acid refolded state as represented below:

$$\mathbf{N \rightarrow U_A \rightarrow A}$$

Where N, $\mathbf{U_A}$ and A represent native heynein at pH 7.0, acid unfolded heynein at pH 2.0 and acid refolded state at pH 0.5, respectively.

In addition, we observed refolding of the $\mathbf{U_A}$-state in presence of 0.5 M KCl, (termed the '$\mathbf{I_1}$-state'), and also in presence of 0.2 M Na_2SO_4 (termed the '$\mathbf{I_2}$-state'). The degree of refolding induced by the sulfate ion is greater than the other anion and the molar CD ellipticity of the sulfate induced state at 222 nm is almost native like (~ –7900 deg cm^2dmol^{-1}) representing the high structural induction.

The temperature induced unfolding of heynein at neutral pH is cooperative and the trend is retained even with decrease in pH, however, the transition midpoints of the denaturation are lowered. The conformational stability of heynein at 25 ºC (ΔG (25ºC)), is assessed to be 9.1 ± 0.2 kcal/mol at neutral pH. It is in good agreement with the ΔG (H_2O) of 8.9 ± 0.2 kcal/mol obtained by GuHCl denaturation of heynein at pH 7.0 (data not shown). At pH 2.0, the ΔG (25 ºC) of heynein is reduced to 3.2 ± 0.2 kcal/mol with a Δ(ΔG) of –5.9 kcal/mol, indicating highly decreased conformational stability, which is consistent with the loss of functional and complete tertiary structural fold. This is strongly suggestive of disruption of major stabilizing interactions an thus the protein is acid unfolded. Although the ΔG (25 ºC) at pH 3.0 is 6.3 ± 0.2 kcal/mol with a Δ(ΔG) of –3.2 kcal/mol and the transition mid-point of heat denaturation of heynein at pH 3.0 is decreased by 10 ºC compared to pH 7.0, no changes can be detected in the CD spectrum at pH 3.0 and the enzyme retains its complete peptidase activity.

The urea induced unfoldings of the refolded $\mathbf{U_A}$-, $\mathbf{I_1}$-, and **A**- states are cooperative. The Δ(ΔG) for the **A**-, and $\mathbf{I_1}$-states are 1.9 and 3.8 kcal/mol respectively relative to the $\mathbf{U_A}$- state, thereby indicating a greater refolding by the chloride anion than protons (i.e. H^+). Interestingly however, the sulfate induced $\mathbf{I_2}$-state follows three-state unfolding kinetics with urea with the mid-point of the second unfolding transition for $\mathbf{I_2}$-state being higher (3.1 ± 0.3M urea) than that of the $\mathbf{U_A}$- (1.23 ± 0.3M urea), **A**- (1.65 ± 0.3M urea) and also the $\mathbf{I_1}$-state (2.08 ±0.3M urea). The lower CD ellipticity at 222 nm and the high transition mid-points of urea denaturation support that the sulfate ions induce higher degree of refolding of the $\mathbf{U_A}$-state compared to the chloride ion and the acid.

Previously, three possible mechanisms have been proposed for stabilization of protein conformation by anions: Debye-Huckel screening effect, ion-pair formation or by affecting the water structure. If the Debye-Huckel screening has the major contributions, effect of various ions would depend on the ionic strength of the solution. Thus, due to a higher degree of observed refolding of the $\mathbf{U_A}$-state in presence of 0.2 M Na_2SO_4 compared with the 0.5 M KCl, this is not the dominant force. In accordance with electro-selectivity series of anions (Gjerde et al., 1980), the ion-pair formation apparently has significant contributions in the anion induced refolding of the $\mathbf{U_A}$-state of heynein. Also, since the anion induced refolding of the $\mathbf{U_A}$-state obeys the Hofmeister series, manipulation of the water structure also contributes in the refolding process. The chloride and sulfate ions being kosmotrops favor the minimization in the protein surface area by inducing folding because of their favorable interaction with surrounding water (Arakawa and Timasheff., 1982).

X-ray crystallographic studies have shown that high stabilities of the related plant cysteine proteases ervatamin B and C are partly due to increased inter-domain interactions which result from the increased hydrogen bonding propensities of the residues flanking the inter-domain cleft (Biswas et al., 2003; Thakurta et al.,

2004). The high stability and cooperative unfolding of heynein may be an indication of similar high inter-domain interactions in the native protein. The observed three-state unfolding of the $\mathbf{I_2}$-state of heynein possibly suggests that in the $\mathbf{I_2}$-state its putative domains may have different stabilities and much reduced inter-domain interactions.

