SOLVENT MEDIATED THERMAL STABILITY AND REFOLDING OF YEAST HEXOKINASE A

Thesis submitted to the Jawaharlal Nehru University in partial
fulfillment for the award of the degree of

Doctor of Philosophy

By

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CERTIFICATE

This is to certify that the present work entitled "Solvent mediated thermal stability and refolding of yeast hexokinase A" submitted to the Jawaharlal Nehru University, New Delhi in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy, embodies original research work carried out in the Centre for Biotechnology, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full, for any degree or diploma of any other university.
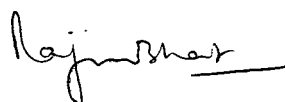
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Dedicated
to my Parents

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Abbreviations

A	Absorbance
ADP	Adenosine diphosphate
ANS	8-anilino-1-naphthalene sulfonic acid
ASA	Accessible surface area
ΔAbs	Change in absorbance
ATP	Adenosine triphosphate
^{o}C	Degree Celsius
CD	Circular dichroism
cm	centimeter
ΔC_p	Excess heat capacity of protein denaturation
D	Denatured state of protein
Da	Dalton
DSC	Differential Scanning Calorimetry
E_A	Activation energy
EG	Ethylene glycol
EXP	Exponentials
Fl	Fluorescence
f_u	fraction unfolded
GdmCl	Guanidinium chloride
GPDH	Glucose-6-phosphate dehydrogenase
ΔG	Gibbs free energy change
ΔH	Enthalpy change
ΔH_{cal}	Calorimetric enthalpy of denaturation of a protein
ΔH_v	van't Hoff enthapy of denaturation of a protein
HK	Hexokinase
I	Intermediate state of protein
K	Equilibrium constant
k	Rate constant of a reaction
kcal mol^{-1}	kilocalories per mole
^{o}K	Degree Kelvin
L	Litre
ln	$\log_e$ (natural log)
M	Molar
m	molality
MG	Molten Globule
mg	milligram
mM	Milimolar
mol	Mole
ml	millilitre
mm	millimeter
N	Native state of protein
nm	nanometer
NADH	Nicotinamide adenine dinucleotide reduced
NAD	Nicotinamide adenine dinucleotide oxidized

NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate oxidized
OD	Optical density
PDB	Protein data bank
pH	$-\log a_{H^+}$
pI	Isoelectric point
pK_a	$-\log K_a$
PPI	Peptidyl prolyl isomerase
T	Temperature
T_m	Midpoint of temperature denaturation
UV	Ulta violet
ε	Extinction coefficient
μg	Microgram
μM	Micromolar
μl	Microlitre
$[\Theta]$	Molar ellipticity

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Introduction

In the process of evolution of life, organisms have adapted to diverse environmental conditions such as high and low temperatures, saline and alkaline conditions and extremes of pressure. The currently known limits for living organisms with respect to various physical variables are $-40\,^{\circ}C$ to $115\,^{\circ}C$ for temperature, < 120 Mpa for pressure, 1 to 11 pH, and water activity of > 0.6 (corresponding to 6 M) (Jaenicke, 2000). To survive under such harsh conditions, organisms have modified the constituent components of the cell such as changing the fluidity of the lipid bilayer, the GC content of nucleic acids and the stability of the proteins, to suit for the particular living conditions.

Proteins are especially interesting, as they constitute the major structural and functional components of all the living organisms. Proteins are heteropolymers of 20 different kinds of amino acids, linked by the peptide bonds. The extraordinary capacity of proteins to be functional in different environmental milieu of external as well as internal conditions lies in the three dimensional structure of their native state, which in turn depends on the linear sequence of the amino acids. How do the protein molecules attain a unique three-dimensional structure from the linear chain of amino acids is still elusive and is known as the "Protein folding problem" (Honig, 1999).

The native state of proteins is a balance of various stabilizing non-covalent interactions like hydrogen bonding, electrostatic interactions, van der Waals interactions, hydrophobic interactions and the covalent linkages like the disulphide bonds and destabilizing configurational entropy. The overall free energy of stabilization of the native state for most proteins is marginal and the native state is stable over the unfolded state by only about 5-10 kcal/mol (Pace, 1975; Dill, 1990). Due to the involvement of different kinds of forces and the marginal stability of the native state, a variety of external conditions can perturb their unique three-dimensional conformation such as temperature, pH, ionic strength, chemical denaturants like urea and GdmCl and pressure, etc. leading to the formation of unfolded or molten globule like states or aggregated species depending on the final balance of the forces. With the advent of the recombinant DNA technology, many commercially important enzymes are cloned and overexpressed in prokaryotic systems, and often form insoluble aggregates known as inclusion bodies (Misawa and Kumagai, 1999). Recovery of active proteins from inclusion bodies is a major hurdle in the biotechnology and pharmaceutical industries. Also, there are growing numbers of evidences of the diseases that are caused by the misfolding of proteins *in vivo* (Hetz and Soto, 2003; Temussi *et al.*, 2003). It is, therefore, necessary to understand the intricate balance of the stabilizing and the destabilizing interactions resulting in the different conformational states of the proteins, and the role of solvent environment in modulating these

forces. Such studies would be interesting not only from an academic point of view but would also find applications in the industrial processes and help generate strategies to counter various conformation related diseases.

NATIVE STATE

A polypeptide chain folds into a unique conformation that is determined entirely by the information included in the sequence of the amino acid residues. According to the thermodynamic hypothesis of protein folding, the three-dimensional structure of a native protein in its physiological milieu is at global minimum (Anfinsen, 1973), which cannot be falsified by experiment, as conformations outside the global minimum cannot be accessed (Baker and Agard, 1994). The folding of a polypeptide chain into a three-dimensional structure is in principle a reversible process. In the native state, each atom occupies a definite position as in a crystal, but differs in that the position of each atom is unique relative to the neighboring atoms representing an aperiodic macroscopic system, which is separated by a large energy barrier from another macroscopic state, the unfolded state (Privalov, 1992).

Native state can be best characterized as the fluctuating ensemble of conformations that involve local unfolding reactions and the stability of each state depends on the balance of factors which involve the gain in conformational entropy due to local unfolding and the solvent interaction with the newly exposed regions (Hilser et al., 1998; Sadqi et al., 1999; Hilser, 2001). The flexibility of the loop regions helps to gain the conformational entropy without losing any enthalpic contributions (Makhatadze and Privalov, 1993, 1996). But for most practical purposes, the native state can be treated as a macroscopic system, which undergoes a first order phase transition upon unfolding (Privalov, 1989).

Native states of globular proteins are compact with a tightly packed hydrophobic core and are biologically active. However, recent studies have changed this view as evident in a recent review (Uversky, 2002a) wherein a new category of proteins, called the "Natively unfolded proteins" are being increasingly discovered which are known to have considerable functional significance. Members of this group are rather unstructured to the extent of random coils without any secondary structure or exist as pre-molten globules with little secondary structure. These intrinsically unstructured proteins are known to undergo order-disorder transition during or prior to biological function. High concentrations of solute such as glucose BSA or ovalbumin have been found to induce structure in unstructured proteins (Dedmon et al., 2002).

Many large proteins organize into domains in its native state. There are diverse definitions for domains; a domain can be defined as a stable unit or a structural unit or a

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genetic unit or a functional unit or an evolutionary unit or a thermodynamic unit or a discrete unit. In multidomain proteins, separate domains appear to maintain their own structures while domains themselves change their position with respect to each other by hinge like motions. The residues within domains experience little change in their position (Garel, 1992; Jaenicke, 1999).

DENATURED STATES

The first theory of protein denaturation was proposed as early as 1931 by Hsien Wu (Wu, 1931). During that period the term denaturation was used loosely and interchangeably to include the changes in solubilities such as precipitation, coagulation and flocculation. Wu defined the term denaturation as "disorganization of natural protein molecule, the change from the regular arrangement of rigid structure to the irregular, and diffuse arrangement of the flexible open chain". As the work on protein stability and folding gained momentum, emphasis gradually shifted from the native to the denatured states. To explain the various physico-chemical properties of the denatured states, many definitions have appeared in the literature over a period of time (Kauzmann, 1959; Tanford 1968, 1970; Dill and Shortle, 1991; Shortle, 1996). Now it is understood that the term 'denatured' refers to any non-native state and the terms 'unfolded' and 'compact' states refer to specific subsets of the denatured conformation depending on whether the radius of gyration of biopolymer is large or small, respectively (Alonso et al., 1991). But the question of how close is the denatured protein to that of a random coil state still persists and its implications in the folding kinetics as well as the stability of proteins are immense and increasingly becoming important (Shortle, 1996; Baldwin and Zimm, 2000; Hammarstrom and Carlsson, 2000).

As the population of the denatured state under native conditions is very low, various methods have been employed to study the denatured states such as low and high temperature, pressure, acidic and alkaline pH, organic cosolvents, and chemical denaturants like urea and guanidinium chloride (GdmCl). They denature proteins by different mechanisms and to various extents, e.g., temperature and pH denaturation has been found to be incomplete, whereas at high concentrations of urea and GdmCl, many proteins approach random coil state (Tanford, 1968, 1970; Tanford and Aune, 1970; Ahmad and Salahauddin, 1979). Some workers have also used fragmentation of proteins to study the denatured states under native conditions (Wang and Shortle, 1995). Though the mechanistic details of protein denaturation by urea and GdmCl are still obscure, these agents exert their action by binding to the protein as evident from apparent partial volume effects (Lee and Timasheff, 1974; Prakash et al., 1981) and heat effects (Makhatadze and Privalov, 1992), or indirectly by changing the solvent

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water properties (Schellman, 1987). However, recent simulation studies show that urea denaturation involves both, direct and indirect effects, by binding and changing the water structure, respectively (Bennion and Daggett, 2003). However, urea and GdmCl increase the surface tension of water thus making cavitation more difficult (Breslow and Guo, 1990). Several methods have been developed to calculate the free energy of stabilization of a protein (Ahmad & Bigelow, 1982, 1986, 1990; Schellman and Gassner, 1996). GdmCl at low concentrations stabilizes RNase T1, which has been attributed to binding of guanidinium ion to the protein and results in non-coincidence of the free energy values with that of urea denaturation, although the transition is two-state in both (Mayr and Schmid, 1993). Urea at low concentrations is also observed to stabilize the molten globule state of papain in terms of increased transition temperature (Edwin et al., 2002).

From the folding point of view, it is well known that the denatured state ensemble is under equilibrium of fast and slow folding species, which arise due to proline isomerization (Schmid et al., 1993). The denatured proteins under different denaturing conditions are thermodynamically indistinguishable, as evident by similar dependence of enthalpy on the Tm for different agents of denaturation (Pfeil and Privalov, 1976 a, b). Also, the partial specific heat capacities of the denatured proteins match well with the estimated values by adding up the individual contributions from amino acids assuming completely unfolded polypeptide chain (Makhatadze & Privalov, 1990). For the compact denatured proteins, heat capacity of denaturation can have positive contributions from the non-covalent intra-protein interactions (Lazaridis and Karplus, 1999).

The ensemble averaged radius of a protein is a function of chain length and depends on the solvent conditions, which can be good or poor or theta. Due to the heterogeneous nature of the polypeptide chain, no solvent is good for all parts of the chain leading to various degrees of compactness in the denatured state (Dill and Shortle, 1991; Kataoka and Goto 1996). Theoretical studies indicate that energetically favorable local side chain and backbone interactions restrict the ensemble of conformations available in the denatured state to a relatively small subset as compared to the entire conformational space (Shortle, 2002). Furthermore, local steric clashes, which can extend beyond nearest chain neighbors, 3-6 residues apart in the case of polyalanine and even more for bulkier side chains, eliminate the large number of backbone conformations in the Ramachandran map (Pappu et al., 2000). Due to the preferential distribution of phi and psi angles in the denatured states even under random coil like situation, formation of secondary structural elements may be favored (Smith et al., 1996) that can melt cooperatively as in the case of tryptophan synthase (Saab-Rincon et al., 1993) or non-cooperatively as in α-lactoglobulin (Schulman et al., 1997). Also, clusters of

hydrophobic groups without any regular secondary structural elements exist, which can be native like, where these sites probably would act as folding initiation sites or they could be non-native like, where these sites have to unfold before attaining proper fold (Wang and Shortle, 1995; Freund et al., 1996). Some of these compact structures are stable as isolated fragments, suggesting that local interactions are responsible for its stability whereas in some other cases they lose structure when isolated as fragments indicating that long range interactions are responsible for its stability (Wang and Shortle, 1997). Experimental evidence for the persistence of native like topology in 8 M urea by residue dipolar couplings suggests that long range ordering occurs well before a folding protein attains compact conformation (Shortle and Ackerman, 2001). Network of hydrophobic clusters have been found in the denatured and reduced lysozyme that are stabilized by single residue tryptophan62 by non-native long-range cooperative interactions. Mutating this residue to glycine leads to the disappearance of all the clusters suggesting the role of non-native interactions in preventing the aggregation during early folding steps (Klein-Seetharaman et al., 2002; Baldwin, 2002).

MOLTEN GLOBULE STATE

Tanford (1968) first showed the existence of partly folded states of proteins by temperature and pH denaturation. Later, Kuwajima et al., (1976) established the existence of two well resolved cooperative transitions, viz., one transition corresponding to aromatic amino acids while the other to the peptide backbone for GdmCl induced denaturation of α-lactalbumin. The observed intermediate structure showed the native like secondary structure but lacked the tertiary interactions, which was named as molten globule by Ohgushi and Wada (1983). After observing molten globule states in a number of proteins, the existence of such state is more of a rule than an exception now.

Molten globule (MG) states are compact and their volume increases by $50\pm8\%$ relative to the native state compared to 3.3 ± 0.3 fold increase in 6M GdmCl (Ptitsyn, 1992). However, large angle scattering shows little difference when compared to the native state indicating the presence of rather native like core contacts and suggests that expansion is not uniform and the core remains packed little loosely than in native state, while the outer shell is much more expanded (Ptitsyn, 1995).

Far UV CD spectra and amide I region of infrared spectra (Dolgikh et al., 1985) show substantial secondary structure comparable to the native state, whereas near UV CD and NMR spectra show a virtual absence or the presence of very few interactions (Kuwajima, 1989) for MG states. There is also a marked difference in the mobilities of the aliphatic and the aromatic side chains. Mobilities of smaller aliphatic side chains coincide with the near UV CD whereas

the mobilities of the bulkier and asymmetric aromatic side chains coincide with the far UV CD. These exposed hydrophobic groups bind to non-polar molecules such as ANS much more strongly than either the native or the unfolded states (Semisotonov et al., 1991). In a number of proteins by using H→D exchange it has been shown that the residual structure in MG state has native like contacts and it retains the overall architecture of the native state (Ptitsyn, 1995).

Although, the molten globule state shows cooperative transitions to the fully unfolded or the fully folded state by chemical denaturants, at the same time the absence of thermal transitions for the MG states of some proteins has led to the controversy that whether molten globule represents a universal thermodynamic state (Pfeil, 1998). It has been argued that in the molten globule some portion (sub domain) remains native like while the other portion is unstructured and hence the transition does not embrace the whole molecule, which is a necessary condition for all or none transition (Privalov, 1996). On the other hand, experimental evidences like the presence of bimodal distribution for MG to unfolded state transition (Uversky et al., 1992) and the molecular weight dependency of the cooperativity (Ptitsyn and Uversky, 1994) support the view that MG state is a true thermodynamic state.

Molten globule state has been shown to be a productive on-pathway kinetic intermediate during protein folding and the structure of the equilibrium molten globule has been observed to be similar to the kinetic intermediate formed during the folding process (Baldwin, 1993; Arai et al., 1998). The stability of the burst phase intermediate studied by unfolding transition curve is identical to the stability of the equilibrium molten globule state, supporting the view that equilibrium molten globule and its kinetic counterpart are identical (Arai and Kuwajima, 1996). The molten globule states have also been shown to play a significant role in a number of physiological processes and in certain diseases (Ptitsyn, 1995; Ren et al., 1999; Morrow et al., 2002).

FORCES RESPONSIBLE FOR CONFORMATIONAL STABILITY OF PROTEINS

Since the proteins are only marginally stable at room temperature, all kinds of molecular interactions contribute either positively or negatively to protein stability (Alber, 1989). Depending on the distance between the interacting atoms, these interactions can be either long ranged or short ranged and depending on the location of interacting atoms along the polypeptide chain, these interactions can be either local or non-local. A local or non-local interaction can arise from either long ranged or short ranged interactions (Dill, 1990).

Electrostatic interactions

The contribution of electrostatic interactions to protein stability can be readily ascertained by the dependency of stability on pH and salt concentration, while pH affects the ionization, salts screen the charges. When a positive charge encounters a negative charge in solution, the complex formed is known as an "ion pair". The strength of the interaction depends on the distance between the two charges and the dielectric constant of the medium. The dielectric constant reflects the screening effect of surrounding medium on the apparent interaction energy between the two charges when compared to their interaction energy in vacuum. Since ionizable groups are constrained in the folded protein, the distance between the charged groups will be less in the folded proteins than in the unfolded protein. Since the dielectric constant of water is much higher (D=80) than the interior of the protein (D=2 to 4), the effective dielectric constant for each pair of charges is lower in the folded protein than in the unfolded protein. For these reasons the contribution of the charged residues should be more in the native protein than in unfolded protein (Tanford, 1961; Imoto, 1983). Significant component of the electrostatic free energy difference between the native and the denatured states is due to a small number of amino acids whose pKa's are shifted anomalously in the native state (Pace et al., 1990; Hu et al., 1992; Yang and Honig, 1993). By using Finite Difference Poisson-Boltzmann (FDPB) method, pKa values can be computed quite reliably (Yang et al., 1993). By using FDPB to account for the solvation effects, free energy determinants of secondary structure formation have been calculated (Yang and Honig, 1995a, b; Yang et al., 1996).

Experimental measurements have confirmed that the pairing of oppositely charged groups could be stabilizing (Fairman et al., 1996). An ion pair was found to have interaction energy of approximately 3 kcal/mol (Fersht, 1972) and a salt bridge in T4 lysozyme has been estimated to contribute 3-5 kcal/mol to the free energy of folding (Anderson et al., 1990). Neutralization and charge reversal of surface charges using site directed mutagenesis was found to be destabilizing in staphylococcal nuclease (Schwehm et al., 2003). Electrostatic interactions at protein-protein interfaces are important for the specificity and stability of the complex (Sheinerman et al., 2000). However, surface charges have been found to play no role in the stability and folding of ubiquitin (Loladze and Makhatadze, 2002). Altering the pI of Ribonuclease Sa affects the pH of minimum solubility but not the pH of maximum activity and maximum stability (Shaw et al., 2001). These studies suggest the important role electrostatic interactions play in protein stability.

Hydrogen bond

A hydrogen bond occurs when a hydrogen atom is shared between two electronegative atoms. A hydrogen bond is primarily a linear arrangement of a donor, hydrogen, and an acceptor and is comprised of electrostatic, dispersion, charge-transfer, and steric repulsion interactions (Pauling, 1960; Vinogradov and Linnell, 1971). The hydrogen bond is generally described as an electrostatic dipole-dipole interaction as it results from the interaction of two permanent partial charges (Burley and Petsko, 1988). The strength of a hydrogen bond, which depends on the electronegativity and orientation of the bonding atoms, is about 2-10 kcal/mol (Pauling, 1960). From the adhesion energy between two lipid surfaces that can form hydrogen bonds, Tareste et al., (2002) have deduced the energy of a single hydrogen bond in water corresponding to about 0.5 kcal/mol.

Mirsky and Pauling were the first to suggest that a hydrogen bond could form between C=O and NH groups of the peptide backbone which led to the discovery of α-helix, parallel and antiparallel sheets (Pauling et al., 1951). The importance of hydrogen bonds in protein stability can also be demonstrated by the fact that almost every group capable of forming a hydrogen bond in a folded protein does so. Several high-resolution protein crystal structures also reveal that about 90% of all groups capable of forming hydrogen bonds form at least one such a bond. On an average the mean distance between nitrogen and oxygen is about 0.297 nm (Baker and Hubbard, 1984; Stickle et al., 1992).

However, the exact contribution of hydrogen bonds to the stability of the folded protein is still doubtful. Kauzmann (1954, 1959) has argued that hydrogen bonds are probably not the dominant forces that fold proteins in water, since there is no basis that intrachain hydrogen bonds in the folded state would have lower free energy than the ones with water in the unfolded state. In support of this view, the distributions of hydrogen bond angles in proteins were observed to be about the same as in small model compounds (Baker and Hubbard, 1984). Folded protein must contain many hydrogen bonds, which would collectively stabilize the folded state. Solvent denaturation also indicates that hydrogen bonding is not the dominant force (Singer, 1962; Edelhoch and Osborne, 1976). Solvents that form strong hydrogen bonds to the peptide backbone should compete effectively and unfold proteins and those solvents that do not affect the hydrogen bonding should not unfold the protein. One important observation in this regard is that small amounts of detergents are known to unfold proteins (Tanford, 1968). Also, the effectiveness of tetraalkylammonium salts to denature proteins depends on the number of methylene groups. These results indicate that it is the hydrophobic interaction rather than the hydrogen bonding that predominantly governs the protein stability (Dill, 1990).

Mutational studies have indicated that hydrogen-bonding groups affect stability by an amount that differs considerably depending on the site as well as the nature of the mutation (Serrano *et al.*, 1992; Yamagata *et al.*, 1998; Peterson *et al.*, 1999). By mutating tyrosine residues to phenylalanine, Pace and coworkers (2001) observed that polar group burial makes a substantial contribution to protein stability. The hydrogen bonding and van der Waals interactions of polar groups in the tightly packed interior of folded proteins are more favorable than similar interactions with water in the unfolded protein. (Wales and Fitzgerald, 2001).

van der Waals interactions

van der Waals interactions arise from interactions among fixed or induced dipoles. In neutral molecules dipole moment arises due to the random fluctuation of electrons (London, 1937; Murphy, 1995). In addition, van der Waals interactions generally include those interactions resulting from the unfavorable spatial overlap of the electronic orbitals (Burley and Petsko, 1988). The energy of interaction for the van der Waals forces is very sensitive to the distance between the two atoms. The energy for the van der Waals forces can be approximated by the Lennard-Jones potential energy function, which combines the inverse twelfth power of repulsive terms with an inverse sixth power of attractive terms. The separation distance at which the interaction between the atoms is most favorable is known as the van der Waals distance (Creighton, 1993). The importance of van der Waals interactions for protein stability depends on the difference in the packing densities of the folded and the unfolded protein. The tight packing of the hydrophobic core results in an increase in the van der Waals interactions between non-polar groups. van der Waals interactions have an enthalpic contribution to the net stability of proteins and are not completely compensated by the van der Waals interactions between polar solvent and apolar atoms exposed upon unfolding (Privalov, 1992; Griko *et al.*, 1994). A large number of mutational studies also indicate the positive contribution of van der Waals interactions to protein stability (Alber, 1989; Kellis *et al.*, 1989; Matthews, 1993, 1995, 1996). However, it is difficult to quantitate the contribution due to changes in the packing and other interactions occurring at the same time.

Hydrophobic interactions

Solutes that are neither charged nor have donors or acceptors of hydrogen bond are expelled from liquid water. This phenomenon has been termed as hydrophobic effect (Kauzmann, 1959), and is analogous to the partitioning of oil in water. Since Kauzmann's predictions, large numbers of protein crystal structures have become available. They show

that a predominant feature of globular protein structures is that the non-polar residues are sequestered into a core to avoid contact with water (Chothia, 1974, 1976; Guy, 1985). The hydrophobic effect is one of the most important contributors for the folding and stability of proteins (Pace et al., 1996; Tanford, 1997). The observation of positive ΔCp for the transfer of non-polar solutes to water and for protein denaturation gave additional support to the concept of hydrophobic stabilization of proteins (Tanford, 1962; Brandts, 1964). When a protein folds, apolar residues partition into the interior of the protein and to quantitate this effect many hydrophobicity scales have been proposed (Nozaki and Tanford, 1971; Wolfenden et al., 1981; Kyte and Doolittle, 1982; Fauchere and Pliska, 1983; Rose et al., 1985). These scales are based on transfer free energies of non-polar side chains to water from micelles, liquid or vapor phases of non-polar solvents, intended to represent the protein interior. More sophisticated models based on contribution of individual atoms have been proposed (Eisenberg and McLachlan, 1986). Significant differences exist between these scales. Karplus (1997) argued that hydrophobicity scales estimated from transfer free energy from non-polar solvent to water for polar side chains vary much more than non-polar side chains. This has been reasoned to arise from the heterogeneous nature of the protein interior, i.e., no solvent can mimic the protein interior for the polar side chains while non-polar solvents fairly represent well for the non-polar side chains. Tryptophan has been scaled as highly hydrophobic by Nozaki and Tanford (1971), while Wolfenden et al., (1981) treated it as hydrophilic. The presence of double bonds between the ring carbon atoms results in segregation of partial electronic charges due to a π-electron cloud covering the face of the aromatic ring, leaving hydrogen atoms on the edge of the ring positively charged (Burley and Petsko, 1988) and rendering them psuedo non-polar (Makhatadze and Privalov, 1995). The hydrophobic effect of aromatic and aliphatic groups thus appears to be based on different mechanisms. In the case of aromatic groups, it is provided by strong van der Waals interactions between these groups and hydration effect decreases its value, whereas in the case of aliphatic groups, van der Waals interactions are enhanced by the hydration effect. At 25°C, the normalized per square angstrom hydrophobic effect of aromatic groups is about 120 $Jmol^{-1}$ and for aliphatic groups it is about 180 J mol^{-1} (Privalov and Makhatadze, 1993). The calculated hydrophobic effect value for the average non-polar groups of RNase A and myoglobin amounts to 170 J mol^{-1} A^{-2} at 25 °C (Privalov, 1995). Hydrophobic interaction increases with temperature (Baldwin, 1986) and cold denaturation is considered to be due to the loss of hydrophobic interactions with decreasing temperature (Privalov, 1990). At high temperatures the entropy of hydration of non-polar groups decreases to zero and the Gibbs energy of the hydrophobic effect becomes completely enthalpic. Thus hydrophobic effect is

not entropic in nature as usually considered. This effect is entropic in nature at low temperatures but enthalpic in nature at high temperatures (Baldwin, 1986; Privalov and Gill, 1988). The entropic and enthalpic effects of non-polar hydration have been related to the different types of water structures present at low temperatures and high temperatures (Tsai et al., 2002). In this proposal, at low temperatures hexagonal, low density, enthalpically favored ice structure (Ice Ih) prevails and exposure of non-polar surfaces further optimizes H-bond network, which compensates for the entropy loss thus leading to cold denaturation. At higher temperatures, however, high-density liquid water predominates (Ice II), exposure of non-polar surface area reduces entropy more than the enthalpic gain from the ordering of water molecules.

There has been some disagreement about the meaning of the hydrophobic effect (Tanford, 1979; Ha et al., 1989; Karplus, 1997). The traditional definition involves the transfer free energy of non-polar solutes from organic solvents to water (Kauzmann, 1959; Tanford, 1997). In the standard definition, hydrophobicity is primarily associated with the disruption of water-water cohesive forces due to the presence of non-polar solute. Indeed the magnitude of the hydrophobic effect is closely related to the free energy associated with opening an empty cavity in water (Lee, 1985). Based on the definition of transfer from vapor phase to water, Privalov and coworkers argued that hydrophobic effect makes only small contribution to protein stability (Privalov and Gill, 1988; Privalov and Makhatadze, 1993; Makhatadze and Privalov, 1993).

COSOLVENT MEDIATED PROTEIN STABILIZATION

Although overall information about the polypeptide chain folding into three-dimensional structure is contained in the amino acid sequence, yet the solvent environment plays an important role for the formation of the structure and the overall stability of the protein (Ben-Naim, 1995; Dill et al., 1995). Proteins are sensitive to the changes in the solvent environment and a wide variety of factors influence the stability and function of proteins such as pH, ionic strength, temperature, and pressure. Proteins are evolved to function in aqueous environment and the role of water in mediating the folding of polypeptide chain and its effect on the stability of three dimensional structure of proteins has been most puzzling, essentially due to the uncertainty of the water structure itself (Israelachvili and Wennerstrom, 1996; Barron et al., 1997; Franks, 2002). This situation becomes even more complex in a multicomponent system where in addition to protein and water, a third component is also present.

Over the years scientists have observed that many plants, animals and microorganisms have adapted to harsh environmental stresses such as high and low temperatures and salinity, etc., by accumulating small molecular weight compounds known as osmolytes at high concentrations (Yancey *et al.*, 1982; Timasheff, 1992, 1993). Nature has selected these osmolytes on the basis that these compounds should not affect the enzyme activity or any other cellular processes. A variety of compounds, which protect the organisms against external stress usually fall into different classes like polyols, sugars, free amino acids and their derivatives and methylamines (Yancey *et al.*, 1982). When used in molar concentrations, these compounds also provide effective way for protein stabilization (Arakawa and Timasheff, 1985; Taneja and Ahmad, 1994; Kaushik and Bhat, 1998). These compounds are used at high concentrations and may occupy as much as 40% of the solvent volume, thus rendering the two solvent components equivalent, and are thus, referred to as cosolvents (Timasheff, 1998).

Polyhydric compounds or polyols, sugars, and their oligomers have long been known to be protein stabilizers (Gerlsma, 1968, 1970). Glycerol, xylitol, sorbitol, mannitol, inositiol, glucose, trehalose and their polymeric compounds like dextran have been observed to provide stability to varying extents depending on number of –OH groups and their stereochemical properties (Back *et al.*, 1979; Uedaira and Uedaira, 1980; Kaushik and Bhat, 1998, 2003). Using spin-lattice relaxation times Uedaira and Uedaira (2001) have measured the dynamic hydration number (nDHN) for 9 polyols which is indicative of thermal motions around a polyol molecule, the values of nDHN increases with an increase in the number of OH groups and depends on the conformation of the isomers. The value of nDHN for inositol was the largest. Trehalose, a disaccharide of glucose has been observed to be an effective stabilizer of protein structures (Kaushik and Bhat, 2003). Trehalose has been found to provide complete protection against freeze thawing (Carpenter and Crowe, 1989) and also increase the reversibility of protein denaturation. Therefore, it has been used extensively in pharmaceutical formulations and some commercial applications (Colaco *et al.*, 1992). In few cases these polyols and sugars have been found to induce conformational changes in acid denatured proteins to form molten globule like states (Davis-searles *et al.*, 1998; Kamiyama *et al.*, 1999; Morar *et al.*, 2001). However, polyols have been found to decrease the transition temperature as well as denaturation enthalpy of DNA melting. The values of these thermodynamic parameters depend on both the nature and concentration of the solute. The overall destabilization of DNA molecule has been related to the different capability of polyhydric alcohols to interact with the polynucleotide solvation sites replacing water, and to the

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modification of the electrostatic interactions between the polynucleotide and its surrounding atmosphere of counter ions (Wang *et al.*, 1993; Del Vecchio *et al.*, 1999).

It has been established now that cosolvents mediate their stabilizing effect by altering the structure and properties of water at the protein water interface. Timasheff and coworkers have shown that stabilizing cosolvents such as polyols are preferentially excluded from the vicinity of protein molecules and in turn lead to preferential hydration of proteins (Gekko and Timasheff, 1981a; Arakawa *et al.*, 1990; Timasheff, 2002)

According to Timasheff (Timasheff, 1998), the thermodynamic principles involved in evaluation of preferential interaction parameters are presented as follows:

Addition of any solute lowers the water activity equivalent to

$-RTm_{cs}\Phi^{cs}/55.56$

where m_{cs} is molal concentration of the cosolvent and Φ^{cs} is the osmotic coefficient. Lowering of water activity in turn lowers the vapor pressure from that of pure water and gives rise to the osmotic pressure across the semi permeable membrane.

When hydrated protein is introduced into aqueous cosolvent solution, the immediate effect is the perturbation of the chemical potential of the cosolvent by the protein

$((\delta\mu_L/\delta m_P)_{m_L} = (\delta\mu_P/\delta m_L)_{m_P}$ where m is the molal concentration of a given component, P is protein, L is ligand or cosolvent and μ is the chemical potential). This in turn perturbs the chemical equilibrium in the vicinity of the protein. To restore the chemical equilibrium, the chemical potential of the cosolvent in the vicinity of the protein domain must be changed by an identical amount but with opposite sign to that of the perturbation. This can be accomplished by adjusting the concentration of the cosolvent, m_L, around the protein by the increment $(\delta m_L/\delta m_P)_{\mu_L} = -(\delta\mu_P/\delta\mu_L)_{m_P}$. Thus, there is mutual perturbation of the chemical potential of the cosolvent and the protein. This results in a change in the solvent composition in the immediate domain of the protein by increasing or decreasing the number of water or cosolvent molecules. Therefore, $(\delta m_L/\delta m_P)_{\mu_L}$ can be positive or negative. Negative values indicate preferential exclusion of the cosolvent, which means preferential hydration. The value of preferential hydration can be smaller than or equal to or greater than protein hydration, i.e., both protein hydration and preferential hydration are two independent aspects and the two quantities are not related directly (Timasheff, 2002).

The Wyman linkage relationship $((\delta \log K/\delta \log a_L) m_P = \Delta\upsilon_L$, where K is equilibrium constant, a_L is the activity of the ligand, and $\Delta\upsilon_L$ is the difference in binding of the ligand to the two end states) allows measurement of the modulation of reactions such as protein denaturation by cosolvents. It is suggested that cosolvent effect on denaturation reaction depends on the interactions of the native state and the denatured state with the solvent

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medium. This may involve a balance between preferential exclusion and binding depending on the type of the cosolvent (Timasheff, 1992, 1995). It has been suggested that when protein-solvent interactions are unfavorable and are not affected by the chemical nature of the protein surface, it would always lead to protein stabilization. On the other hand, if the chemical nature of the protein surface does matter in the ternary system, addition of a cosolvent either stabilizes or destabilizes the native state, even though it may be a strong precipitant of the native protein and the measurement of the interactions of the protein in either of the states would not give any insight about the solvent effect on the protein stability (Timasheff, 1995). Cosolvents such as sugars and polyols fall into the former category while 2-methyl 2, 4 pentenediol (MPD) belongs to the latter category. MPD destabilizes proteins even though it is a strong precipitant, owing to its binding to the non-polar groups of the protein (Arakawa *et al.*, 1990).

Cosolvents that are independent of the chemical nature of the protein surface, preferential exclusion occurs by two mechanisms, steric exclusion and increase in water surface free energy by the cosolvent. In steric exclusion mechanism, the radius of the cosolvent being much greater than that of water, a shell with some volume is created around the protein. This shell is enriched with water resulting in the preferential exclusion of the cosolvent (Bhat and Timasheff, 1992). The other mechanism involves the perturbation of the surface tension of water by cosolvents resulting in the distribution of solvent components at the protein-water interface. When the cosolvent raises the surface tension, it is depleted from the surface layer, which results effectively in preferential hydration; when it lowers the surface tension it is accumulated at the protein surface. For the class of cosolvents whose interaction depends on the chemical nature of the protein surface, exclusion takes place by a mechanism of 'solvophobic effect'. If the affinity of protein sites is greater for water than for the cosolvent, the most prominent outcome of such an effect is preferential exclusion. Solvophobic compounds such as glycerol are known to exclude from protein surface due to its repulsion from the non-polar regions of the protein while MPD appears to be repelled from the charges on the protein surface (Arakawa *et al.*, 1990). However, the total thermodynamic interaction between a protein and a cosolvent system is given by the sum of all such interactions. The net effect may be positive, negative, or zero, depending on the magnitude of the various compensating interactions.

Identification of part or parts of a protein responsible for the exclusion of cosolvents is helpful in understanding the forces responsible for stabilization. The measurement of transfer Gibbs energy (Δg^{o}_{tr}) gives the preference of that functional group for interaction with the cosolvent species relative to its preference for interaction with water (Nozaki and Tanford,

1971), and is similar to the transfer of non-polar groups to water used to measure their hydrophobicity. Δg°_{tr} of peptide backbone from water to protecting osmolyte solutions was found to be positive suggesting that osmolytes interact unfavorably with the peptide backbone, i.e., relative to the interaction of water with peptide backbone, osmolyte solution interacts unfavorably with the backbone (Qu *et al.*, 1998). This unfavorable interaction has been termed as the "osmophobic effect". Because the peptide backbone is highly exposed to osmolyte in the denatured state, the osmophobic effect preferentially raises the free energy of the denatured state, shifting the equilibrium in favor of the native state (Bolen and Baskakov, 2001).

PROTEIN FOLDING

The folding of a polypeptide chain under native conditions is a spontaneous and a reversible process that is directed by the information encoded in the amino acid sequence. The establishment of kinetic mechanisms for unfolding and refolding of a particular protein are first steps in the elucidation of the folding pathway followed by characterization of intermediates and transition states between them in terms of energetics and structure. Over the years kinetic folding mechanisms of many small globular proteins have been worked out thoroughly. Based on the results of such studies as well as insights gained from theoretical and computational studies various models have been postulated to explain the folding mechanisms.

Models of Protein Folding

FRAMEWORK MODEL

According to this model local sequence information is sufficient to predict the secondary structure of the protein and the formation of native secondary structure precedes the formation of tertiary interactions. The existence of on-pathway folding intermediates is a strong evidence in support of this model where intermediates are present along the folding pathway. This model also argues that the difference between proteins, which fold with and without populating any intermediates lies not in the folding mechanism but only in the stability of intermediates (Baldwin and Rose, 1999a, b). Folding reactions with successive kinetic intermediates, in which late intermediates are folded more than the early intermediates, indicate that folding is hierarchical (Baldwin, 1995).

NUCLEATION-CONDENSATION AND DOCKING MODEL

The classical nucleation model postulated that some neighboring residues of sequence would form native secondary structure that would act as a nucleus from which the native

structure would propagate in a stepwise manner (Wetlaufer, 1973, 1990). The local nucleus is weak otherwise it could slow down the folding and is stabilized by long-range interactions to give a large extended nucleus. The formation of tertiary structure is a necessary consequence of the secondary structure. This model explains essentially the folding mechanism of small proteins, which fold in a two-state fashion (Fersht, 1997).

HYDROPHOBIC COLLAPSE MODEL

This model suggests that a protein would rapidly collapse around its hydrophobic side chains and then rearrange from restricted conformational space occupied by the collapsed structure and the cooperativity arises due to the assembly of the non-polar residues into a good core. In this model, the secondary structure would be directed by the native-like tertiary interactions. This model also supports the existence of intermediates along the folding pathway (Chan et al., 1995).

DIFFUSION-COLLISION MODEL

This model considers a protein molecule to be divided into several micro domains small enough to search all conformations rapidly. Several of the micro domains coalesce together to form the structural entity that is stable (Karplus and Weaver, 1976). It assumes that coalescence of the micro domains occurs only if both the partners have at least partly native structure and correct orientation (Karplus and Shakhanovich, 1992). Intermediates can occur along the folding pathway if the stability of some of the microdomains increases (Islam et al., 2002).

THE NEW VIEW OF PROTEIN FOLDING

The new model uses the concept of funnels and energy landscapes to describe the folding of a protein molecule. A point in the multidimensional energy surface, which is characterized by local minima and maxima, represents each conformation and each state can be well represented by an ensemble. The slow or fast folding of a given protein depends on the ruggedness of the energy surface. The transition states are conceptually considered as bottlenecks, affecting the folding rate, and the model does not specify any fixed structures to the transition states (Dill and Chan, 1997).

Fast events in protein folding

The advent of the rapid reaction techniques as well as their customization using a variety of spectroscopic techniques has allowed to monitor the folding of proteins in sub millisecond to sub microsecond timescales (Bieri and Kiefhaber, 1999; Roder and Shastry, 1999). The early events primarily involve polypeptide chain collapse and secondary structure formation.

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To set the upper limit for protein folding, Hagen and coworkers (Hagen et al., 1997) estimated the rate of the polypeptide chain collapse to be about 10^6/s under folding conditions. The collapse of a polypeptide chain has been observed to follow single exponential relaxation kinetics suggesting a two-state behavior and a barrier-limited process unlike the collapse of homoploymers, which follow multiphasic kinetics (Hagen and Eaton, 2000). Plaxco and coworkers (Plaxco et al., 1999) used stopped-flow X-ray scattering to demonstrate that protein L apparently folds directly to the native state in the absence of a chain collapse. It is possible that the collapsed states of some proteins are less stable than the denatured state and hence the collapse has to be coupled with subsequent folding. Thus, sequence composition, topology, and local structural propensity may augment or retard the rate of collapse, perhaps giving rise to the spectrum of folding behavior experimentally observed (Ferguson and Fersht, 2003).

The diffusion rates for the collision of reporter groups separated by 3-4 residues range from 17-42 nanoseconds (Bieri et al., 1999) possibly indicating the upper limit for the nucleation of the secondary structure, since one turn of an α-helix or a β-hairpin formation also requires 3-4 residues (Ferguson and Fersht, 2003). Helix formation is substantially faster than β-hairpin ($\tau_{1/2}$~ 100-200 ns and 6 μs respectively) (Munoz et al., 1997; Lapidus et al., 2002). The collapse of polypeptide chain can be described by a single exponential phase while α-helix formation can be multiphasic (Thompson et al., 1997; Huang et al., 2002). By using site specific labeling of synthetic peptides it has been observed that the N-terminal and the central residues fold at the same rate while C-terminal residues unfold faster suggesting that the helix unfolding begins at C-terminus with the loss of one or more hydrogen bonds (Huang et al., 2002). Folding rate constant of α-helical peptides is less dependent on viscosity than that of the β-hairpins under the conditions in which equilibrium constant and activation energy are unaffected by the viscogenic effect (Jas et al., 2001).

Slow events in protein folding

The intrinsically slow events, which limit the rate of protein folding, are the formation of tertiary interactions (Forge et al., 1999), disulphide bonds formation (Creighton, 1992) docking of domains (Garel, 1992; Matagne et al., 1997) or cofactors (Elove et al., 1994), and the cis-trans isomerization of prolines.

Since the cis and trans forms of X-Pro peptide groups are almost isoenergetic, with trans form being slightly favorable, unfolded proteins and small peptides exhibit a mixture of cis and trans forms in equilibrium with about 10-30% in cis form (Zimmerman and Scheraga, 1976; Grathwohl and Wuthrich, 1981). On the other hand, either completely cis or trans forms

of X-Pro peptide groups are observed in native proteins because, the almost equal energy between the cis and trans forms is overwhelmed by favorable interactions with the neighboring groups in the native proteins. About 0.15% nonproline cis peptide bonds (Pappenberger et al., 2001) and approximately about 7% of all prolyl peptide bonds in cis form have been found in native proteins by X-ray crystallography (Stewart et al., 1990). Rare exceptions of cis-trans equilibrium of X-Pro peptide bonds in the native state of proteins have been observed in staphylococcal nuclease (Evans et al., 1987), calbindin (Chazin et al., 1989) and tendamistat (Pappenberger et al., 2003). Both experimental (Steinberg et al., 1960) and theoretical (Pincus et al., 1983) observations indicate that there is a high-energy barrier (~20 kcal/mol), limiting the rate of interconversion between the two forms because of the partial double bond character of the peptide bond (Corey and Pauling, 1953). Cis-trans isomerization can be catalyzed by acid, which acts by protonating the carbonyl oxygen (Steinberg et al., 1960), or by using the enzyme peptidylprolyl isomerase, which apparently acts by disrupting the double bond character of the peptide, bond (Schmid et al., 1993). Flanking amino acid side chains have been found to affect the isomerization rates, especially histidine and tyrosine, which are known to reduce the rate by several folds by deprotonating their side chains (Reimer et al., 1998).