Strikingly, the $\mathbf{U_A}$-state of heynein converts into a predominantly β-sheet rich structure upon addition of methanol in the solvent. The substantial decrease in the CD ellipticity at 215 nm and increase in the tryptophan fluorescence intensity support refolding of the $\mathbf{U_A}$-state into a β sheet-rich conformation (termed the '**O**-state'). The conformational switch to β sheet-rich structure is a characteristic feature of amyloid formation (Patel et al., 2009; Patel and Liebman 2007; Stefani and Dobson, 2003), thus polypeptide chain of heynein may have amyloidogenic propensity. Notably, similar methanol driven conformational conversion to β sheet-rich structure at low pH is also seen with the cysteine proteases ervatamin B and C (Sundd et al., 2000).

The observed conformational transitions of heynein can be summarized schematically as in **Figure 8**. It is possible that some of these obtained equilibrium intermediates of heynein bear structural likeness to the on-pathway intermediates in the *in vivo* folding of this protein.

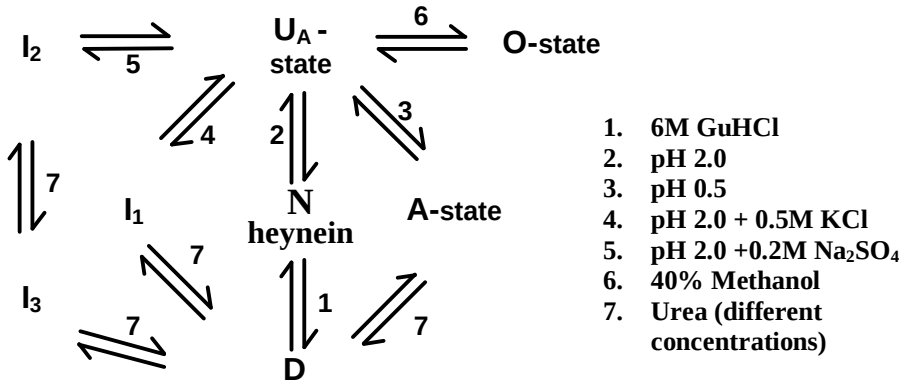

Figure 8: Summary of *in vitro* Conformational Transitions in Heynein: N, Native State of Heynein; D, Denatured State; U_A, Acid Unfolded State, A, Acid Refolded State; I1, Chloride Refolded State; I_2, Sulfate Refolded State; O, Methanol Induced State.

VI MATERIALS AND METHODS

Urea and GuHCl were procured from Sigma Chemical Company, USA. ANS was from Aldrich, USA. All other chemicals were of high purity. For spectroscopy, urea and GuHCl concentrations were determined by refractometry (Pace et al., 1989), and samples were filtered through 0.45 μm filters.

Purification of Heynein and Protease Assay

Heynein was purified to homogeneity by the method of Patel and Jagannadham (Patel and Jagannadham, 2003). Protein concentration was determined using its extinction coefficient ($\varepsilon^{1\%}_{280}$ = 23.2) and its protease activity on denatured azoalbumin was assayed as described previously (Patel and Jagannadham., 2003).

Spectroscopy

Fluorescence spectra were recorded on Perkin-Elmer LS 50B spectrofluorimeter equipped with a constant temperature cell holder. For tryptophan fluorescence, the sample was excited at 292 nm and its fluorescence emission was monitored between 300-400 nm. ANS fluorescence was recorded between 400-600 nm after excitation at 380 nm. Slit widths for excitation and emission were 10 and 5 nm respectively and the protein concentration was 1 μM for all experiments. All spectra were recorded at 25 ^{o}C unless stated otherwise.

Circular dichroism (CD) spectra were recorded on a JASCO 500A spectropolarimeter, pre-calibrated with 0.1% d-10-camphorsulfonic acid solution (Cassim and Yang., 1969). Secondary structure was probed using far-UV CD between 200-260 nm using 0.1 mg/mL protein and 1 mm path length cuvette. For estimating tertiary structure, CD spectrum of 1mg/mL protein was recorded in the wavelength 260-320 nm using 10 mm path length cuvette. CD spectra were corrected for baseline contributions before calculating the mean residue ellipticity [θ] using:

$$[\theta] = \theta_{obs} \times MRW/10c.l$$

where θ_{obs}, *c*, and l represent the observed ellipticity in degrees, protein concentration in g/mL and the path length of the light in cm respectively. Mean amino acid residues weight (MRW) was taken as 112. α-helical content was

calculated by the method of Chen et al (Chen et al., 1972) using the equation:

$$\% \alpha \text{ -helicity} = (\theta_{222} - \theta_{min}) / (\theta_{max} - \theta_{min}) \times 100$$

where, θ_{222} is the molar ellipticity at 222 nm. θ_{min} and θ_{max} are the minimum (2340) and maximum (30, 300) values of molar ellipticities at 222 nm, respectively calculated from unordered and helical fractions of five proteins(Chen et al., 1972).