After unfolding, X-Pro peptide bonds isomerize to give an equilibrium mixture of U_F (fast folding) and U_S (slow folding) forms due to correct or wrong isomers with respect to the native state (Brandts et al., 1975), the isomerization being the rate-determining step for the U_S forms. The proportion of U_S form is higher for proteins that have cis proline isomers in the native state, e.g., RNase A (Garel and Baldwin, 1973) and RNase T1 (Kiefhaber et al., 1990). Native states, which have all trans prolines, also show sizeable proportion of U_S forms (Tan et al., 1997). To determine the cis-trans equilibrium at specific X-Pro peptide bonds in the denatured proteins, attempts have been made by using isomer specific proteolysis (Lin and Brandts, 1983) and NMR (Alder and Scheraga, 1990). Levitt (1981) suggested the existence of three classes of prolines, Type I prolines slightly destabilize native state in their incorrect isomeric form, and hence do not affect folding, Type II prolines allow the folding but at a diminished rate in the incorrect isomeric form while Type III destabilize the native state entirely, and hence these incorrect proline isomers block folding.

In some cases cis-trans isomerization of X-Pro peptide bonds has been used to probe the local environment of the X-Pro isomerizing peptide bond (Tan et al., 1997; Wedemeyer et al., 2002). The local structural environment of the X-Pro peptide bonds has been found to significantly influence the isomerization rates (Bhat et al., 2003). Theoretical as well as experimental methods have been established to show kinetic coupling between protein folding

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and isomerization and folding has been found to depend on the number of prolines in the cis isomeric form (Kiefhaber et al., 1992; Kiefhaber and Schmid, 1992). Proline isomerization has been shown to be the rate-limiting step for the folding of a number of proteins (Eyles and Gierasch, 2000; Kamen and Woody 2002a,b; Wu and Matthews, 2002). Although prolyl isomerization reaction should be observable for all the proteins containing proline residues, the amplitudes for the experimentally observed slow folding reactions are much less than expected from model compound data (Schindler et al., 1995).

Kinetic intermediates

Accumulation of a kinetic intermediate requires that the free energy barrier between a conformation and the next state on the folding pathway is higher than the one before it, i.e., a kinetic intermediate must precede the rate-determining step or transition state (Chamberlain and Marqusee, 2000). Some proteins fold very fast within the sub millisecond timeframe, suggesting that a population of observable intermediates is not required for efficient folding (Huang and Oas, 1995; Schindler et al., 1995). Some believe that these intermediates simply represent the off-pathway misfolded structures, due to the wrong ligandation of cofactor (Yeh and Rousseau, 1998) or incorrect proline isomerization or transient aggregation (Silow and Oliveberg, 1997), from which a protein must unfold before refolding to the native conformation (Fernandez-Recio et al., 2001).

Proteins fold quickly, usually in the time range of seconds to minutes. Because of the ephemeral nature, a detailed study of the kinetic intermediates is difficult. As a result of which more focus has been shifted to studying the equilibrium molten globule states. The general features of kinetic intermediates resemble those observed in partially folded proteins such as the equilibrium molten globule states, which possess significant amounts of secondary structures but little fixed tertiary interactions and compact structure as compared with the unfolded proteins. However, there is a diversity of the molten globule state in terms of where it accumulates maximally along the kinetic folding pathway from the unfolded state. When it accumulates during the late stages of refolding, it is more structured and corresponds to the structured molten globule formed by addition of stabilizing anions at equilibrium (Arai and Kuwajima, 2000). Those intermediates that accumulate during burst phase have been known as the pre-molten globule state (Uversky and Ptitsyn, 1996). In many proteins it has been shown that the first regions to fold are also the most stable regions of equilibrium intermediates (Chamberlain and Marqusee, 2000). For many proteins, however, the kinetic intermediates appear native like (Arai and Kuwajima, 2000; Nishimura et al., 2002), supporting the direct hierarchical mechanism of folding (Raschke and Marqusee, 1997;

Chamberlain and Marqusee, 2000). In such cases intermediates increase the rate of folding by reducing the conformational space available.

Folding of multidomain proteins

Although many definitions exist for the term 'domain', a more relevant definition from structural point of view is: a domain is a part of the polypeptide chain that forms a compact globular structure with significant number of interactions within itself compared with other parts of the chain (Janin and Wodak, 1983). The problem of studying the stability and folding of the multidomain proteins is the use of probe to monitor the structural changes, since no single probe can monitor the global native character, except the functional probes such as catalytic efficiency or binding affinity (Jaenicke, 1999).

Even though the domains can exist as independent folding units, the rate of folding of an isolated domain takes place at the same rate or is faster than in the intact protein. The slower refolding in the intact protein could be due to the unfavorable interactions with the rest of the protein (Garel, 1992; Jaenicke, 1999; Sato et al., 2000). The folding kinetics of multidomain proteins is complex and in most cases two kinetic phases can be observed. An initial rapid phase is characterized by large changes in the several physical properties such as the formation of secondary structure and hydrophobic regions within the timescale of seconds. This step is followed by slower steps characterized by little changes in the physical properties associated with the appearance of functional properties. The slower phase occurs on the minutes to hours timescale. Several results suggest that the slowest step is the pairing of the domains which is sensitive to the viscosity of the solvent (Jaenicke, 1999). The occurrence of the slow step enables the intermediates to populate which tend to aggregate to give off-pathway products (Goldberg et al., 1991; Kiefhaber et al., 1991). Using coat and tail-spike proteins from Salmonella bacteriophage P22 as models, homogeneous aggregates rather than mixed populations of aggregates have been obtained (Speed et al., 1996). In the multidomain complex of annexin I, folding of domains has been shown to follow a sequential and hierarchical pathway, in which the folding of one domain regulates the folding of the other domains (Cordier-Ochsenbein et al., 1998). The folding of the multidomain protein low-density lipoprotein receptor in vivo proceeds in a nonvectorial manner with N-terminus domain being the last to fold (Jansens et al., 2002).

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HEXOKINASE A

Hexokinase is a member of the kinase family of tissue specific isozymes, which is the first enzyme in the glycolytic pathway, catalyzing the transfer of a phosphoryl group from ATP to glucose to form glucose-6-phosphate and ADP with the release of a proton.

In Yeast, two isozymes named PI and PII (Hexokinase A & B (Womack *et al.*, 1973)) are known with 76% overall homology of the amino acid sequence (Kuser *et al.*, 2000). PI and PII can be converted to SI and SII forms, respectively by trypsin digestion or during purification by endogenous proteases resulting in the loss of the first 11 residues at the N-terminus (Colowick, 1973). The P-forms are predominantly dimeric with molecular weight of 100 kDa and the S-forms tend to exist mainly as monomeric 50 kDa forms (Schmidt and Colowick, 1973). Crystal structure is available for both the hexokinase isozymes, for hexokinase PI complexed with glucose (Bennett and Steitz, 1980a, b) and for hexokinase PII complexed with ortho-toluoylglucosamine (OTG), a competitive inhibitor (Steitz, 1971). Both of these crystal structures have missing residues, since at that time amino acid sequence was not available and the side chains were deduced from the electron density with only 30% of the amino acid sequence homology between the primary structure of the crystallographic models and the one obtained from cDNA sequence (Kuser *et al.*, 2000). Recently PII without any bound ligands with correct amino acid sequence has become available (Kuser *et al.*, 2000). Also the crystal structure of PI isozyme complexed with glucose has been elucidated (Steitz TA, personal communication). Both the isozymes share similar α/β fold, and the polypeptide chain is distinctly folded into two domains of unequal size, the large and the small domain. The two domains are closely integrated, i.e., without conspicuous hinge between the two domains. The secondary structure is composed of 14 α-helices and 13 β-strands with the large domain containing most of the helices and six stranded and five-stranded mixed β-sheet forming the interface at the active site cleft of the large and the small domain, respectively. Comparison of the two crystal structures of hexokinase with and without bound glucose reveals the conformational changes involved upon binding of the substrate due to the domain movement and suggests the induced fit mechanism of enzyme catalysis. These domains are separated by a large cleft forming the active site. The large domain consists of residues 19-76 and 212-457, while the small domain consists of residues 77-211 and 458-486. Glucose binding is attributed to rigid body movement of domains which involves 4 peptide segments i.e. L1-L4 (L1: 87-92, L2: 115-124, L3: 158-163 and L4: 174-178) which move forward to form the glucose binding site. Asp 211, the catalytic base forms a H-bond with the OH group at the 6th position of glucose. Asn 237, Glu 302 and Glu 269 are at H-bonding distance with O4, O3 and O of glucose. Also Thr 172 and Lys 173 are at H-bonding distance of glucose.

Comparison of hexokinase sequence from various sources reveals conserved glycine residues apart from the active site residues. These glycine residues are located at the end of the secondary structural elements and are probably involved in the flexibility of the molecule necessary for the domain movements (Kuser *et al.*, 2000). Calorimetric studies have shown that glucose binding is entropy driven and binding enthalpy is zero (Takahashi *et al.*, 1981). Kuser and coworkers (Kuser *et al.*, 2000) have observed a conserved hydrophobic channel in the small domain, with one end opening into the active site and the other end being on the surface of the protein, which may help to pump protons released during catalysis in order to facilitate the release of the products.

Thermal denaturation of yeast hexokinase B by DSC has revealed two partially overlapped transitions, which correspond to the melting of the two structural domains in the native conformation that possess different thermal stabilities and are denatured more or less independently (Takahashi *et al.*, 1981). The binding of glucose enhances the domain-domain interactions and gives rise to a single transition upon heating in a DSC experiment (Takahashi *et al.*, 1981; Catanzano *et al.*, 1997). Using solution X-ray scattering studies, it has been found that the radius of gyration decreases by about 0.95 A^o upon binding to glucose (McDonald *et al.*, 1979). It has also been observed that hexokinase undergoes large dehydration/rehydration reactions during turnover, which implies a significant contribution of solvation to the energetics of the conformational changes (Rand *et al.*, 1993) and the hydrophobic effect alone has been found to contribute to the observed energetics of glucose binding (Bennett and Steitz, 1978). Extensive thermal stability studies have been carried out on yeast hexokinase A in our lab using DSC in the presence of various cosolvents such as polyols, amino acids, and carboxylic acids. These studies have revealed the enhanced thermal stability of the protein in the presence of cosolvents as well as the merger of the two transitions to varying extents depending on the concentration and the nature of cosolvents similar to that observed in the presence of glucose (Tiwari, 1999).

Hexokinase being one of the important enzymes in bioenergetics, its malfunction has been implicated in a number of diseases in humans. Reduction in the activity of hexokinase in humans causes hemolytic anemia (Magnani *et al.*, 1985, Peters *et al.*, 2001) and cardiomyopathy (Barrie *et al.*, 1979). Mutations in glucokinase are associated with the early onset of non-insulin dependent diabetes mellitus (Vionnet *et al.*, 1992; Gupta *et al.*, 1997) and in hexokinase it causes non-spherocytic hemolytic anemia (van Wijk *et al.*, 2003). Hexokinase has been a target for the development of efficient inhibitors in the search of new drugs against diseases caused by trypanosomas (Wilson *et al.*, 1997). Type II hexokinase-mitochondrial interactions were observed to promote tumor cell growth and survival of many cancers and

hexokinase has been suggested as the ideal target for therapeutic intervention (Smith, 2000; Pedersen et al., 2002). Modifications of the catalytic activity of hexokinase have been suggested to play a role in the pathogenesis of the Alzheimer's disease (Sorbi et al., 1990). Hexokinase has been used for the construction of biosensors capable of detecting glucose (Cheng and Stevens. 1997). Recently, it has been shown that hexokinase plays a role in sensing and maintaining the glucose levels and in signal transduction to regulate the expression of genes in plants (Moore et al., 2003; Frommer et al., 2003).

All these characteristics of hexokinase make it an interesting multidomain system for studying the conformational changes induced by various perturbants. Despite this, relatively few biophysical studies have been carried out on this protein. We have selected yeast hexokinase A as a model protein for investigating the structure-stability relationship of the enzyme. The results of the study are likely be useful in understanding these aspects, which have long term implications for understanding its function as well as abnormalities caused by its deficiency.

OUTLINE OF THE PRESENT RESEARCH PROBLEM

Interactions between charged groups in proteins affect their structure, thermodynamic stability and biological function (Allewell and Oberoi, 1991). Electrostatic interactions affect the stability of the native state (Dao-pin et al., 1991), binding of ligands (Linse et al., 1988) and pKa values of the ionizable groups (Russel et al., 1987). pH and ionic strength together are excellent parameters to study the electrostatic effect in proteins. Hexokinase is the classical example for the "induced fit" mechanism of catalysis (Koshland, 1959). In order to understand the role of electrostatic interactions in domain stability and its relation to the domain movements, in the present work (chapter 3) we have studied extensively the effect of pH on the thermal stability of yeast Hexokinase A using Differential Scanning Calorimetry and its structural properties by spectroscopic probes like circular dichroism and fluorescence to investigate the various conformational states that populate at different pH conditions. Attempt has also been made to relate the calorimetric profiles obtained at different pH conditions with the structural aspects that are crucial for domain movements in hexokinase A.

It is of general interest to study the protein conformational changes using a variety of perturbants such as chemical denaturants such as urea and GdmCl, and organic solvents such as trifluroethanol and DMSO, etc., most of which induce non-native conformations. In few cases stabilizing osmolytes have been shown to induce molten globule like states from the acid denatured protein (Davis-Searles, 1998; Kamiyama et al., 1999; Bongiovanni et al., 2002), compact structure in random coil proteins (Qu et al., 1998) and cooperative transitions

in natively unfolded proteins (Baskakov and Bolen, 1998a). Osmolytes have been found to stabilize the native state of proteins by raising the chemical potential of the denatured state relative to the native state (Arakawa *et al.*, 1990). However, not much information is available on the effect of cosolvents on the MG↔N transition, which involves compaction of the hydrophobic core, formation of tertiary interactions and appearance of the unique native conformation. In chapter 3 we have shown that hexokinase A at pH 2.5 has properties similar to that of the molten globule like state. In chapter 4, we have used polyols and sugars to study their effect on the molten globule like state and the stability of the structures attained using DSC. The underlying conformational changes have been probed by various spectroscopic techniques. Thermal denaturation studies have been carried out as a function of cosolvent concentration, using far UV CD, absorbance spectroscopy as well as DSC in order to understand the contribution of the secondary and the tertiary interactions to the thermal transitions observed.

Folding of large and multidomain proteins is often complicated by propensities of side reactions such as aggregation, cis-trans isomerization of prolines and docking of the domains or subunits (Jaenicke, 1999). In most of the conditions studied above, hexokinase A is irreversible upon denaturation. To find out the possible causes of the irreversibility and to optimize the conditions for proper folding, we have made an attempt to study the refolding of hexokinase A denatured by 8 M urea, 6 M GdmCl and acid, both in the presence as well as absence of the reducing agent, DTE (chapter 5). Further, folding kinetic experiments by manual mixing of the denatured protein have been carried out using activity assay and intrinsic as well as ANS fluorescence as probes to understand the nature of the rate limiting step for the refolding of hexokinase A. Finally we have carried out some preliminary experiments using the enzyme peptidyl prolyl isomerase (PPI) to show that the rate limiting step could be due to cis-trans isomerization of some X-Pro peptide bond as observed in many proteins (Kamen and Woody, 2002a, b; Wu and Mattews, 2002).

Materials & Methods

MATERIALS

Yeast hexokinase used in these experiments was obtained from Sigma Chemical Co., USA (H 5625), which is a mixture of isozymes PI and PII. The isozymes were separated by using the procedure of Womack *et al.*, (1973), which involves the separation based on the differential adsorption of hexokinase isozymes to hydroxyapatite, and the PI fraction was eluted by 0.1 M potassium phosphate, pH 6.8.

Tris (hydroxymethyl) amino-methane (Tris), sorbitol, xylitol, trehalose, glucose, β-nicotinamide adenine dinucleotide phosphate (NADP), adenosine 5'-triphosphate, urea, guanidinium chloride (GdmCl), dithioerythritol (DTE), and 8-anilino-1-naphthalene sulfonic acid (ANS) were procured from Sigma Chemical Co., USA. Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim, Germany. Glycerol, ethylene glycol, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were from Merck, India. Glycine, acetic acid, and sodium acetate were from Sisco Research Laboratories Pvt. Ltd. India. The chemicals used were of the highest purity grade available and were used without any further purification. Either Glass double distilled or Milli-Q water from Millipore set-up was used to make the buffers. The pH of the buffer solutions was adjusted on a Control Dynamics pH meter model APX 175, by adding hydrochloric acid or sodium hydroxide solutions. The pH standards used for calibrating the pH meter were from Sigma Chemical Co. (USA).

METHODS

Preparation of buffers and solutions

0.025, 0.05, 0.06 and 0.1 M potassium phosphate, pH 6.8 buffers were used for the separation of hexokinase isozymes. The following solutions were prepared for the pH dependent studies (Chapter 3): 20 mM glycine-HCl or NaOH, for pH 2.5, 3.0, 3.5. 10.5, 11.0, 11.5, 12.0, and 12.5; 20 mM acetate for pH 4.0, 4.5, and 5.0; 20 mM Tris for pH 8.5, 9.0; 20 mM Ampso for pH, 9.5, 10.0; 20 mM glycine, pH 3.5, containing 50, 100, and 200 mM NaCl for studies of the salt effect on hexokinase A stability carried out by DSC. All the polyols of given concentration were dissolved in 20 mM glycine and the pH was adjusted to 2.5 using HCl (Chapter 4). For the denaturation of hexokinase A (Chapter 5), either 20 mM Tris pH 7.8 containing 8 M urea or 6 M GdmCl for the chemical denaturation or 20 mM glycine pH 2.5 for acid denaturation, containing either no DTE or 1mg/ml DTE was used, and the protein was refolded in 100 mM Tris pH 7.8 containing 0, 0.25, 0.5, 1.0, and 2.0 mg/ml DTE. For refolding kinetics monitored by fluorescence, 100 mM Tris, pH 7.8 containing 1 mg/ml DTE was used as the refolding buffer. For the activity assays 100 mM $MgCl_2$ solution and 13 mM NADP solution in double distilled water, 500 mM glucose solution and 16 mM ATP solution

in 100 mM Tris, pH 7.8, and 1 mg/ml glucose-6-phosphate dehydrogenase in 100 mM Tris, pH 7.8 were prepared. The preparation of the solutions and the setting of the pH were carried out at 25 °C. All the solutions were filtered through a 0.22 μm membrane filter (Sartorius, Germany) to remove any dust particles and the protein solutions were centrifuged at 15000 rpm (Heraeus, Germany) for 15 min to remove any aggregated species. Yeast hexokinase A concentration was calculated based on the specific absorptivity of 0.885 cm^2 mg^{-1} (Colowick, 1973).

Separation of isozymes PI and PII

We followed the procedure of Womack *et al.*, (1973) for the separation of the isozymes pI and PII, which is based on the differential adsorption to hydroxyapatite. First the column was filled with about 15-20 grams of hydroxyapatite equilibrated with 0.025 M potassium phosphate, pH 6.8 and the column was washed for about 1 hour using 0.025 M potassium phosphate, pH 6.8 buffer. The protein was loaded onto the well equilibrated column followed by washing to remove any unbound protein by 0.025 M potassium phosphate, pH 6.8. PII fraction was eluted by 0.05 M potassium phosphate, pH 6.8 and again the column was washed with 0.06 M potassium phosphate to remove any traces of PII. Finally PI fraction was eluted by 0.1 M potassium phosphate, pH 6.8. The PI fraction was concentrated using a Centricon concentrator (Amicon Inc., USA) and the specific activity of the enzyme was determined using glucose and fructose as substrates to ascertain purity. The ratio of the activities obtained for the substrates fructose/glucose was within the limits of 2.3 to 3.1. The concentrated protein solution was stored at 4 °C without any stabilizing additives and used within 3-4 days.

Differential Scanning Calorimetry

For DSC studies as a function of pH (Chapter 3), the protein was equilibrated with the requisite buffers by using Centricon concentrators (Amicon Inc., USA). The buffer was passed through the protein solution in the centricon tube for at least three times to ascertain proper equilibration. For studying the effect of polyols on the molten globule state (Chapter 4), the concentrated protein (~60-80 mg/ml) in water (~pH 6.0) was directly diluted in the buffer containing the polyol (pH 2.5) and the volume was adjusted with 20 mM glycine, pH 2.5 to give the final required protein concentration of 2 mg/ml as well as the desired concentration of the cosolvent. The pH was checked again for any variations in the pH. No change in the pH was usually observed. The protein solution was then incubated for at least 4-6 hours at 10 °C to allow for attaining the equilibrium. The blank was also prepared in the same way for loading into the reference cell of the calorimeter. Since hexokinase A unfolding

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was irreversible at high concentrations, the native protein (in water) was diluted into 20 mM glycine, pH 2.5 containing the polyols rather than first equilibrating the protein with 20 mM glycine, pH 2.5 followed by the addition of polyols at pH 2.5. Here we assume that the protonation changes are faster than the conformational changes of the protein and the cosolvents essentially affect the subsequent conformational changes depending on the concentration and the nature of the polyol used. The same procedure was also used for the spectroscopic studies of the effect of polyols on the pH 2.5 state of hexokinase.

The protein and the buffer solutions were degassed for 15 minutes prior to loading into the DSC cells. The instrument used for the calorimetric studies was MC-2D from Micorcal Inc., Northampton, USA. The machine was calibrated with the temperature standards provided by the company. A scan rate of 1 K min^{-1} was used and the data were acquired through an in-built data translation board DT 2801 in a 486 DX personal computer using ORIGIN software. The data were analyzed using the non-two state model provided in the Origin ^{TM} software supplied along with the machine. The detailed set-up and the operational procedure of the DSC has been described in detail elsewhere (Tiwari, 1999).

Circular Dichroism studies

Circular dichroism spectra were recorded on a Jasco J-815 spectropolarimeter. The instrument was calibrated with (+)-10-camphor sulfonic acid. In general, an average of 4-6 scans were used and the data were presented as mean residue ellipticity [MRE] normalized to the number of residues expressed in deg cm^2 dmol^{-1}. All the spectra as well as the urea denaturation transition curves were acquired at 18 °C. For the transition curves, data were recorded for at least three samples and the mean was used for plotting. Data were obtained at an interval of 0.1 nm and a scanning speed of 50 nm/min. Nitorgen gas was continually flushed at the rate of 5 lit. min^{-1}. Higher flow rates were used below 200 nm to minimize the noise in the far UV region. 0.2 cm and 1cm pathlength cuvets were used in the far and near UV regions, respectively. The protein concentrations for the far UV and the near UV spectra acquisition were 200 μg/ml, and 1.0 mg/ml, respectively. Thermal denaturation was carried out at a rate 1 K. min^{-1} at 222 nm using 0.2 cm pathlength cuvets.

The data were normalized using the equation

$$[\Theta] = M_0 \, \Theta / 10 lc \qquad\qquad (1)$$

where Θ is the observed ellipticity in millidegrees, M_0 is the mean residue weight of the protein, c is the protein concentration in g/cm^3, and l is the path length of the cuvet in centimeters. A molecular weight of 50 kDa and the total number of 468 residues for yeast hexokinase A (since crystal structure shows only 18 to 486 residues) was used in the calculation.

Fluorescence studies

Tryptophan as well as ANS fluorescence studies were carried out to characterize the intermediates as well as to determine the transition curves. Fluorescence measurements were carried out on a Cary Varian Eclipse spectrofluorimeter interfaced with peltier temperature controller. 1 cm path length cuvets were used to record the spectra of the samples under constant stirring. A protein concentration of 30 μg/ml for the intrinsic fluorescence and 10 μg/ml for the ANS fluorescence was used. For intrinsic fluorescence, excitation wavelength of either 280 or 295 nm was used, with an excitation slit width of 5 nm and an emission slit width of 10 nm. The emission was recorded from 300 to 450 nm. For ANS fluorescence, an excitation wavelength of 400 nm was used and the emission was measured from 410 to 600 nm with slit widths of 5 nm for excitation and 10 nm for emission. All the spectra and the transition curves were measured at 18 °C. ANS concentration was kept 50 μM throughout and the concentration of ANS was determined in a UV-spectrophotometer using an extinction coefficient of 7800 mol^{-1} cm^{-1} at 372 nm (Lakowicz, 1999).

Refolding experiments

Protein solutions were diluted, to give a final protein concentration of 1 mg/ml, into a freshly prepared denaturing buffer containing either 8 M urea or 6 M GdmCl, 20 mM Tris, pH 7.8 or 20 mM glycine, pH 2.5, with or without 1 mg/ml DTE, and incubated at 25 °C for 1 hour to allow the denaturation reaction to complete. Either 10 μl or 30 μl of the denatured protein was diluted into the refolding buffer to achieve final volume of 1 ml (100 mM Tris, pH 7.8) under constant stirring, for monitoring activity or fluorescence based refolding kinetics, respectively. The refolded protein was incubated at 25 °C for various periods of time and aliquots were taken periodically for activity assays, or the denatured protein was diluted directly into the fluorescence cuvet for measuring fluorescence intensity as a function of time. Similarly, native and denatured base lines were determined at the same protein concentrations to calculate the % refolding yield or the activity of the native protein (i.e., without denaturation), which was taken as 100%. For the refolding kinetics monitored by ANS fluorescence, the protein solutions were diluted 100 fold in the presence of ANS into the fluorescence cuvet. Refolding kinetics were measured at least in triplicates and the data were fit using the exponential fit using the equation:

$$Y = Y_0 + A*(1-EXP(-B*X)) \qquad (2)$$

where Y is the observed value, Y_0 is the initial value, A is the amplitude, B is the rate constant of the reaction, X is time in mins. All the folding kinetics data except ANS fluorescence kinetic data were fit to a single exponential whereas the ANS fluorescence data were fit to a sum of two exponentials.

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Measurement of accessible surface area

Accessible surface areas (ASA) of the residues and the distances between the residues in hexokinase A were calculated using inbuilt programs of Insight II module of Molecular Simulations Inc., on a Silicon Graphics O2 workstation. The method of Lee and Richards (1971) was used to calculate the ASA using a probe size of 1.4 A°. The PDB code for the crystal structure used was, 1IG8 (Kuser *et al.*, 2000) for the open conformation while for the closed conformation coordinates provided by Prof. T. A. Steitz, Yale university, USA were used.

Activity Assay

Hexokinase activity was assayed by the glucose-6-phosphate dehydrogenase coupled method (Shill and Neet, 1975). The assay is based upon the reduction of NAD^+ through coupled reaction with glucose-6-phosphate dehydrogenase (GPDH) and is determined spectrophotometrically by measuring the increase in the absorbance at 340 nm. Yeast hexokinase A catalyzes the phospharylation of glucose by ATP in presence of Mg^{+2}.

$$Glucose + ATP \xrightarrow{\text{Hexokinase + Mg+2}} Glucose\text{-6-phosphate} + ADP \qquad (3)$$

$$Glucose\text{-6-phosphate} + NADP \xrightarrow{\text{GPDH}} Gluconate\text{-6-phosphate} + NADPH^+ + H^+ \quad (4)$$

Glucose-6-phosphate dehydrogenase (~17 μg/ml) and hexokinase (~170ng/ml) A were used in concentrations enough to lead to a $\Delta A/min$ of 0.3 to 0.6.

The compositions of the activity assay mixture were:

100 mM Tris, pH 7.8	800 μl
$MgCl_2$	67 μl
Glucose	33 μl
ATP	33 μl
NADP	33 μl
GPDH	17 μl

In the sample cuvet, 17 μl of hexokinase and in the reference cuvet 17 μl of Tris buffer were added and the solutions mixed thoroughly. The final volume of the solution was 1 ml each and increase in absorbance at 340 nm was measured in a Hitachi spectrophotometer at 25 °C. Activity of the native enzyme was taken as 100% and the refolding yield as a function of time was calculated relative to the native activity. Percentage error in the activity measurements was less than 5%.

pH Dependent Conformational Changes
of Yeast Hexokinase A

INTRODUCTION

pH and salts have profound influence on the stability, structure, and function of many globular proteins. pH is known to influence electrostatic interactions by altering the degree of ionization of the ionizable side chains leading to a change in the net charge as well as the charge distribution of a protein. At extremes of pH, proteins denature because of accumulation of repulsive like-charges (Linderstrom-Lang, 1924). The pH dependency of protein stability can also be explained on the basis of ionization of charged residues to different extents in the native and the denatured states (Yang and Honig, 1993). On the other hand, salt ions modulate the electrostatic interactions by either binding to specific sites, which are specific for the particular ion or by screening the charges, which is a function of the ionic strength. Also, in physiological conditions, ions and protons impart minor conformational changes in proteins, which are essential for function (Garcia-Moreno, 1995). Thus, pH and ionic strength together are excellent parameters for the study of conformational changes in proteins and for understanding the energetics of protein stability modulated by electrostatic interactions.

Unlike many small globular proteins, the multidomain and multisubunit proteins usually unfold by significantly populating the intermediates (Jaenicke, 1999). Acid and base denaturation does not always lead to complete unfolding resulting in the formation of intermediate states, well known as the "molten globule" state (Fink *et al.*, 1994). Due to the similarity of molten globule like states to the kinetic folding intermediates, much attention has been devoted to the study of equilibrium molten globule like states, as it is easy to study compared to the kinetic intermediates which are transient in nature (Arai and Kuwajima, 2000). Domains are known to be independent folding units, having their own structural stability. The interactions between such domains, which are necessary for function of the protein, can alter their stability depending on the pair-wise interaction free energy at the interface (Brandts, 1989). Little is known about the relationship between domain-domain interactions and the thermal stability of proteins, and the various conformational states a multidomain protein can adopt under different conditions.

Yeast hexokinase A is a two-domain protein, with a deep cleft between the domains that forms the active site. The interdomain region of hexokinase A consists of one beta sheet from each domain sandwiching two helices that are oriented perpendicularly, in which the two helices undergo shear motion to close the cleft upon binding the substrate glucose. In the present work, we have studied extensively the effect of pH on the thermal stability of yeast hexokinase A using Differential Scanning Calorimetry and its structural properties by using spectroscopic probes like circular dichroism and fluorescence to investigate the various

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conformational states that populate at different pH conditions. The results have been discussed in terms of domain stability and the formation of intermediates at extremes of pH. Attempt has also been made to relate the calorimetric profiles at different pH conditions with the structural aspects that are crucial for domain movements in hexokinase A.

RESULTS

Thermal denaturation

Thermal denaturation curves of hexokinase A determined by scanning calorimetry as a function of pH have been presented in Fig. 1. The corresponding thermodynamic parameters deduced from these transitions have been listed in Table 1. From Fig. 1 it is evident that at alkaline pH protein unfolding results in two independent thermal transitions whereas only a single transition has been observed in the acidic pH range. Thermal denaturation profile of the protein monitored by ellipticity at 222 nm also matches with the nature of transitions observed by DSC at acid and alkaline pH (Fig. 2). The common temperature function of ellipticity observed for heat-denatured states of hexokinase A, both at acidic and alkaline pH, suggests that the extent of unfolding is similar in both the conditions and the single cooperative transition observed at acidic pH involves melting of both the domains. Thermal denaturation at acidic pH is completely irreversible while at alkaline pH the transitions are reversible by 25-30%, as estimated from the enthalpy change observed upon reheating of the sample, relative to the enthalpy change of the first heating cycle (data not shown). Two independent thermal transitions for melting of yeast hexokinase A in the alkaline pH region have been reported previously from our lab and by others (Takahashi *et al.*, 1981; Catanzano *et al.*, 1997; Tiwari, 1999). The two transitions have been assumed to result from the melting of the two domains, with the first transition corresponding to the smaller domain and the second transition corresponding to the larger domain (Tiwari, 1999). Thermal denaturation monitored by a decrease in the fluorescence intensity at 330 nm at pH 8.5, reveals only a single cooperative transition that shows correspondence to the first transition on the temperature axis with the biphasic transition observed by DSC and CD, followed by a linear decrease in intensity with increasing temperature (Fig. 2). Yeast hexokinase A contains four tryptophans, three of which are buried in the hydrophobic core in the smaller domain while the larger domain contains only a single partially exposed tryptophan. Comparison of the temperature denaturation curves at pH 8.5 monitored by different techniques reveals that the correspondence of single transition, as observed by fluorescence, with the first transition observed in CD or DSC experiments suggests that the first transition is likely to be due to the
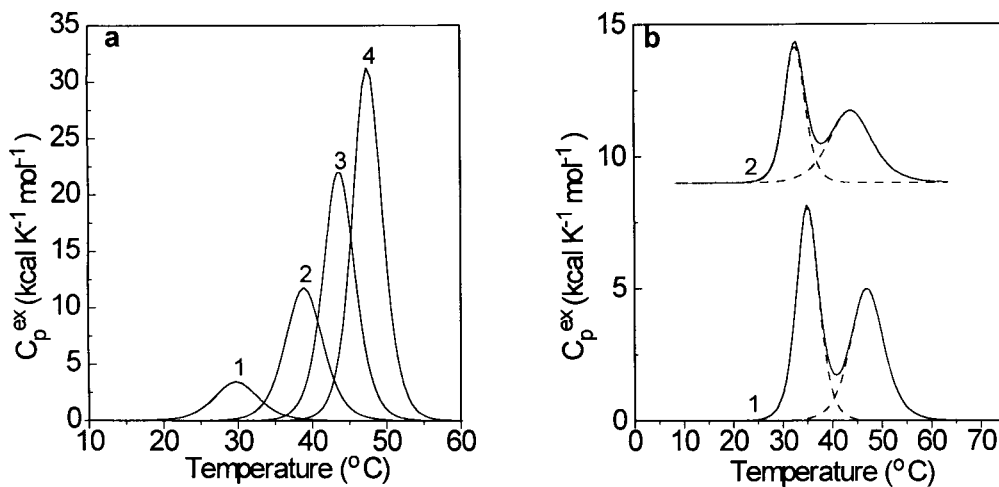
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Fig. 1: Effect of pH on the thermal stability of hexokinase A monitored by Differential Scanning Calorimetry. The protein concentration used was 1.0 mg/ml. In the acidic pH range (a), the numbers represent the pH of the buffer, 1: 3.0; 2: 3.5; 3: 4.0 and 4: 4.5. In the alkaline pH range (b), the numbers represent the pH of the buffer, 1: 9.0 and 2: 10.0.
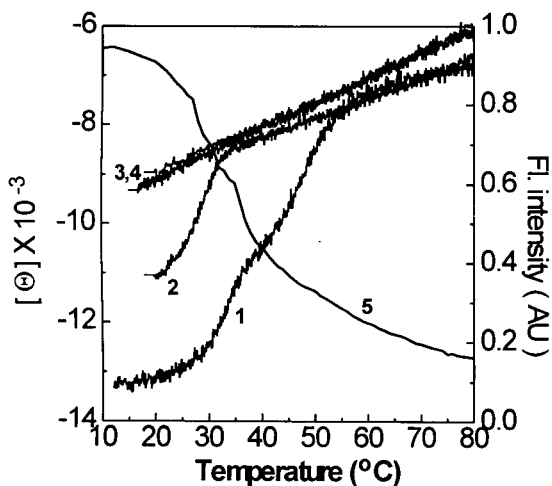

Fig. 2: Temperature denaturation curves for hexokinase A at different pH values monitored by CD at 222 nm (1-4) and fluorescence intensity at 330 nm, excited at 280 nm (5). The numbers represent the pH values of buffers, 1: 8.5; 2: 3.0; 3: 2.5; 4: 2.0 and 5: 8.5.
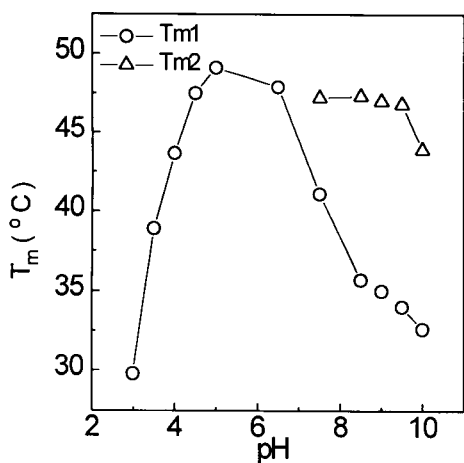
Fig. 3: Dependence of the transition temperature, T_m of hexokinase A on the pH of the buffer. Circles represent the T_m of either the first transition, where two transitions are observed (at alkaline pH), or the single transition (at acidic pH), while the triangles represent the T_m of the second transition at alkaline pH. Data points for pH 6.5, 7.5, 8.5 have been taken from Tiwari (1999).

C$_p^{ex}$ (kcal K^{-1} mol^{-1})

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30 ⟶ 200 mM

20 ⟶ 100 mM

10 ⟶ 50 mM

0 ⟶ 0 mM

10   20   30   40   50   60

Temperature (°C)
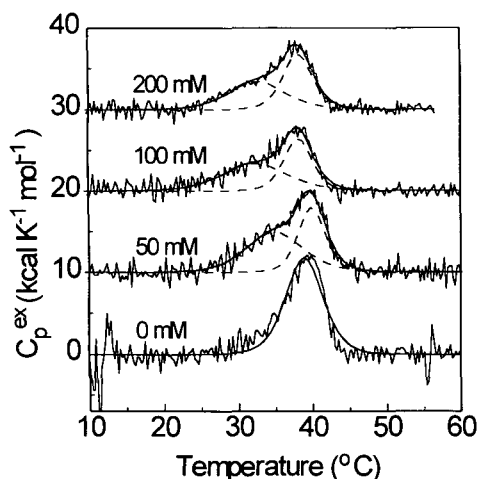
Fig. 4: Effect of NaCl on the stability of hexokinase A monitored by differential scanning calorimetry, at pH 3.5. The numbers represent the concentration of NaCl in millimoles. For 0 mM NaCl, the data were fit to a single peak, while for the rest of the thermograms fitting function used two peaks. The protein concentration used was 1.5mg/ml.

Table 1: Thermodynamic parameters for the denaturation of hexokinase A as a function of pH determined by DSC.

pH	T$_{m1}$ (°C)	T$_{m2}$ (°C)	Δ H$_{cal1}$ kcal/mol	Δ H$_{cal2}$ kcal/mol	Δ H$_{v1}$ kcal/mol	Δ H$_{v2}$ kcal/mol	ΔH$_{cal1}$/ ΔH$_{v1}$	ΔH$_{cal2}$/ Δ H$_{v2}$
3.0	29.7±0.2		27±2		92±8		0.29	
3.5	38.9±0.1		78±3		116±6		0.67	
4.0	43.6±0.1		125±3		141±4		0.88	
4.5	47.5±0.1		166±3		154±4		1.07	
5.0	49.1±0.1		131±3		175±5		0.74	
9.0	35.0±0.1	47.0±0.1	48±1	43±1	127±3	93±4	0.37	0.46
9.5	34.0±0.1	46.8±0.1	40±1	34±1	124±4	82±4	0.32	0.42
10.0	32.6±0.1	43.9±0.2	28±1	30±1	136±5	71±3	0.20	0.43

Table 2: Effect of NaCl on the thermodynamic parameters for the denaturation of hexokinase A determined by DSC.

Conc. of NaCl (mM)	T$_{m1}$ (°C)	T$_{m2}$ (°C)	Δ H$_{cal1}$ kcal/mol	Δ H$_{cal2}$ kcal/mol	Δ H$_{v1}$ kcal/mol	Δ H$_{v2}$ kcal/mol	ΔH$_{cal1}$/ ΔH$_{v1}$	ΔH$_{cal2}$/ Δ H$_{v2}$
0	38.9±0.1		78±3		116±6		0.67	
50	35.0±0.8	39.9±0.4	49±9	38±8	74±9	163±18	0.66	0.23
100	32.4±0.8	38.2±0.1	39±7	33±6	64±8	154±16	0.60	0.20
200	32.7±0.8	38.1±0.1	34±7	35±6	74±10	147±14	0.45	0.23

melting of the smaller domain of hexokinase A, since it harbors three buried tryptophans out of four. As shown in Fig. 3, the T$_m$ values decrease at extremes of pH in general and T$_{m1}$ corresponding to the smaller domain decreases drastically compared to T$_{m2}$ corresponding to the larger domain in the alkaline pH range, suggesting the differences in the intrinsic stabilities of the two domains.

At acidic pH, thermal transition consists of a single peak indicating the merger of the two domains, with the first transition getting shifted to the T_m of the second transition. The pI of Yeast hexokinase A is 5.1 and at pH 5.0, it shows maximum stability in terms of the mid point of transition (T_m), but at the pH values near the pI the transition is considerably distorted by aggregation of the protein. The maximum stability in terms of T_m observed at a pH close to pI suggests that the charged groups are essentially exposed on the surface, since for proteins with buried charge groups, the maximum stability is usually not at their pI (Stigter and Dill, 1990). At pH 4.5 and 4.0, $\Delta H_{cal}/\Delta H_v$ is close to unity suggesting a two state unfolding with both the domains melting cooperatively. At pH 3.5 and 3.0, $\Delta H_{cal}/\Delta H_v$ ratio is less than unity possibly due to the existence of considerable amounts of the partially or completely denatured species. At pH 2.5, we did not observe any transition suggesting that pH 2.5 state is an acid denatured state.

In order to understand the electrostatic contribution to the domain merger, thermal denaturation at pH 3.5 was carried out in the presence of 50-200 mM NaCl (Fig. 4). Addition of the salt leads to a slight destabilizing effect. In addition, the transition peak observed was asymmetric toward the pre-transition zone and the whole transition could be deconvoluted into two transitions. Therefore, it appears that in the presence of the salt the domains have a tendency to melt independently. Salts at lower concentrations are effective in screening the electrostatic interactions and this adds further support to the role electrostatic interactions play in the domain merger. The destabilizing effect is saturating at 100 mM salt and further increase to 200 mM has essentially the same effect. Due to strong aggregation problems, higher salt concentration could not be used at pH 3.5. In the deconvoluted thermograms, the first peak is less cooperative while the second peak is sharper. This type of trend is reverse as compared with the thermal transition observed at alkaline pH (Fig 1b).