ANS Binding Assay

A stock solution of ANS ($\varepsilon 350_{nm} = 5000\ M^{-1}cm^{-1}$) was prepared in dimethyl sulfoxide (Stryer et al., 1965). Protein was incubated with ANS (molar ratio 1:100) for 30 minutes in dark before assessing the extrinsic fluorescence of Protein-ANS complex by recording fluorescence spectra (400-600 nm) after excitation at 380 nm.

Conformational Transitions with pH

Effect of pH on the structure of heynein was monitored by activity assays, far UV CD, near UV CD and fluorescence spectroscopy, after 24 hours incubation at a given pH at 25 ^{o}C. To obtain various pH ranges, the different buffers used were: 0.05 M KCl-HCl (pH 0.5-1.5), 0.05 M glycine-HCl (pH 2.0-3.5), 0.05 M sodium acetate (pH 4.0-5.5), 0.05 M sodium phosphate (pH 6.0-7.5), 0.05 M tris-HCl (pH 8.0-10), and 0.05M sodium carbonate (pH 10.5-12).

Unfolding of heynein

Thermal unfolding of heynein was monitored by incubating at different temperatures for 15 minutes prior to the spectra collection. The fraction of unfolded protein (f_U) was calculated using the equation:

$$f_U = (y_F - y)/(y_F - y_{U)}$$

where, y_F and y_U represent the value of y characteristic of folded and unfolded states respectively. The conformational stability of the protein was calculated using the equation:

$$\Delta G = - R\ T \ln K = - R\ T \ln (y_F - y)/(y - y_{U)}$$

where ΔG, R, T, and K respectively represent the free energy change, gas constant, absolute temperature and equilibrium constant for the unfolding process. The values of ΔG (H_2O) and ΔG (25 ^{o}C) were obtained by extrapolation to the no denaturant concentration and to 25 ^{o}C respectively.

REFERENCES

[1] Arakawa, T. and Timasheff, S.N. (1982). "Preferential Interactions of Proteins with Salts in Concentrated Solutions." *Biochemistry*. **21**: 6545-6552.

[2] Barrick, D., Hughson, F.M. and Baldwin, R.L. (1994). "Molecular Mechanisms of Acid Denaturation. The Role of Histidine Residues in the Partial Unfolding of Apomyoglobin." *J. Mol Biol*. **237**: 588-601.

[3] Becktel, W.J. and Schellman, J.A. (1987). "Protein Stability Curves." *Biopolymers*. **26**: 1859-1877.

[4] Biswas, S., Chakrabarti, C., Kundu, S., Jagannadham, M.V. and Dattagupta, J.K. (2003). "Proposed Amino Acid Sequence and the 1.63 A X-ray Crystal Structure of a Plant Cysteine Protease, Ervatamin B: Some Insights into the Structural Basis of its Stability and Substrate Specificity." *Proteins*, **51**: 489-697.

[5] Bychkova, V.E., Pain, R.H. and Ptitsyn, O.B. (1988). "The 'Molten Globule' State is Involved in the Translocation of Proteins Across Membranes?" *FEBS Lett.* **238**: 231-234.

[6] Cassim, J.Y. and Yang, J.T. (1969). "A Computerized Calibration of the Circular Dichrometer." *Biochemistry*. **8**: 1947-1951.

[7] Chen, Y.H., Yang, J.T. and Martinez, H.M. (1972). Determination of the Secondary Structures of Proteins by Circular Dichroism and Optical Rotatory Dispersion." *Biochemistry*. **11**: 4120-4131.

[8] Dobson, C.M. (2003). "Protein Folding and Misfolding." *Nature*. **426**: 884-890.

[9] Dubey, V.K. and Jagannadham, M.V. (2003). "Differences in the Unfolding of Procerain Induced by pH, Guanidine Hydrochloride, Urea, and Temperature." *Biochemistry*. **42**: 12287-12297.

[10] Edwin, F. and Jagannadham, M.V. (1998). "Sequential Unfolding of Papain in Molten Globule State." *Biochem Biophys Res Commun* **252**: 654-660.

[11] Edwin, F. and Jagannadham, M.V. (2000). "Single Disulfide Bond Reduced Papain Exists in a Compact Intermediate State." *Biochim Biophys Acta*. **1479**: 69-82.

[12] Fink, A.L., Calciano, L.J., Goto, Y., Kurotsu, T. and Palleros, D.R. (1994). "Classification of Acid Denaturation of Proteins: Intermediates and Unfolded States." *Biochemistry*. **33**: 12504-12511.

[13] Gjerde, D.T., Schmuchler, G. and Fritz, J.S. (1980). "Anion Chromatography with Low-conductivity Eluents-II." *J. Chromatogr*. **187**: 35-45.