Spectroscopic Studies

NEAR UV CD

The characteristic spectrum of native state consists of three negative peaks at 268, 277 and 286 nm and a positive peak at 291 nm (Fig. 5). In acidic conditions the negative and positive peaks respond differently to the changes in pH. The intensity of the negative ellipticity approaches zero with a decrease in the pH, whereas the intensity of the positive peak increases with decrease in the pH reaching a maximum at pH 3.5 and then decreases below this pH. The positive peak could be specific to tryptophan's environment, since tryptophan absorbs in this region. The differential changes observed with the positive peak at
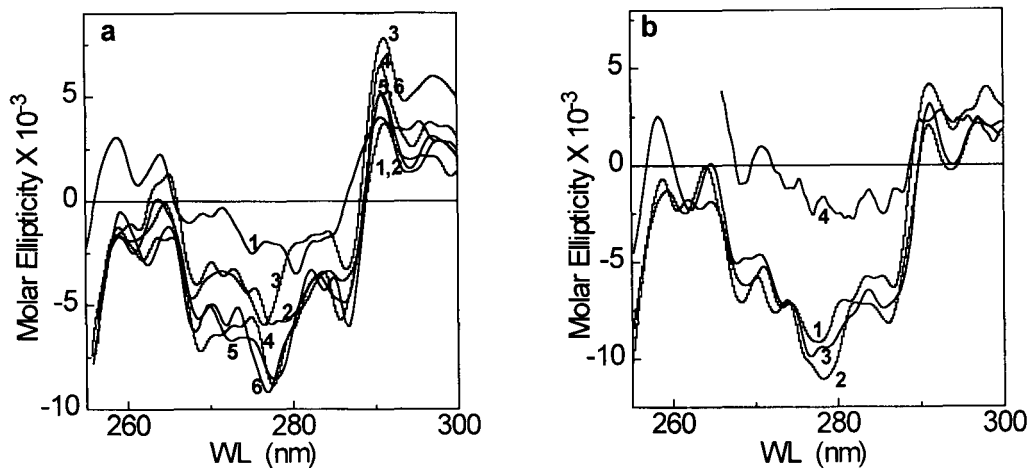
Fig. 5: Near UV CD spectra of hexokinase A as a function of pH at 18°C. The data were converted to molar ellipticity units (deg cm^2 dmol^{-1}). In acidic pH range (a), the numbers represent pH of the buffers, 1: 2.5; 2: 3.0; 3: 3.5; 4: 4.0; 5: 4.5 and 6: 5.0. In alkaline pH range (b), the numbers represent pH of the buffers, 1: 8.5; 2: 9.5; 3:10.0 and 4: 11.0.
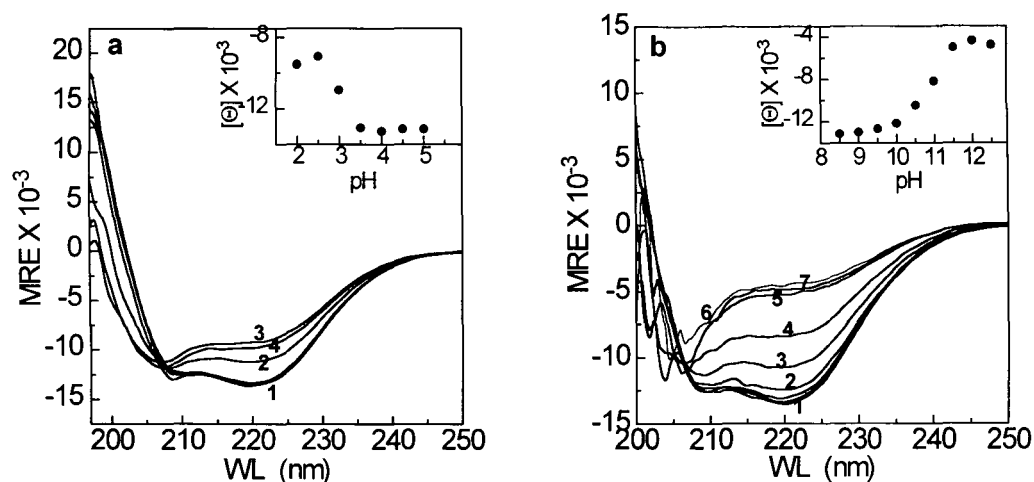

Fig. 6: Far UV CD spectra of hexokinase A as a function of pH at 18°C. The ellipticity values have been normalized to per residue molar ellipticity units (deg cm^2 dmol^{-1}). In both panels a and b, the insets show the ellipticity values at 222 nm plotted against the pH. In acidic pH range (a), the numbers represent pH of the buffers, 1: 5.0, 4.5, 4.0 and 3.5; 2: 3.0; 3: 2.5 and 4: 2.0. In alkaline pH range (b), the numbers represent pH value of buffers, 1: 8.5, 9.0 and 9.5; 2: 10.0; 3: 10.5; 4: 11.0; 5: 11.5; 6: 12.0 and 7: 12.5.

Table 3. α -Helix content of hexokinase A as a function of pH, calculated from the CD data.[†]

pH	5.0	4.5	4.0	3.5	3.0	2.5	2.0		
% α-Helix	35.7	35.7	36.0	35.4	28.4	22.2	23.7		
pH	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0	12.5
% α-Helix	35.7	35.1	34.1	32.4	26.8	19.3	8.8	6.7	8.0

[†]Method of Chen et al., (1972) was used to evaluate the helical content.

291 nm might, thus, be related to a change in the tryptophan environment as a result of lose of some tertiary interactions. In alkaline conditions, the native spectrum is preserved up to pH 10.0 while at pH 11.0, hexokinase A loses most of its tertiary interactions. At 258 nm there is a considerable increase in the ellipticity with an increase in the pH. This could be due to the ionization of the tyrosine residues.

FAR UV CD

In the far UV region, the native hexokinase A shows a characteristic double minima at 208 and 222 nm (Fig. 6), and belongs to an α/β class of proteins (Kuser *et al.*, 2000). The calculated secondary structure elements are listed in Table 3 using the method of Chen *et al.*, (1972). The native secondary structure is preserved up to pH 3.5 in the acidic conditions. However, at pH 2.5 it looses 38% helicity with no minimum observed at 222 nm. At pH 2.0, there is a slight increase in the helicity, thus suggesting that the pH 2.5 state is an acid unfolded state (U_A). Visible aggregates were observed below pH 1.0 at high protein concentration, which is a characteristic of the acid induced folding.
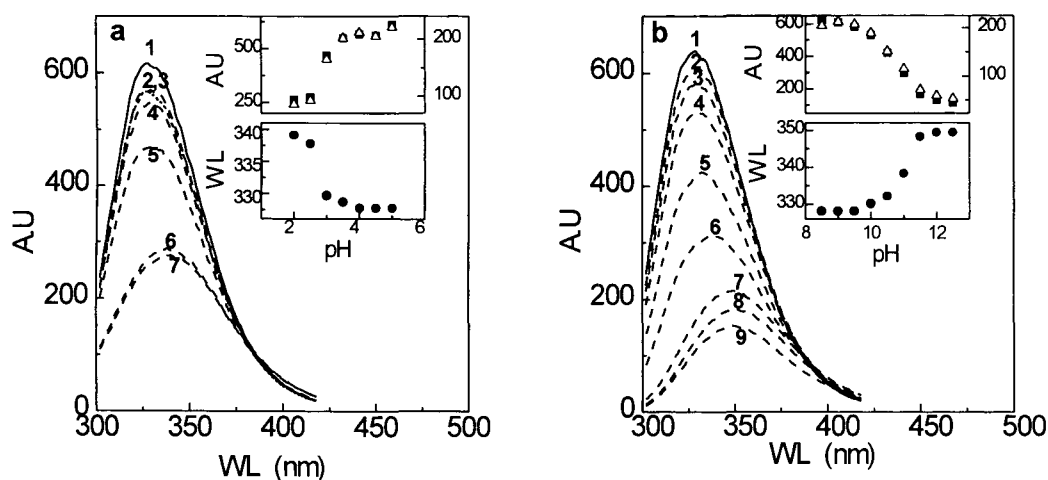
Fig. 7: Fluorescence emission spectra of hexokinase A excited at 280 nm as a function of pH at 18°C. In both panels a and b, the upper insets shows the emission intensity at 330 nm, excited at 280 nm (■) and at 295 nm (Δ), while the lower insets shows the emission maximum wavelength plotted against pH. In acidic pH range (a), the numbers represent pH of the buffers, 1: 5.0; 2: 4.5; 3: 4.0; 4: 3.5; 5: 3.0; 6: 2.5 and 7: 2.0. In alkaline pH range (b), the numbers represent pH of the buffers, 1: 8.5; 2: 9.0; 3: 9.5; 4: 10.0; 5: 10.5; 6: 11.0; 7: 11.5; 8: 12.0 and 9: 12.5.

In alkaline conditions hexokinase A loses secondary structure at pH >10.0 and reaches a plateau at pH 12.0. As compared with the acid denatured state, the alkali denatured state is more unstructured and it loses 77.5% of helicity. The acid and alkali denatured states lose the characteristic minimum at 222 nm suggesting an increase in the β-sheet content. In both low

and high pH, the denaturation shows an isodichroic point at 207 nm suggesting that the process is a two-state.

FLUORESCENCE

Hexokinase A has 16 tyrosine residues distributed all over the protein and 4 tryptophan residues of which three are located in the small domain and one in the large domain. The accessibility of Trp ranges from completely buried to partially solvent exposed (fractional solvent accessible surface area of residues was calculated by Lee and Richards (1971) method for crystal structures with glucose bound form are Trp 69: 0.01; Trp 128: 0.025; Trp 174: 0.025 and Trp 441: 0.192).

Fluorescence emission was measured either upon excitation at 295 nm to follow the changes in the environment of the Trp residues specifically, or upon excitation at 280 nm, in which emission arises from both Tyr and Trp residues as well as due to the result of energy transfer from Tyr to Trp residues. Thus, the excitation wavelength of 280 nm links the Tyr probes distributed through out the protein with fluorescence emission from the four Trp residues.

Emission spectra of the native state have an emission maximum of 330 nm, suggesting that the tryptophan residues are buried in a non-polar environment. The emission maximum of the acid denatured protein is red shifted to 340 nm and that of the alkali denatured protein shifted to 350 nm (Fig. 7) indicating that the solvent exposure of tryptophans in the acid and the alkali denatured protein takes place to varying extents. This data is in accordance with the far UV CD data, which shows more secondary structure content for the acid denatured protein as compared with the alkali denatured protein. The emission intensity of the protein also decreases upon deviation from the native state due to solvent quenching of the tryptophan fluorescence.
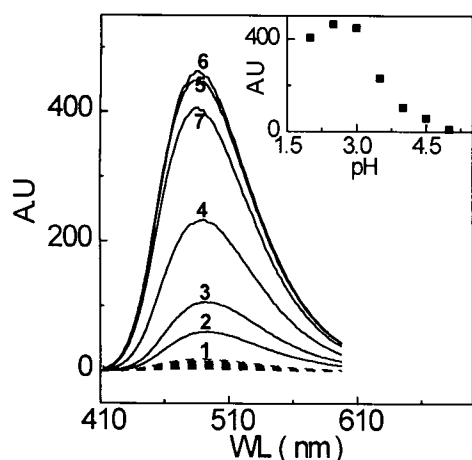

Fig. 8: Fluorescence spectra of ANS binding of hexokinase A as a function of pH, excited at 400 nm at 18°C. The inset shows the intensity at 480 nm plotted against pH. The numbers represent the pH values of buffers, 1: 5.0 and 8.5 to 12.5 with an interval of 0.5 units; 2: 4.5; 3: 4.0; 4: 3.5; 5: 3.0; 6: 2.5 and 7: 2.0.

ANS FLUORESCENCE

ANS has been shown to bind to hydrophobic regions of partially unfolded proteins that become exposed to solvent (Semisotnov et al., 1991). The results of ANS binding are shown in Fig. 8. ANS binding was observed only at the acidic conditions with an increase in the emission intensity as well as a concomitant shift in the emission maximum indicating the presence of exposed hydrophobic groups. ANS binding studies also support the near UV CD absorption data in the acidic conditions that show partial loss of tertiary interactions at pH 3.5 without any secondary structure loss. Alkali denatured protein does not bind ANS, suggesting complete unfolding as evidenced by far UV CD and emission maximum at pH 12.5.
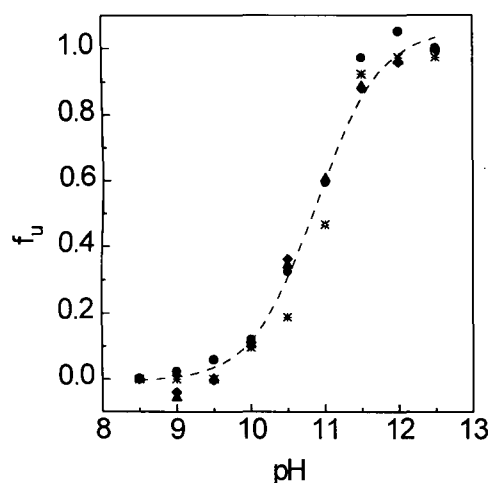
Fig. 9: Normalized spectroscopic data represented as fraction of protein unfolded as a function of pH. The symbols represent ellipticity at 222 nm (●); emission intensity at 330 nm excited at 280 nm (▲); emission intensity at 330 nm excited at 295 nm (♦); emission wavelength maximum (*). The broken line shows the sigmoid fit to the data.

Table 4: Number of positively or negatively charged side chains within the given radius of the COO⁻ group of the negatively charged residues in the active site of hexokinase A‡.

Residue[†]	Fraction ASA[§]	< 4A° +ve	< 4A° -ve	<6A° +ve	<6A° -ve	<8A° +ve	<8A° -ve	<10A° +ve	<10A° -ve
ASP86 C/O	0.208/ 0.177	-/1	-/-	1/1	-/-	3/2	2/2	3/3	3/3
ASP211 C/O	0.116/ 0.211	-/-	-/-	1/-	-/-	1/-	3/4	1/2	4/4
GLU269 C/O	0.009/ 0.318	-/-	-/-	1/-	1/-	2/-	2/2	2/2	2/2
GLU302 C/O	0.008/ 0.113	-/-	-/-	1/-	1/-	2/1	1/1	4/3	2/1
ASP417 C/O	0.265/ 0.256	-/-	-/-	-/-	-/1	-/1	2/2	2/3	4/4
GLU457 C/O	0.357/ 0.354	-/-	-/-	-/-	-/-	-/-	1/2	2/2	3/3
ASP458 C/O	0.297/ 0.369	1/1	-/-	1/1	-/1	1/1	1/4	2/2	3/4

‡These numbers were calculated using both crystal structures of hexokinase isozymes, PI and PII representing the closed (C) and open (O) conformations.

§ Fractional Accessible Surface Area (ASA) of the acidic residues listed in the left column in both, closed (C) and open (O) conformation were calculated using Lee and Richards, (1971) method using a probe size of 1.4 A°.

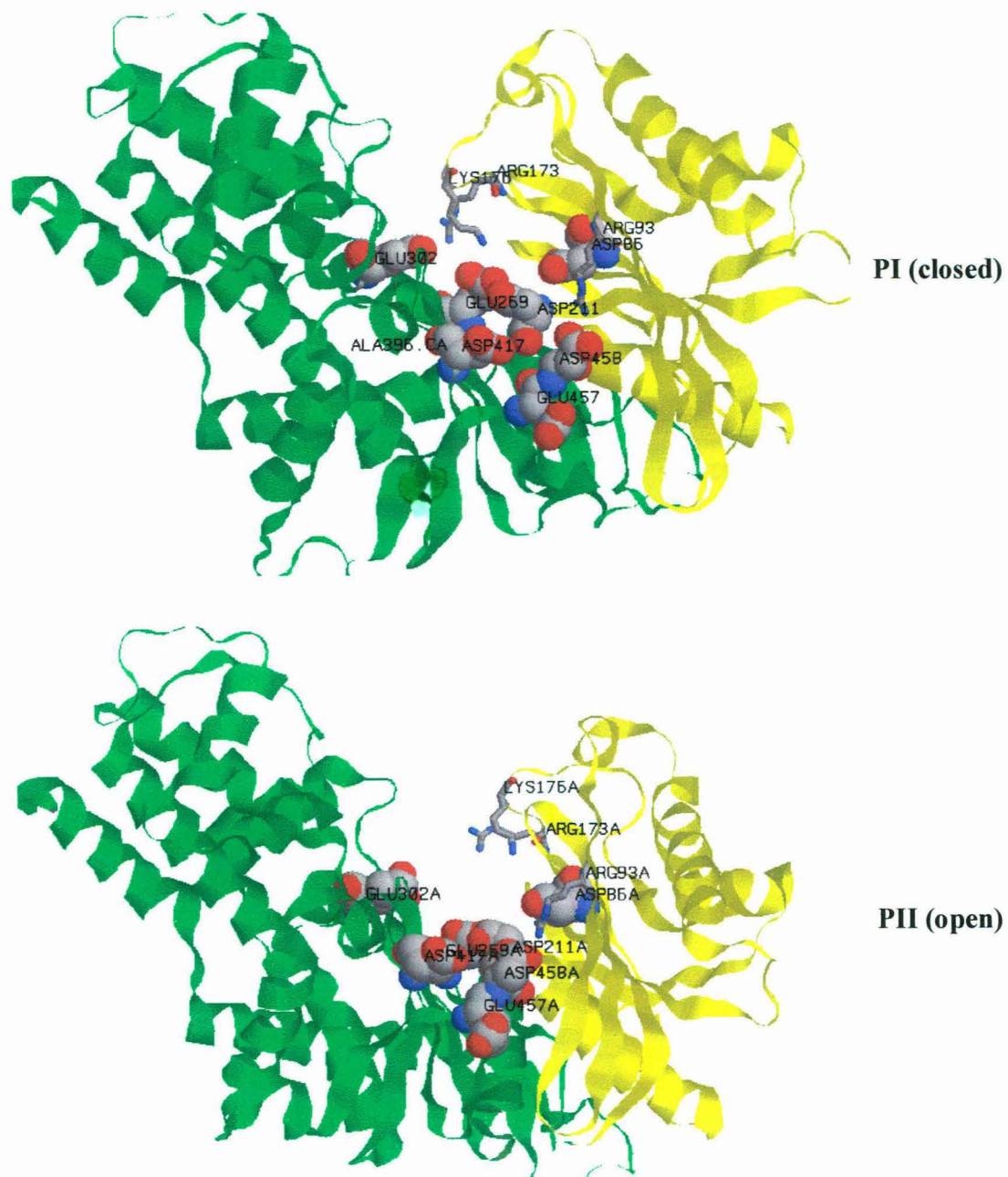† These residues are located at the active site cleft between the two domains

41

Fig. 10: Ribbon diagrams showing the closed (glucose bound, PI form) and open (without glucose, PII form) conformations of yeast hexokinase isozymes. The two domains are displayed in different colours, green (larger domain) and yellow (smaller domain). The acidic residues between the two domains have been shown as spacefill, while the basic residues have been shown in sticks mode. The PDB code for the open conformation is 1IC8. The structure of the closed conformation was drawn from the coordinates supplied by Prof. T.A. Steitz, Yale University, USA.

DISCUSSION

pH dependence of domain interactions and the effect of salt

From Figs. 1a and b, it appears that yeast hexokinase A exists in two alternative conformations depending on the pH. Hexokinase A is a two-domain protein and its calorimetric profile at pH 8.5 consists of two transitions attributed to the melting of the two domains independently (Takahashi *et al.*, 1981; Tiwari, 1999). At low pH, the thermal transition consists of a single peak with $\Delta H_{cal}/\Delta H_v$ ratio of unity similar to the results at pH 8.5 in the presence of glucose or NaCl, where the thermal transition consists of a single cooperative transition wherein the $\Delta H_{cal}/\Delta H_v$ is unity (Takahashi *et al.*, 1981). This suggests that at low pH, the two domains could be interacting with each other as in the presence of glucose. In Fig. 3, at alkaline pH T_{m1} changes drastically from pH 7.5 to 8.5 while T_{m2} is more or less constant. The inter-domain interactions that are responsible for the single cooperative transition at low pH, are stabilizing the smaller domain to a greater extent than the larger domain. This is in agreement with the model of Brandts *et al.*, (1989), which suggests that in multidomain proteins the interdomain interactions stabilize the domain with a lower T_m.

Hexokinase rapidly loses its activity below pH 6.5 with no enzymatic activity obtained at pH 4.0 (Sols *et al.*, 1958). However, using spectroscopic techniques we observed no conformational changes in this pH range. Although the pH range of 6.5 and below falls in the region of the pK_a of the histidyl residues, Grouselle *et al.*, (1973) showed that histidyl residues have no role in binding to the substrate or in catalysis. Viola and Cleland, (1978) have used the pH variation of the enzyme kinetic parameters, V_{max} and V/K to elucidate the chemical mechanism of enzyme action. From the pH dependency of V_{max} and V/K profile for glucose, they observed cooperative protonation of at least 5 carboxyl residues with pK_a ranging from 6.44± 0.07 to 6.87±0.01 and 6.59± 0.05 to 6.05± 0.04, respectively depending on temperature. The protonation of these carboxylic acids also showed high ionization enthalpy. They attributed the high ionization enthalpy to conformational changes associated with the protonation and the elevated pK_a observed for carboxyl residues to their location in deep cleft with limited accessibility to the solvent. New crystal structures of hexokinase PII in the absence of glucose with the correct amino acid sequence (Kuser *et al.*, 2000) and the structure of hexokinase A with the revised sequence (TA Steitz, personal communication) reveals the presence of 7 acidic residues (Asp 86, Asp 211, Glu 269, Glu 302, Asp 417, Glu 457, Asp 458) in the interdomain region of the active site cleft, which are conserved among different species (Kuser *et al.*, 2000). (See Fig. 10 and Table 4 for distances and ASA). Out of these

residues, Asp 211, Glu 269 and Glu 302 are closest (< 6A°) to the basic residues, Arg 173 and Lys 176 respectively in the liganded form, probably stabilizing the closed conformation by electrostatic interactions or hydrogen bonding to the substrate glucose (Table 4). These basic residues are present in small domain and stay far apart from the acidic residues in the unliganded or open conformation (crystal structure of PII). The above mentioned interactions along with additional interactions from the active site cavity being occupied by glucose might be favoring interactions between the two domains, consequently resulting in the single transition observed by DSC at the alkaline pH in the presence of glucose as reported by Takahashi et al., (1981). In the open conformation, the two acidic residues, Asp 417 and Asp 458 are close to each other (<6 A°) than in the closed conformation, and might be resulting in net repulsive interactions. These repulsive forces along with the absence of favorable interactions between the two domains could be responsible for the two separate transitions observed in the alkaline pH range.

Gerstein et al., (1994) classified the domain motions in hexokinase to be of shear type in which three moving layers x, b, a (small domain) move against three static layers X, B, A (large domain). The a and A layers consists of two helices oriented perpendicularly which participate in major shear motion. The acidic residues listed in Table 4 are located in a, A, b, B and X layers (Fig. 10). In the light of results obtained by Viola and Cleland (1978) and the model for domain movement suggested by Gerstein et al., (1994), one of the reasons for the single peak observed in the calorimetric profile at acidic pH could be due to protonation of the acidic residues, which would eliminate the repulsive forces between the domains. Single cooperative transition in DSC observed at pH 6.5 (Tiwari, 1999), coincides well with the observation of Viola and Cleland, (1978) of the elevated pKa value of 6.5 for carboxylic acids in hexokinase active site.

The binding enthalpy of glucose to hexokinase has been reported to be zero with an indistinguishable heat capacity change (Takahashi et al., 1981; Catanazano et al., 1997). The relatively indistinguishable ΔC_p change upon binding glucose could be due to cancellation effect of burial of nonpolar and charged groups (Takahashi et al., 1981). However, the contact surface area upon the formation of enzyme-glucose ([E.G]) complex is reduced which further undergoes a conformational change to form the active enzyme-glucose complex ([E.G]'). The compaction has been assumed to be due to the hydrophobic effect, which predicts that the active (closed) conformation of hexokinase could form in the absence of glucose but that would create an empty cavity, which might destabilize the closed conformation (Bennett and Steitz, 1978). Thus, protonation of acidic residues at the base of the cleft might favor the

active enzyme conformation in the absence of glucose, i.e., closed form but with an empty cavity.

At pH 3.5, DSC profile shows a single transition with a T_m of 38.9 °C. Also, the tertiary CD shows loss of some interactions while substantial ANS binding was observed by fluorescence. Addition of 50 mM NaCl leads to the destabilization and broadening of the transition towards the pre-transition zone. Deconvolution into two transitions shows that the first transition is getting more destabilized than the second transition. This suggests that interdomain interactions are stabilizing the less stable domain more than the stable domain (peak 2). However, enthalpy change is not much dependent on salt concentration (Table 2). Salt-induced destabilization with no enthalpy change has also been observed in a fragment representing the leucine zipper of GCN4 at low concentrations, whereas there is stabilization at higher concentrations (Kenar et al., 1995). Due to the lack of significant dependency of transition enthalpy on salt concentration, salt effect was primarily attributed to be entropic (Kenar et al., 1995).

Irreversibility, strong aggregation, mild effect of ionic strength, and weak signal at pH 3.5 restrained us from carrying our detailed salt effects. Takahashi et al., (1981) have observed that in 0.2 M NaCl at pH 8.5 the high temperature melting domain (T_{m2}) shifts toward the low temperature melting domain (T_{m1}) to give rise to a single transition, which is opposite to that of the glucose binding effect. At pH 3.5 also, the less cooperative transition (T_{m1}) was destabilized to a larger extent than the sharper transition (T_{m2}) in the present study, which suggests that the salt has a destabilizing effect for the large domain. Although from this limited data it would be inappropriate to arrive at the mechanism of salt action, it can be fairly assumed that salts would affect the pK_a of the acidic residues, which show a shift from their intrinsic pK_a values in addition to the screening of some favorable charge-charge interactions. Salts are known to modulate the pK_a values such that with increasing salt concentration the pK_a shifts observed in a folded protein drift toward the intrinsic value (Antosiewicz et al., 1994; Abe et al., 1995; Garicia-Moreno, 1995). It could thus, be possible that NaCl affects the above mentioned acidic residues that are close to each other and show an elevated pKa of 6.5 as suggested by Viola and Cleland (1978).

Acid denaturation

The calorimetric profiles show a single transition at low pH with $\Delta H_{cal}/\Delta H_v$ ratio close to unity at pH 4.5 and 4.0 suggesting the cooperative melting of the two domains in a two-state fashion. No change was observed in the spectroscopic properties indicating that the

protein is in the native state at these pH values. However, at pH 3.5 near UV CD shows a sudden enhancement of the positive peak at 291 nm along with a reduced negative ellipticity, while there is no change in the far UV CD spectra. The loosening of the tertiary interactions might be exposing the hydrophobic groups, which is manifested by ANS binding. Intrinsic fluorescence results show a decrease in the intensity at 330 nm without any change in the emission maximum suggesting the loosening of the tertiary interactions. At pH 3.0, the near UV CD shows no change in the negative ellipticity compared to pH 3.5 state but a complete loss of the positive peak and the enhanced ANS binding, which is associated with the partial loss of the secondary structure, suggesting that some portions of the molecule melt cooperatively while some other portions melt non-cooperatively. At pH 2.5, the protein loses most of the tertiary interactions and it shows no transition upon heating in DSC, suggesting that the pH 2.5 state is an acid unfolded state, U_A. U_A has high secondary structure content as shown by far UV CD and it has an emission maximum of 340 nm where as urea unfolded protein has an emission maximum of 353 nm suggesting incomplete exposure of tryptophans to the solvent. ANS binding as measured by fluorescence intensity at 480 nm is maximum for this state, suggesting that it could be a molten globule like state.

The melting of the pH 2.5 and pH 2.0 state either by DSC or by CD at 222 nm shows no cooperative transition, suggesting the lack of tertiary interactions in these states. Acid denaturation of hexokinase A in low ionic strength buffers was incomplete and involves a conversion from the native state to a partially collapsed state with little tertiary interactions but high secondary structure content which binds to ANS. Formation of a molten globule like state at low pH has been attributed to the failure of electrostatic repulsions to overcome the favorable hydrophobic interactions (Fink et al., 1994). While Uversky (Uversky, 2002b) has argued that it is the bulk hydrophobicity versus the charge ratio that determines the formation of a molten globule state.

Acid denaturation of yeast hexokinase A is akin to the type II proteins such as α-lactalbumin and carbonic anhydrase which directly transform from native state to the molten globule state without any detectable unfolded species. The transformation is characterized by a low amplitude change in the $[\Theta]_{222}$ values while it loses complete tertiary interactions (Fink et al., 1994). The pH 2.5 state of hexokinase A probably represents a molten globule like structure with high secondary content and low tertiary interactions. The fluorescence data also show intermediate values for the emission maxima as well as the intensity compared to that of the native or the completely denatured states.

Alkali denaturation

At pH 9.0 and above, calorimetric profiles show two transitions that unfold more or less independently. The base-induced transition begins around pH 10.0, which is close to the pK_a of tyrosine ionization. Far UV CD of alkali-denatured protein also shows less helicity compared to the acid denatured state. The emission maximum shifts to 350 nm for the alkali denatured protein compared to 340 nm for the acid denatured state, suggesting that the alkali denatured protein is more unfolded than the acid denatured protein. These results suggest that the alkali-denatured protein is similar to a pre-molten globule form observed in other proteins (Rami and Udgaonkar, 2002; Ptitsyn et al., 1995) in the sense that it is less ordered than the molten globule form and more structured than the chemically unfolded protein. Since no enhancement of ANS fluorescence was observed during alkali denaturation even in the presence of salts at pH 12.5 (data not shown), it suggests that each domain apparently unfolds in a two-state manner without populating any intermediates. Alkali denaturation appears to be a two-state process since the conformational changes monitored by different probes coincide well with each other with an apparent midpoint of pH 10.8 (Fig. 9).

In conclusion, the results of the studies carried out show that domain-domain interactions in hexokinase are influenced by pH and might be controlled by the acidic residues located at the base of the active site, consequently altering the thermal stability of the domains. These results suggest that DSC is a powerful technique to understand the nature of domain-domain interactions in proteins when used in conjunction with their structural information. Acid-denatured state of yeast hexokinase A has properties similar to that of the molten globule state, while the alkali-denatured protein is less structured than the acid denatured protein and probably represents a pre-molten globule form.

Effect of Polyols on the Molten globule State of Yeast Hexokinase A

INTRODUCTION

Several organisms accumulate a variety of low molecular weight organic compounds to counter the effects of stress conditions such as salinity, high temperature, dehydration and freezing (Stewart and Lee, 1974; Yancey et al., 1982; Csonka, 1989; Cayley et al. 1992; Santoro et al., 1992; Timasheff, 1992). These substances, collectively known as osmolytes, fall into different categories, like polyols, sugars, salts and amino acids and have been shown to provide stability to enzymes both in vivo (Singer and Lindquist, 1998) and in vitro (Sanotro et al., 1992; Timasheff, 1995).

The mechanism of stabilization of proteins by the action of cosolvents, has been worked out in detail for sugars (Back et al., 1979; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Lin and Timasheff, 1996; Kaushik and Bhat, 2003), polyols (Gerlsma, 1968; Gekko and Morikawa, 1981a, b; Gekko and Timasheff, 1981a, b; Gupta and Bhat, 1995; Xie and Timasheff, 1997a, b; Kaushik and Bhat, 1998; Radha et al., 1998), amino acids (Arakawa and Timasheff, 1983, 1985; Santoro et al., 1992, Gopal and Ahluwalia, 1993; Taneja and Ahmad, 1994; Sabulal and Kishore, 1995) and methyl amines (Kita et al., 1994; Wang and Bolen, 1997; Baskakov et al., 1998; Baskakov and Bolen, 1998b; Zou et al., 2002). Based on these studies, now it is possible to conclude that the mechanism of action of these stabilizing cosolvents is under thermodynamic control, i.e. the stabilizing action is the direct consequence of mutual perturbation of chemical potentials of the protein and the cosolvent. These stabilizing cosolvents have been found to invariably lead to the greater preferential hydration of the denatured state compared to the native state as a result of which the denatured state being more unfavorable compared to the native state, drives the equilibrium towards the native state, resulting in the stabilization of the protein.

The exclusion of cosolvent from the immediate domain of proteins is a function of the surface properties of the protein like the solvent exposed peptide bond (Bolen and Baskakov, 2001) and the non-polar surface area (Gekko and Timasheff, 1981a), which is quite different for the native, the denatured and the molten globule like intermediates. The general mechanism of action of stabilizing cosolvents has been arrived by studying mostly the native state of proteins. In few cases these stabilizing cosolvents have been found to induce molten globule like state in an acid denatured state (Davis-Searles et al., 1998; Kamiyama et al., 1999; Bongiovanni et al., 2002) and a cooperative transition in natively unfolded or thermodynamically unfolded proteins (Baskakov and Bolen, 1998a; Baskakov et al., 1999).

Among various cosolvents, linear chain polyols are interesting in that they are neutral and are available in a series of carbon chain length ranging from 2-carbon ethylene glycol to 6-carbon sorbitol, wherein each successive member of the series has an additional CHOH

group, that is known to drastically alter the stabilizing properties of these cosolvents. Protein stability has been found to increase linearly with the number of -OH groups in these polyols (Tiwari, 1999). A point to be noted here is that a member of this series, ethylene glycol is a destabilizer of native state of proteins, lowering the transition temperature of several proteins (Back et al., 1979).

In this study, we have used solvent perturbation method to study the conformational changes in the pH 2.5 state of yeast hexokinase A, in the presence of cosolvents such as linear polyols, the disaccharide trehalose and the substrate glucose. At this pH, hexokinase A has been shown to exist as a molten globule like state (Chapter 3). Since polyols stabilize native structure by strengthening the hydrophobic interactions, the use of cosolvents under the conditions in which molten globule is predominant should induce the conformational changes on the way to the attainment of the native structure. The use of stabilizing cosolvents is, thus, of crucial importance from the point of view of MG↔N transition, which mainly requires compaction of the hydrophobic core and the formation of the tertiary interactions. It would also be interesting to see whether the MG↔N transition achieved by exploiting cosolvents is cooperative or non-cooperative in nature. To elucidate the effect of cosolvents on the protein structure, we use the terms "structure induction" and "structure protection" interchangeably. Different techniques like calorimetry, far and near UV CD, intrinsic and ANS fluorescence have been employed to study the conformational changes mediated by these cosolvents. The results have been discussed in terms of the native like structure formation from the molten globule state and its relation to the cooperative transitions observed by calorimetry.

RESULTS

Thermal denaturation studies

In the previous chapter we have shown that at pH 2.5, in low ionic strength buffer, yeast hexokinase A exists as a molten globule like intermediate. It retains about 62% of helices as evidenced by molar ellipticity at 222 nm, has largely disordered tertiary interactions, and binds to the non-polar fluorescence dye ANS, which are the characteristics of a molten globule like state. Fig. 1 shows the thermal denaturation of yeast hexokinase A monitored by CD at 222 nm and by DSC at pH 2.5. As is apparent from Fig. 1, the pH 2.5 state of hexokinase A does not show any cooperative transition with increasing temperature. The excess Cp increases monotonously with increasing temperature with positive slope suggesting positive heat capacity change upon melting due to the exposure of nonpolar groups. Molten globule states of apo-myoglobin at pH 4.0 and 3.5 have also been observed to melt without heat absorption, with the excess heat capacity increasing with increasing

temperature devoid of any cooperative transition typical of native proteins (Griko and Privalov, 1994). Many other proteins that show molten globule like intermediates also denature without any enthalpy change upon heating and has led to the controversy about MG state being a true thermodynamic state (Privalov, 1996; Ptitsyn and Uversky, 1994; Pfeil, 1998).
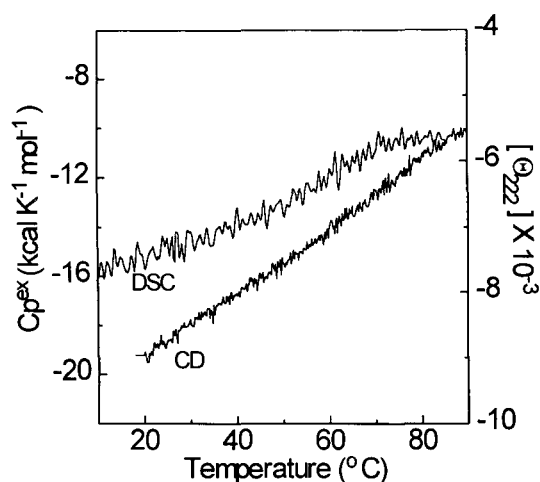

Fig. 1: Thermal denaturation of yeast hexokinase A in 20 mM Glycine, pH 2.5 monitored by excess heat capacity and ellipticity at 222 nm.
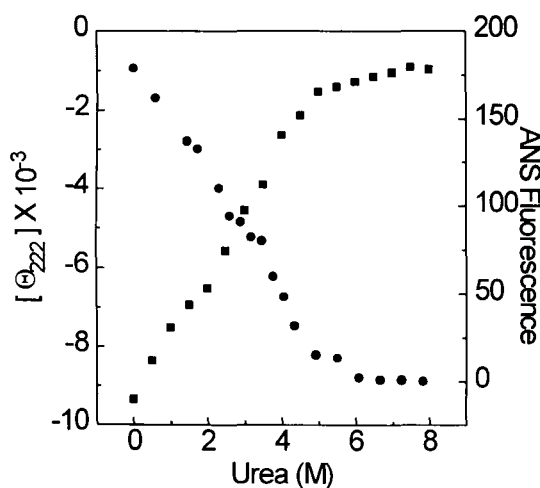
Fig. 2: Urea denaturation of yeast hexokinase A in 20 mM Glycine, pH 2.5 at 18°C, monitored by ellipticity at 222 nm (■) and ANS fluorescence intensity at 485 nm (●).

Fig. 2 shows urea denaturation of the pH 2.5 state of hexokinase A monitored by ellipticity at 222 nm and ANS fluorescence intensity at 485 nm. In contrast to the thermal denaturation, urea denaturation clearly shows cooperative transition without any pre-transition baseline, probably due to the low stability of the MG like state. This transition shows a kink at 2.2 M urea and appears to be slightly biphasic in nature, possibly suggesting the independent unfolding behavior of the two domains of the protein. Unlike temperature denaturation, urea

denaturation is complete with the molar ellipticity value at 222 nm reaching a plateau at around 5.0 M urea. Interestingly, urea transitions monitored by the two probes appear to be mirror images of each other. ANS fluorescence data further suggest that the residual structure has considerably exposed hydrophobic patches even at high concentrations of urea. These results suggest that yeast hexokinase A at pH 2.5 state has many similarities to the molten globule states observed for other proteins (Fink et al., 1994). Thus, it can be fairly assumed that yeast hexokinase A exists as a molten globule like state at pH 2.5.
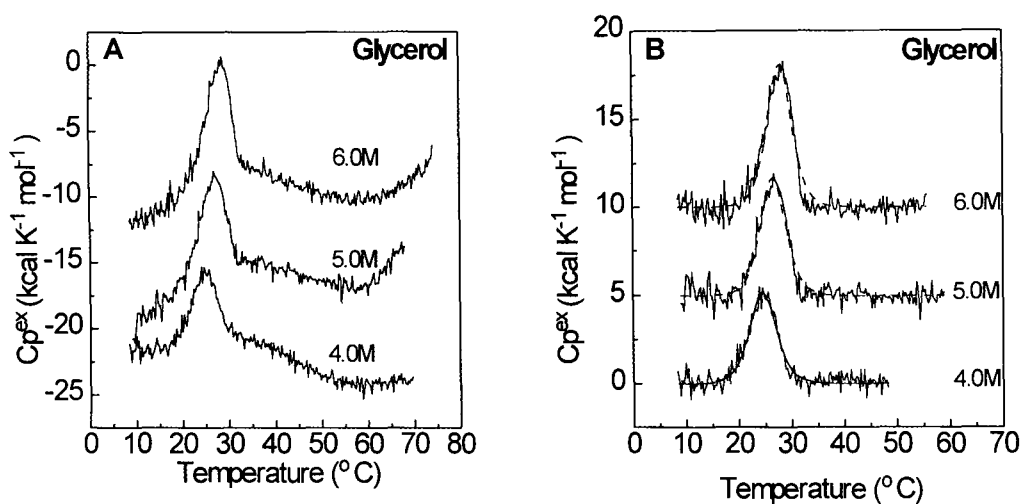
Fig. 3: Effect of different concentrations of glycerol on thermal denaturation of yeast hexokinase A at pH 2.5. A: The raw data after buffer baseline subtraction and concentration normalization. B: Data after deconvolution into a single peak.
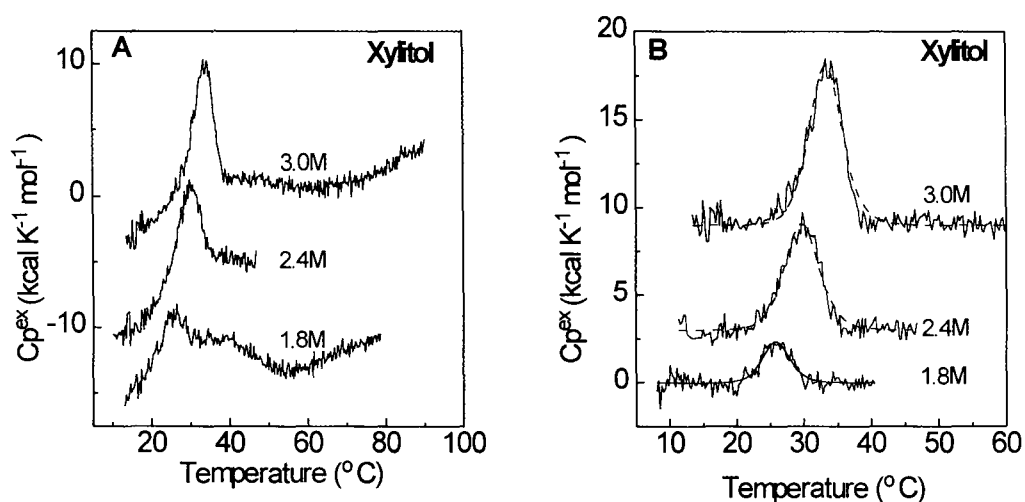
Fig. 4: Effect of different concentrations of xylitol on thermal denaturation of yeast hexokinase A at pH 2.5. A: The raw data after buffer baseline subtraction and concentration normalization. B: Data after deconvolution into a single peak.