[14] Hernandez-Arana, A. and Soriano-Garcia, M. (1988). "Detection and Characterization by Circular Dichroism of a Stable Intermediate State Formed in the Thermal Unfolding of Papain." *Biochim Biophys Acta*. **954**: 170-175.

[15] Huet, J., Looze, Y., Bartik, K., Raussens, V., Wintjens, R. and Boussard, P. (2006). "Structural Characterization of the Papaya Cysteine Proteinases at Low pH." *Biochem Biophys Res Commun*. **341**: 620-626.

[16] Kelly, J.W. (1998). "The Alternative Conformations of Amyloidogenic Proteins and Their Multi-step Assembly Pathways." *Curr Opin Struct Biol*. **8**: 101-106.

[17] 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.

[18] Manavalan, P. and Jonson jr, W. C. (1983). "Sensitivity of Circular Dichroism to Protein Tertiary Structure Class." *Nature*. **305**: 831–832.

[19] Nandi, P.K. (1998). "Evidence of Molten Globule Like State(s) of Interferon Gamma in Acidic and Sodium Perchlorate Solutions." *Int J Biol Macromol*. **22**: 23-31.

[20] Pace, C.N. and Grimsley, G.R. (1988). "Ribonuclease T1 is Stabilized by Cation and Anion Binding." *Biochemistry*. **27**: 3242-3246.

[21] Pace, C.N., Shirley, B.A. and Thomson J.A. (1989). "In Protein Structure: A Practical Approach (eds. T.E. Creighton) IRL Press", *Oxford*, pp 311-330.

[22] Patel, B.K., Gavin-Smyth, J. and Liebman, S.W. (2009). "The Yeast Global Transcriptional Co-repressor Protein Cyc8 can Propagate as a Prion." *Nature Cell Biology*. **11**: 344-349.

[23] Patel, B.K. and Jagannadham, M.V. (2003). "A High Cysteine Containing Thiol Proteinase from the Latex of *Ervatamia Heyneana*: Purification and Comparison with Ervatamin B and C from *Ervatamia Coronaria*." *J. Agric Food Chem.*" **51**: 6326-6334.

[24] Patel, B.K. and Liebman, S.W. (2007). "Prion-proof" for [PIN+]: Infection with in Vitro-made Amyloid Aggregates of Rnq1p-(132-405) Induces [PIN+]." *J. Mol Biol*. **365**: 773-782.

[25] Randall, L.L. and Hardy, S.J. (1995). "High Selectivity with Low Specificity: how SecB has Solved the Paradox of Chaperone Binding." *Trends Biochem Sci*. **20**: 65-69.

[26] Sharma, Y.V. and Jagannadham, M.V. (2003). "N-terminal Domain Unfolds First in the Sequential Unfolding of Papain." *Protein Pept Lett*. **10**: 83-90.

[27] Stefani, M. and Dobson, C.M. (2003). "Protein Aggregation and Aggregate Toxicity: New Insights into Protein Folding, Misfolding Diseases and Biological Evolution." *J. Mol Med*. **81**: 678-699.

[28] Stryer, L. (1965). "The Interaction of A naphthalene Dye with Apomyoglobin and Apohemoglobin." A Fluorescent Probe of Non-polar Binding Sites." *J Mol Biol*. **13**: 482-495.

[29] Sumner, I.G., Harris, G.W., Taylor, M.A., Pickersgill, R.W., Owen, A.J. and Goodenough, P.W. (1993). "Factors Affecting the Thermostability of Cysteine Proteinases from *Carica Papaya*." *Eur J Biochem*. **214**: 129-134.

[30] Sundd, M., Kundu, S. and Jagannadham, M.V. (2000). "Alcohol-induced Conformational Transitions in Ervatamin C. An alpha-helix to Beta-sheet Switchover." *J. Protein Chem*. **19**: 169-176.

[31] Tanford, C. (1968). "Protein Denaturation." *Adv Protein Chem*. **23**: 121-282.

[32] Thakurta, P.G., Biswas, S., Chakrabarti, C., Sundd, M., Jagannadham, M.V. and Dattagupta, J.K. (2004). "Structural Basis of the Unusual Stability and Substrate Specificity of Ervatamin C, a Plant Cysteine Protease from *Ervatamia Coronaria*." *Biochemistry*. **43**: 1532-1540.

[33] Tiktopulo, E.I. and Privalov, P.L. (1978). "Papain Denaturation is not a Two-state Transition." *FEBS Lett*. **91**: 57-58.

[34] Turk, B., Turk, D. and Turk, V. (2000). "Lysosomal Cysteine Proteases: More than Scavengers." *Biochim Biophys Acta*. **1477**: 98-111.

[35] Turk, B., Turk, V. and Turk, D. (1997). "Structural and Functional Aspects of Papain-like Cysteine Proteinases and their Protein Inhibitors." *Biol Chem*. **378**: 141-150.