Figures 3a-7a show the buffer baseline subtracted and concentration normalized calorimetric profiles of hexokinase A denaturation in the presence of glycerol, xylitol,

sorbitol, glucose and trehalose respectively. The corresponding deconvoluted thermograms have been shown in Figures 3b-7b. The data have been fit to a non-two state model using Origin[TM] software and the resulting thermodynamic parameters have been presented in Table 1.
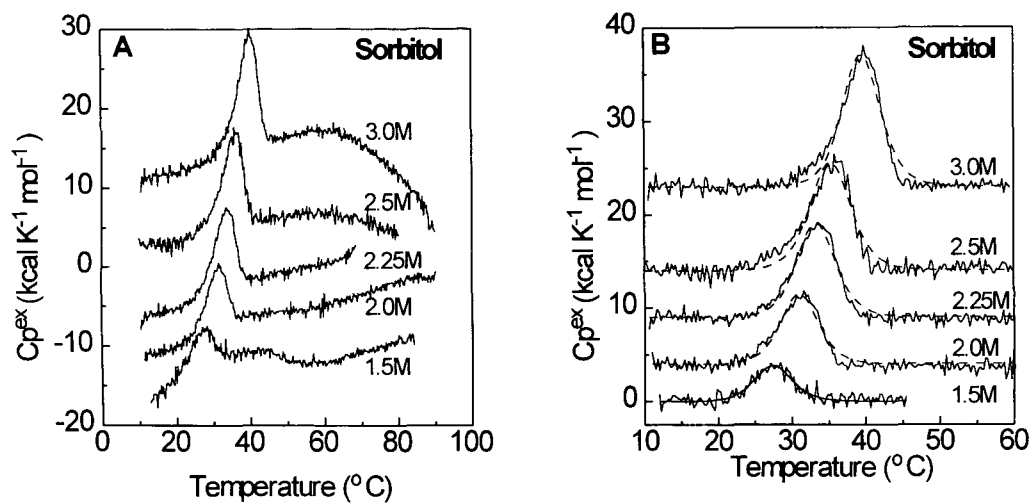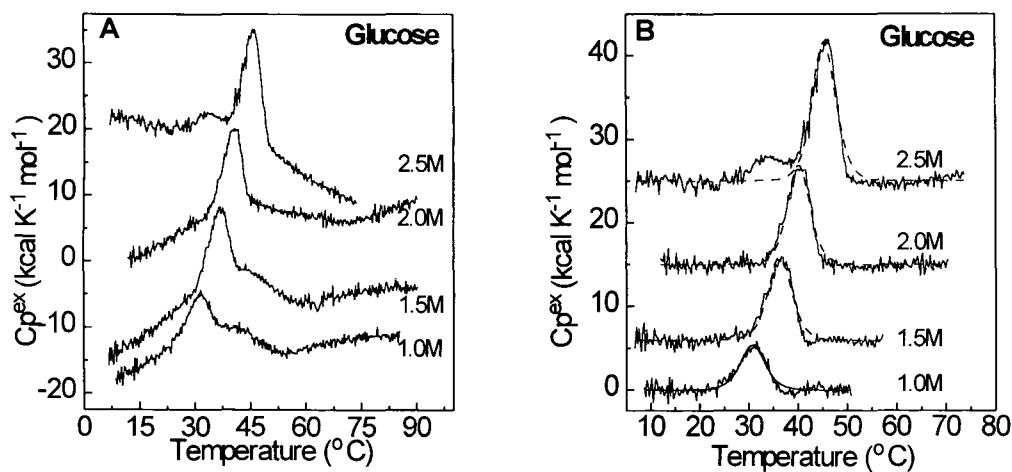

Fig. 5: Effect of different concentrations of sorbitol on thermal denaturation of yeast hexokinase A at pH 2.5. A: The raw data after buffer baseline subtraction and concentration normalization. B: Data after deconvolution into a single peak.

Fig. 6: Effect of different concentrations of glucose on thermal denaturation of yeast hexokinase A at pH 2.5. A: The raw data after buffer baseline subtraction and concentration normalization. B: Data after deconvolution into a single peak.

The data clearly indicate the cooperative melting of hexokinase A in the presence of these cosolvents, whereas the pH 2.5 state in the absence of the stabilizers does not show any transition at all upon heating. The noisy nature of the data especially at low concentrations of the cosolvents is due to the low enthalpy of denaturation. Minimum concentration of a cosolvent required to show a thermal transition is around 3.0 M for glycerol, 1.8 M for xylitol,

1.5 M for sorbitol, (which is equivalent to 27%w/v) and 1.0 M for glucose and trehalose. The enthalpy as well as T_m increases with increasing number of -OH groups in the linear polyol molecules.
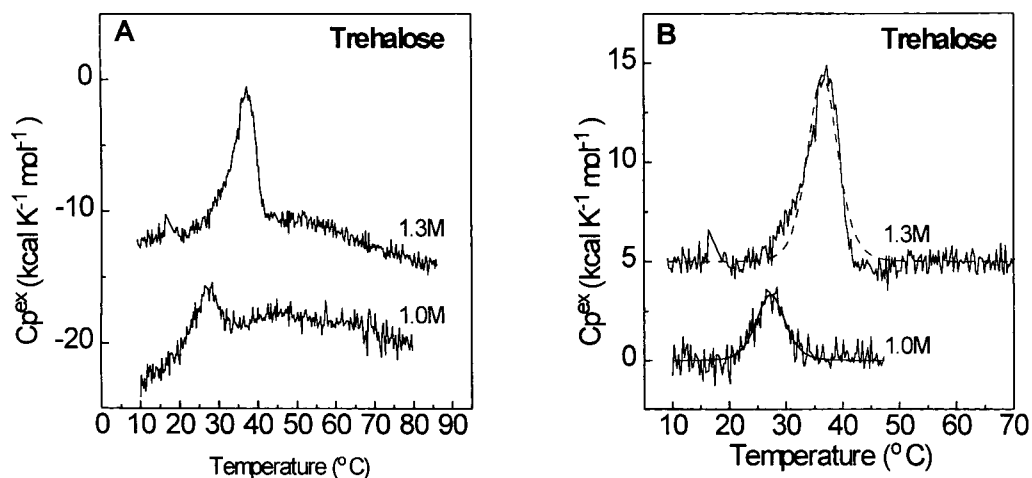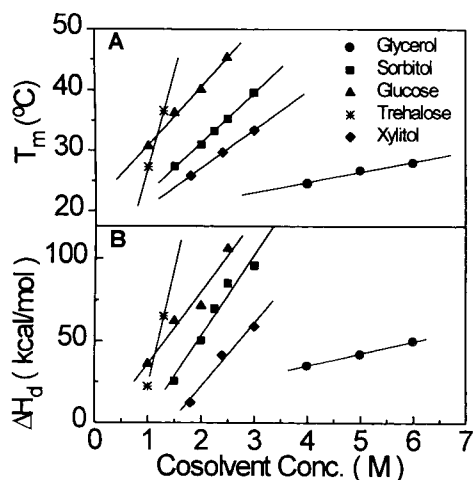

Fig. 7: Effect of different concentrations of trehalose on thermal denaturation of yeast hexokinase A at pH 2.5. **A:** The raw data after buffer baseline subtraction and concentration normalization. **B:** Data after deconvolution into a single peak.

Fig. 8: Dependence of apparent T_m (A) and enthalpy of denaturation, ΔH_d (B) obtained from DSC curves for the denaturation of hexokinase A at pH 2.5 in the presence of various cosolvents, on cosolvent concentration.
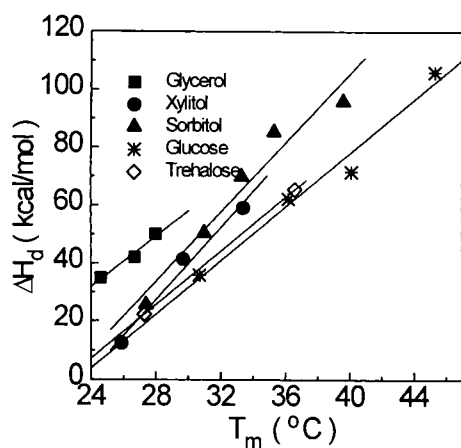

Fig. 9: Dependence of enthalpy of denaturation (ΔH_d) on apparent transition temperature (T_m) obtained from DSC melting curves for hexokinase A denaturation at pH 2.5 in the presence of various cosolvents.
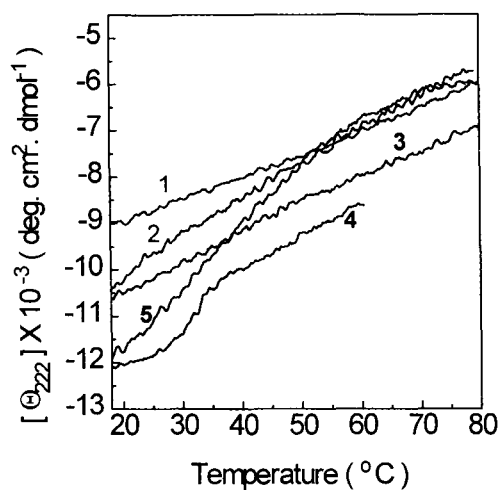
Fig. 10: Thermal denaturation of yeast hexokinase A in the presence of cosolvents, control (buffer) (1), 1.5 M sorbitol (3), 2.5 M sorbitol (4), 4.0 M glycerol (2), 8.0 M EG (5) in 20 mM Glycine pH 2.5, monitored by CD at 222 nm. Protein concentration was 200 μg/ml.
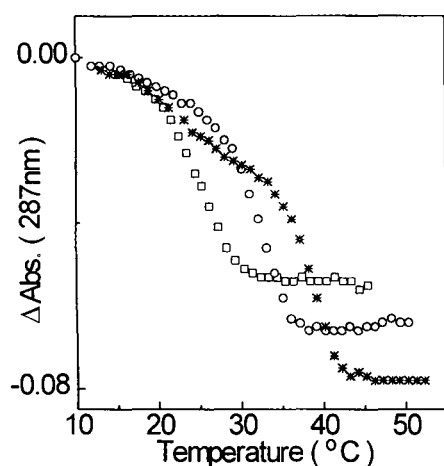

Fig. 11: Thermal denaturation of yeast hexokinase A in the presence of 1.0 M (□), 1.5 M (o), and 2.0 M (x) glucose in 20 mM Glycine, pH 2.5 monitored by change in absorbance at 287 nm. Protein concentration was 1mg/ml.
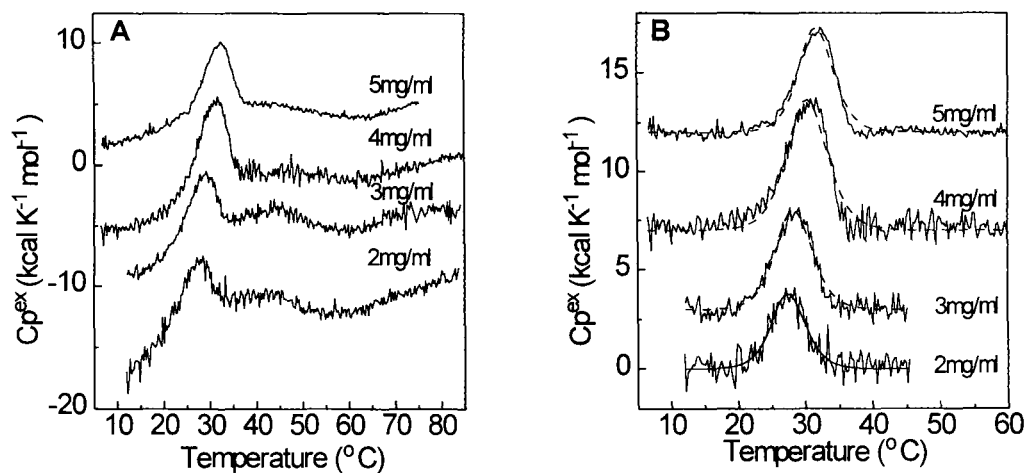

Fig.12: Thermal denaturation of yeast hexokinase A in 20 mM Glycine, pH 2.5, 1.5M sorbitol as a function of protein concentration. A: The raw data after buffer baseline subtraction and concentration normalization. B: Data after deconvolution into a single peak.

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Fig. 8a presents the plots of dependency of T_m and ΔH_d on cosolvent concentration, the T_m and ΔH_d increase linearly with increasing cosolvent concentration, with differing slopes depending on the type of the cosolvent used. The slopes obtained from the plots are, $1.7\,^{\circ}C/M$ for glycerol, $6.3\,^{\circ}C/M$ for xylitol, $8.2\,^{\circ}C/M$ for sorbitol, $9.5\,^{\circ}C/M$ for glucose, and $31.0\,^{\circ}C/M$ for trehalose. Higher concentrations of trehalose were not possible due to its limited solubility. Since glucose is a substrate for hexokinase A, it could be effective in stabilization at lower concentrations by binding to the active site compared to the linear 6-carbon polyol, sorbitol. Enthalpy function also follows a similar trend as compared with the dependency of T_m yielding values of 7.5 kcal/mol/M for glycerol, 38.9 kcal/mol/M for xylitol, 49.1 kcal/mol/M for sorbitol, 44.0 kcal/mol/M for glucose, and 143.6 kcal/mol/M for trehalose (Fig. 8B).
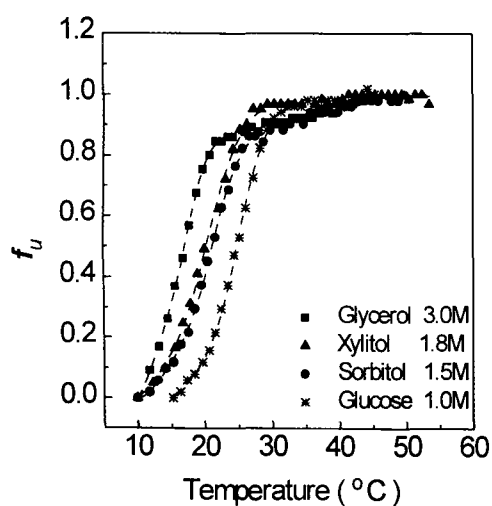

Fig. 13: Thermal denaturation of yeast hexokinase A in the presence of various cosolvents at given concentrations in 20 mM Glycine, pH 2.5, monitored by change in absorbance at 287 nm at a protein concentration of 1mg/ml. The data are plotted as fraction of unfolded protein, f_u. The broken line shows two state fit to the data as described in Kaushik and Bhat, (1998).
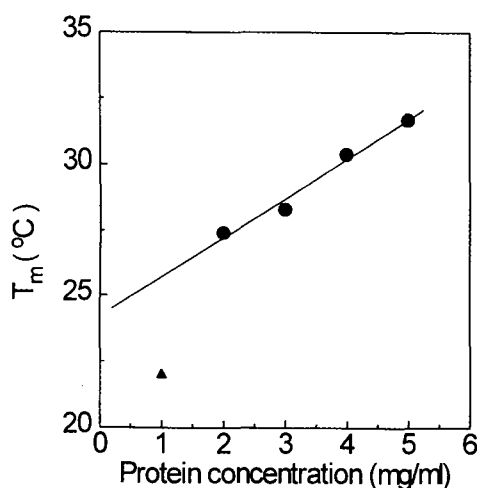

Fig. 14: Dependence of transition temperature (T_m) determined from DSC experiments in the presence of 1.5 M sorbitol, pH 2.5, on protein concentration (●). The ▲ data point indicates the value of the T_m determined from UV absorbance at 287 nm.
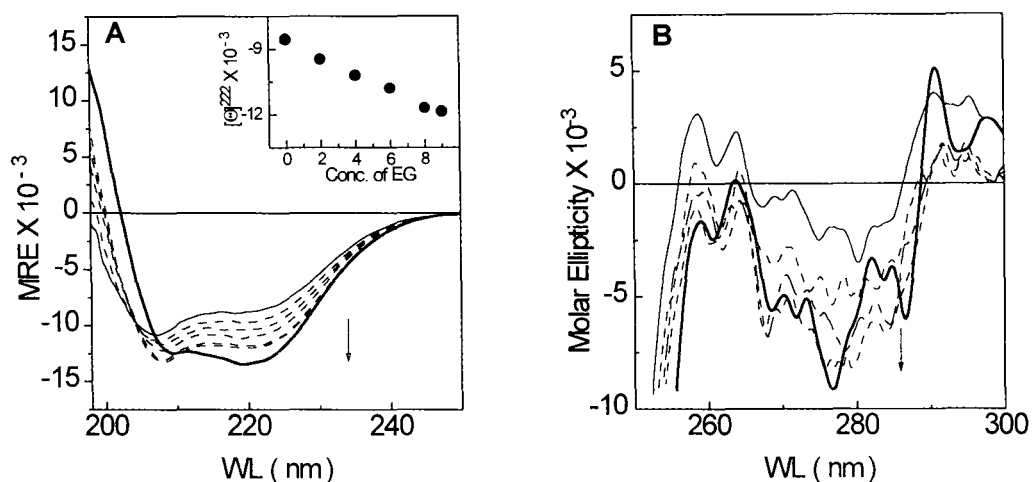
Fig. 15: Far-UV (A) and near-UV (B) CD spectra of yeast hexokinase A in the presence ethylene glycol at pH 2.5. The dotted line shows the spectra in the presence of different concentration of cosolvent and the arrow indicates the direction of increasing concentration of cosolvent ranging from 0-9 M for EG. The thin continuous line is the pH 2.5 state without cosolvent and the thick continuous line is the native state. The data were converted to mean residue ellipticity (MRE) for far UV CD and molar ellipticity for near UV CD. Inset of Fig. 15A shows the MRE values at 222 nm plotted against cosolvent concentration, the units for ellipticity being deg.cm^2. dmol^{-1}.
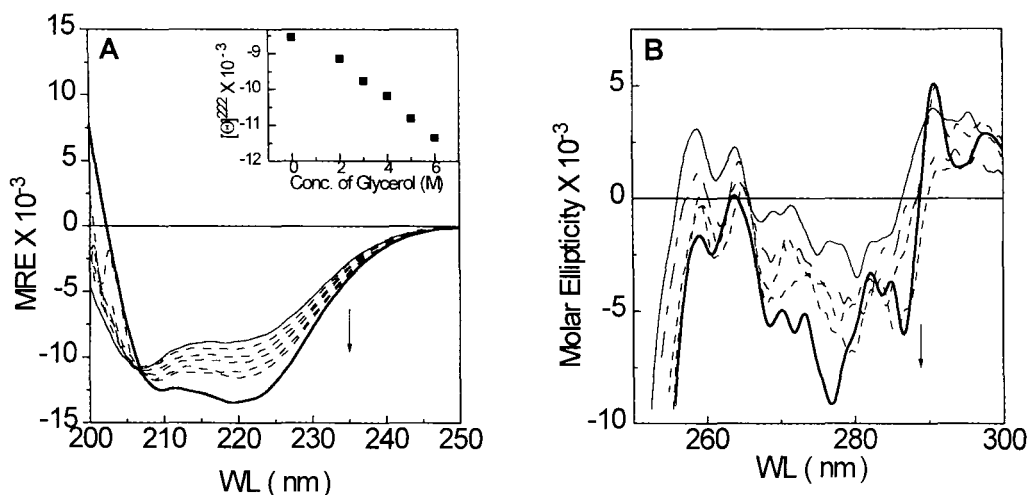

Fig. 16: Far-UV (A) and near-UV (B) CD spectra of yeast hexokinase A in the presence of glycerol ranging from 0-6 M at pH 2.5. The description of the plots is same as given in the legend of Fig. 15.

In Figs. 3A-7A, the post transition base line above 60°C was highly unstable and could be due to the high concentrations of the cosolvents used. The raw data reveal a second broad and less cooperative transition immediately after the first transition, especially at the low concentrations of the cosolvents. This transition diminishes with the increase in concentration of the cosolvents. This type of broad and less cooperative transition has been previously reported for the melting of the MG state of Staphylococcal nuclease in the presence of salt (Carra et al., 1994). To investigate whether the less cooperative second transition represents

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the breaking down of some secondary or tertiary interactions, thermal denaturation was also probed by CD at 222 nm (Fig. 10) and by UV absorbance at 287 nm (Figs. 11 & 13). In contrast to the DSC data, thermal denaturation monitored by UV absorbance shows only one transition with more or less flat post transition baseline and surprisingly, no cooperative transition was observed by CD at 222 nm at 27% w/v of cosolvents. Thus, the absence of any cooperative transition in UV melting curves corresponding to the second transition observed in the DSC suggests that the DSC transition could merely represent the heat capacity changes associated with the hydration of non polar groups with increasing temperature and not a true endothermic transition.

The amount of residual structure retained as evidenced from ellipticity at 222 nm values after denaturation was observed to increase with increasing concentration of sorbitol (Fig. 10), supporting the earlier observations that denaturation in the presence of osmolytes leads to compact denatured states (Ueda et al., 2001) relative to in their absence. On the other hand, ethylene glycol (EG) and glycerol show no effect on ellipticity at 222 nm at higher temperatures, suggesting extensive opening of secondary structure in their presence similar to that observed in the buffer. Interestingly, compared to the all other cosolvents, glycerol showed higher enthalpy of denaturation and lower T_m (Fig. 9). The higher enthalpy could be due to lesser residual structure observed at post denaturation temperatures compared to sorbitol suggesting larger extent of unfolding in the presence of glycerol (Fig. 10).

In Fig. 11, the amplitudes of transition curves (ΔA) monitored by UV absorbance at 287 nm are increasing with increasing glucose concentration (also true for other cosolvents, data not shown).
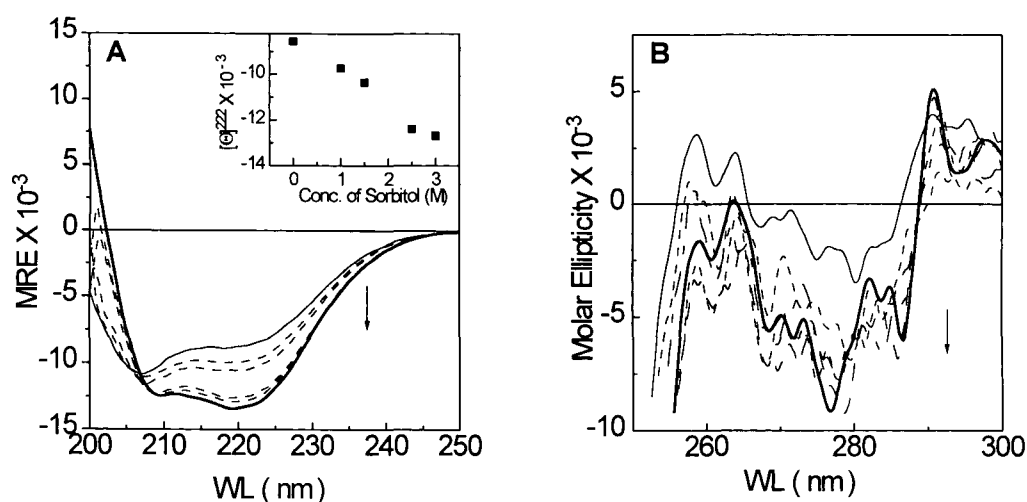

Fig. 17: Far-UV (A) and near-UV (B) CD spectra of yeast hexokinase A in the presence of sorbitol ranging from 0-3 M at pH 2.5. The description of the plots is same as given in the legend of Fig. 15.
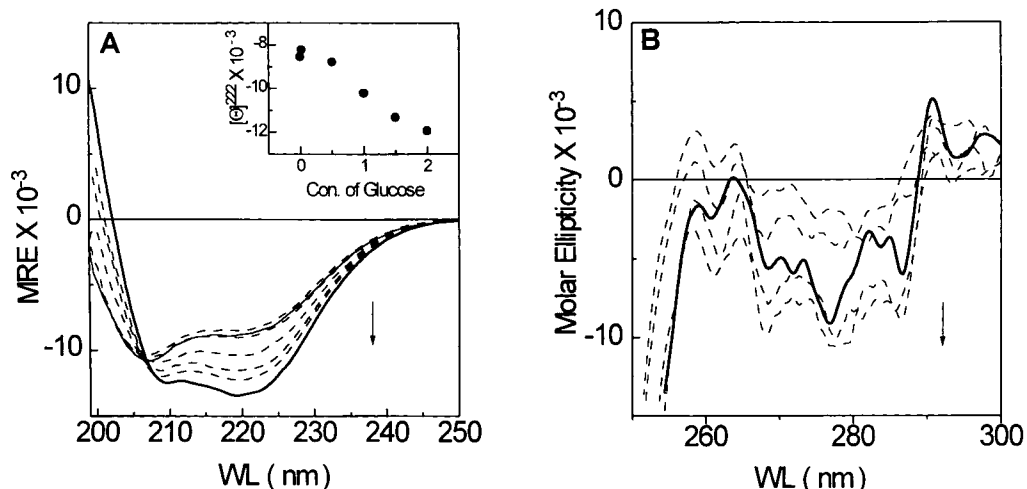
Fig. 18: Far-UV (**A**) and near-UV (**B**) CD spectra of yeast hexokinase A in the presence of glucose ranging from 0-2 M at pH 2.5. The description of the plots is same as given in the legend of Fig. 15.
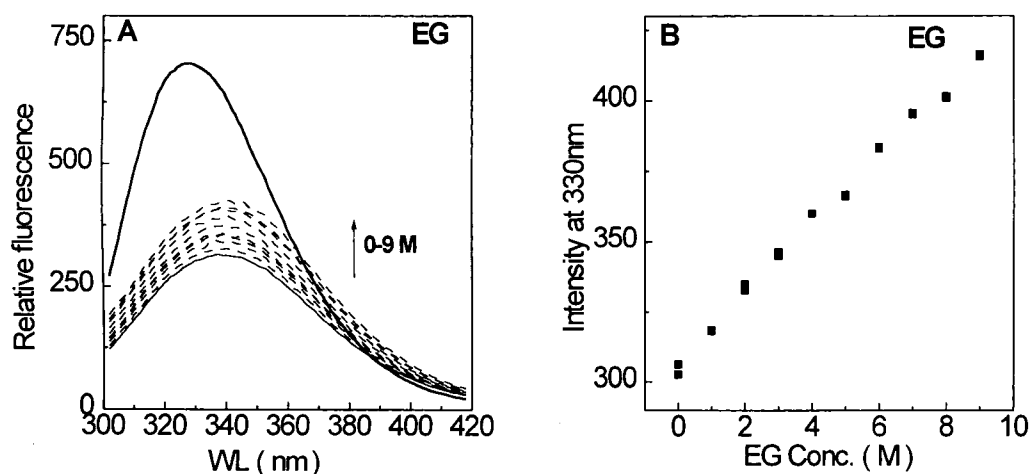

Fig. 19A: Intrinsic fluorescence spectra of yeast hexokinase A in the presence of 0-9 M EG at pH 2.5, excited at 280 nm. The arrow indicates the direction of increasing cosolvent concentrations (dotted lines). The lower most solid line shows the acid denatured state at pH 2.5 and the upper solid line for the native state at pH 5.0. **B:** fluorescence intensity at 330 nm plotted against EG concentration.

This data is qualitatively in agreement with the calorimetric enthalpy change, whereas van't Hoff enthalpy remains almost constant (Table 1). This suggests that at low concentrations of the cosolvents at pH 2.5, hexokinase A exists in two different populations, one melting cooperatively, and the other showing non-cooperative denaturation. Increasing the concentration of the cosolvent appears to shift the population more toward the cooperatively unfolding intermediate state.

Surprisingly, the results in Fig. 10 do not show any cooperative transition in the presence of 1.5 M sorbitol or 4 M glycerol at pH 2.5, although the extent of secondary structure present is higher in the presence of cosolvents as compared to the acid denatured

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state at pH 2.5 alone. However, at 2.5 M sorbitol, a clear sigmoidal transition can be seen. Even though no transition was apparent in the presence of 1.5 M sorbitol or 4 M glycerol probed by molar ellipticity at 222 nm, thermal denaturation monitored by UV absorbance at 287 nm in the presence of 3.0 M glycerol and 1.5 M sorbitol shows a cooperative transition (Fig. 13) indicating the breaking down of tertiary interactions upon heating. Thus, it is apparent that at low concentrations of the cosolvents, the secondary and tertiary interactions are contributing independently to different extents to the stability while at higher concentrations of the cosolvents both the secondary as well as tertiary interactions are contributing together to the stability of the protein. The transitions observed by UV absorbance at 287 nm were irreversible with no clear pre-transition baselines (Fig. 13). Hence, we have used van't Hoff analysis for calculating apparent midpoints of transition only. The apparent T_m values calculated from these measurements are not in agreement with the mid point of transition obtained from DSC melting curves (Table 1). This could be due to the reason that different protein concentrations have been used in these two types of experiments or perhaps due to the reason that UV absorbance changes at 287 nm only reflect local changes in the tryptophan environment. Nonetheless, the mere presence of these transitions supports the DSC data of cooperative thermal denaturation in the presence of cosolvents at pH 2.5. The order of cosolvents in terms of increasing the apparent T_m values is also true for the spectroscopic measurements at 27% w/v of cosolvents (Fig. 13).
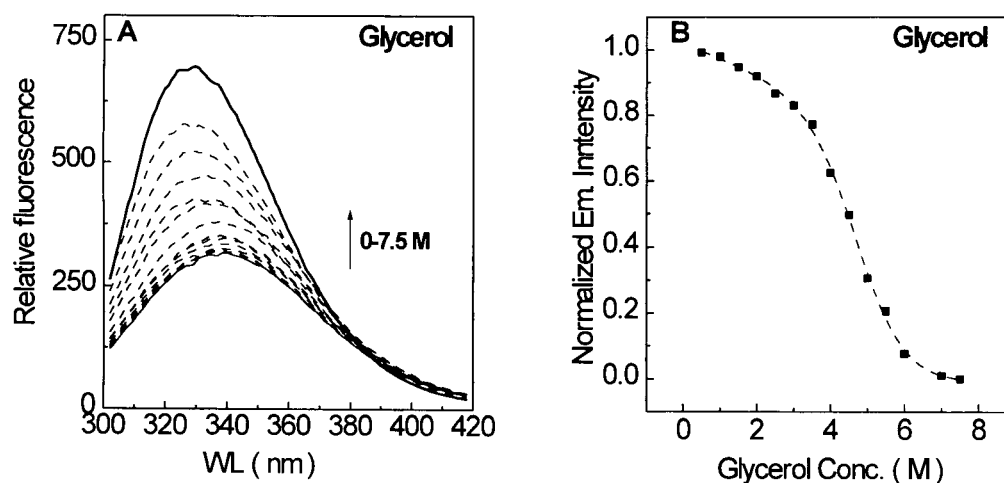
Fig. 20A: Intrinsic fluorescence spectra of yeast hexokinase A in the presence of 0-7.5 M glycerol at pH 2.5, excited at 280 nm. The arrow indicates the direction of increasing cosolvent concentrations (dotted lines). The lower most solid line shows the acid denatured state at pH 2.5 and the upper solid line for the native state at pH 5.0. **B:** Normalized fluorescence emission intensity at 330 nm plotted against glycerol concentration. The broken line is the two-state fit to the data as described in Shirley, (1995).

Fig. 21A: Intrinsic fluorescence spectra of yeast hexokinase A in the presence of 0-3.5 M sorbitol at pH 2.5. **B:** Normalized fluorescence emission intensity at 330 nm plotted against sorbitol concentration. The description of the plots is same as given in the legend of Fig. 20.

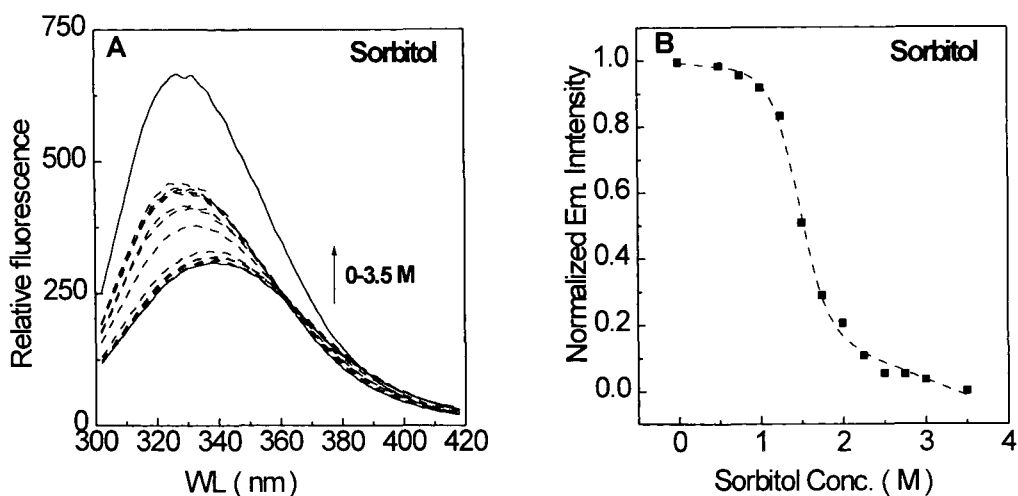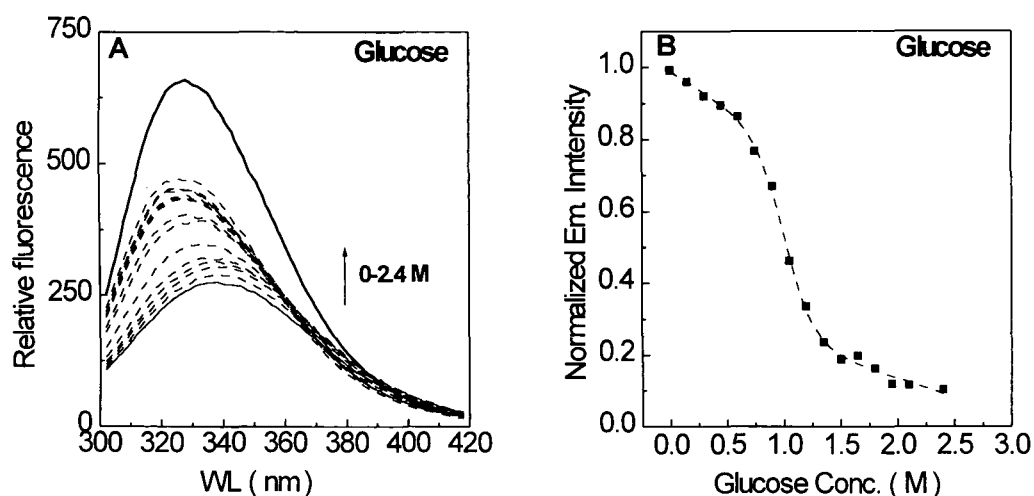**Fig. 22A:** Intrinsic fluorescence spectra of yeast hexokinase A in the presence of 0-2.5 M glucose at pH 2.5. **B:** Normalized fluorescence emission intensity at 330 nm plotted against glucose concentration. The description of the plots is same as given in the legend of Fig. 20.

Aggregation or association of partially folded states that have exposed hydrophobic patches is a well-known phenomenon. Such protein states show concentration dependence of the melting temperature(T_m). Fig. 14 shows the dependence of T_m on the protein concentration in the presence of 1.5 M sorbitol at pH 2.5. The T_m values as measured by DSC (Fig. 12) were found to be linearly and strongly dependent on protein concentration. It could be possible that these folded states are associating and forming soluble multimers that might be converting the ensemble, which is melting less cooperatively, to a cooperatively melting ensemble. It is not clear at the moment that whether the cooperative ensemble consists of such multimers. This type of association-induced structure has also been observed in SNase, where at a fixed

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concentration of the anion, increasing the protein concentration induced a cooperative transition (Uversky et al., 1999). They interpreted these results in terms of the conversion of one form of the intermediate to another form due to the association of flickering structural elements with the matching regions in an intermolecular fashion, which might be stabilizing these flickering structural elements leading to an increase in the secondary structure in the associated states (Uversky et al., 1999). In the case of SNase at sufficiently high protein concentrations, the multimeric intermediates were precipitating out, while for hexokinase A we did not observe any such visible precipitation before or after heating even at 5mg/ml of protein concentration.
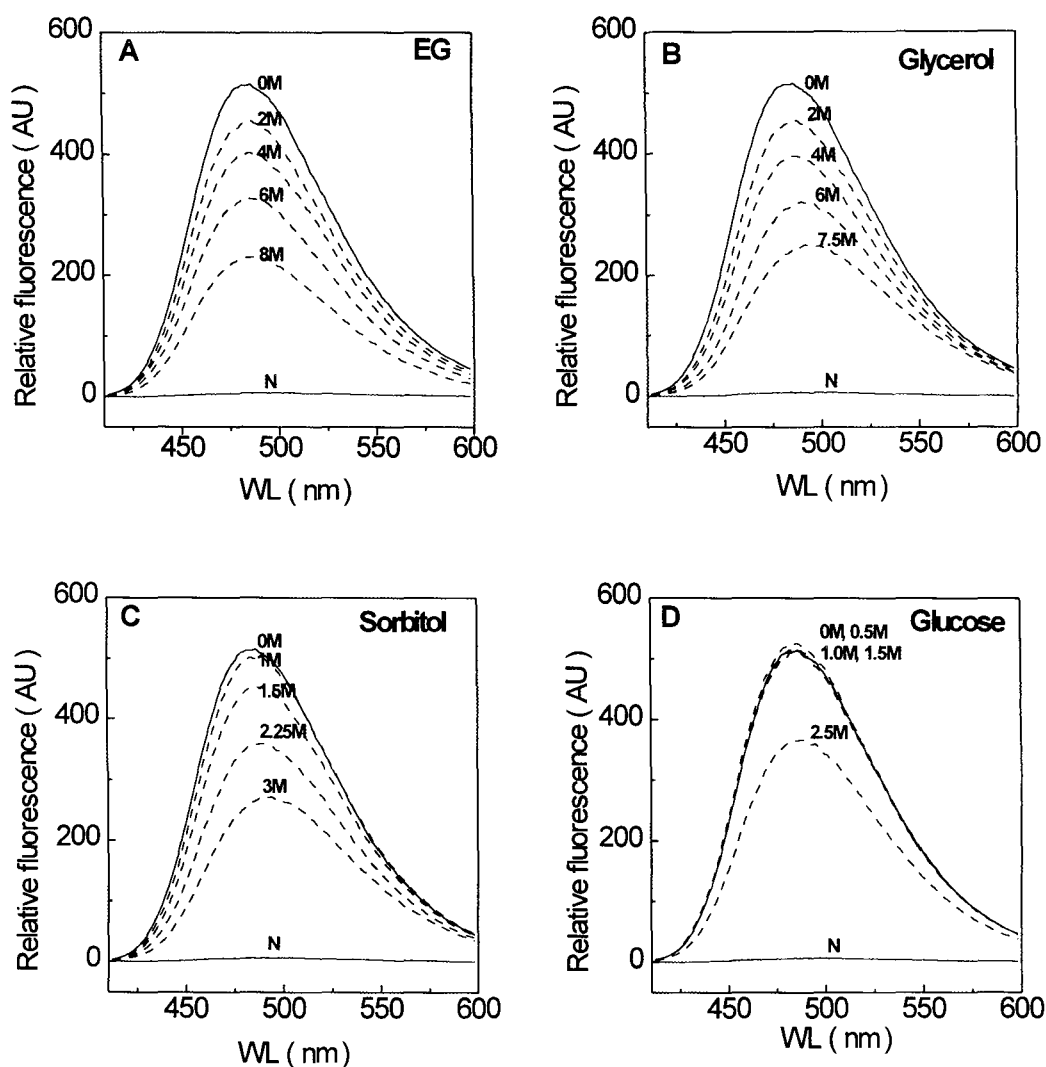
Figs. 23: ANS fluorescence spectra of yeast hexokinase A in the presence of ethylene glycol (A), glycerol (B), sorbitol (C) and glucose (D) in 20 mM Glycine, pH 2.5, excited at 400 nm. The dashed lines are in the presence of various cosolvent concentrations as indicated. The uppermost solid line is for the acid denatured state at pH 2.5 and the lowermost solid line (N) represents the native state in water.

Table 1: Thermodynamic data obtained from DSC experiments, carried out in the presence of various cosolvents in 20 mM glycine pH 2.5.

Concentration (M)	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_v
Glycerol			
4.0	24.6 ±0.2 (18.7±0.4)	34.9 ±3	104.0 ±3
5.0	26.7 ±0.2	42.0 ±1	113.0 ±3
6.0	28.0 ±0.1	50.0 ±1	117.0 ±3
Xylitol			
1.8	25.9±0.2 (21.4±0.5)	12.4±3	134.0±3
2.4	29.8±0.1	41.4±2	107.0±3
3.0	33.4±0.1	59.1±3	115.0±5
Sorbitol			
1.5	27.4 ±0.2 (21.1±0.4)	25.6 ±3	106.0 ±5
2.0	31.0 ±0.1	50.3 ±2	109.0 ±3
2.25	33.3 ±0.1	69.9 ±2	108.0 ±3
2.5	35.3 ±0.1	85.2 ±4	103.0 ±5
3.0	39.6 ±0.1	95.8 ±5	114.0 ±4
Glucose			
1.0	30.7 ±0.2 (25.3±0.4)	35.9 ±3	110.0 ±4
1.5	36.2 ±0.1	62.2 ±2	120.0 ±3
2.0	40.1 ±0.1	71.6 ±4	130.0 ±3
2.5	45.3 ±0.1	106.0 ±5	127.0 ±5
Trehalose			
1.0	27.3 ±0.1	22.3 ±4	107.0 ±6
1.3	36.6 ±0.1	65.4 ±3	110.0 ±3

The T_m values given in parenthesis are determined by two-state fitting to UV melting curves.

Table 2: Dependence of the thermodynamic data obtained from DSC experiments as a function protein concentration in the presence of 1.5 M sorbitol, 20 mM glycine pH 2.5.

Conc. of protein (mg/ml)	Tm (°C)	ΔH_{cal} (kcal/mol)	ΔH_v
2.0	27.4 ±0.2	25.6 ±4	106.0 ±5
3.0	28.3 ±0.2	36.0 ±8	101.0 ±4
4.0	30.4 ±0.3	52.9 ±5	92.1 ±4
5.0	31.7 ±0.2	36.2 ±5	107.0 ±3

Effect of cosolvents on the structure of hexokinase A

We have examined the changes in the structural properties of hexokinase A in the presence of varying concentrations of the cosolvents by using spectroscopic probes such as near and far UV CD, intrinsic and ANS fluorescence. Fig. 15 shows the far and near UV CD spectrum of hexokinase A in the presence of varying concentrations of ethylene glycol.

The amount of the secondary as well as the tertiary structure increases with increasing concentration of EG. The far UV CD spectrum shows the absence of isodichroic point, which indicates that the formation of structure is non-two state process. It may be pointed out that EG does not induce cooperative thermal transition of the protein even at high concentrations when studied by DSC (data not shown). The near UV CD spectrum clearly shows the increase in magnitude of negative ellipticity at 278 nm with increasing EG concentration. However, there is no enhancement of the positive ellipticity at 292 nm as seen in the case of the native state. The intrinsic fluorescence data (Fig. 19) is in agreement with the far UV CD data. The emission intensity increases linearly with increasing concentration of EG without any blue shift in emission maximum. The pH 2.5 state shows emission maximum at 340 nm, whereas the native protein has emission maximum at 330 nm. The increase in intensity at 340 nm in the presence of various EG concentrations is also small whereas ANS fluorescence shows substantial decrease relative to the pH 2.5 state (Fig. 23A). It is known that neither the native nor the completely unfolded protein should show ANS fluorescence. From our data it is not very clear whether EG is stabilizing or destabilizing the tertiary interactions in the pH 2.5 state of the protein, whereas it is clearly increasing the secondary structure content in a non-two state manner.

Fig. 16 shows the far UV CD spectra of hexokinase A in the presence of glycerol at pH 2.5. Glycerol also increases the secondary structure content of hexokinase A as evident from the dependence of MRE values at 222 nm plotted in the inset of Fig. 16A. In contrast to the data for EG, the spectra in the presence of glycerol at pH 2.5 state clearly show the presence of an isodichroic point at 206 nm suggesting that this process is a two state. Near UV CD shows substantial increase in the negative and positive ellipticities with increasing glycerol concentration (Fig. 16B). Intrinsic fluorescence results also show enhancement of emission intensity as well as a blue shift in the emission maximum wavelength toward 330 nm (Fig. 20A), suggesting that at high concentrations of glycerol, the tryptophans are getting buried in the hydrophobic environment. Interestingly, the emission intensity plotted against glycerol concentration shows a sigmoidal transition unlike that for EG, suggesting cooperative structural changes. ANS fluorescence shows a decrease with increasing glycerol concentration (Fig. 23B), showing approximately 45% intensity relative to the pH 2.5 state at 7.5 M concentration, where as in Fig. 20B the transition appears to be completed at 7.5 M glycerol. From the above data it is clear that the ensemble undergoing transition is far from the native state structure.

Similar to the action of glycerol, sorbitol also enhances secondary and tertiary structure as evidenced by data in Fig. 17. At high concentrations (3.0 M) the near UV CD

spectrum is very close to that for the native state at pH 5.0 suggesting that the native secondary as well as tertiary structure content is almost attained. The far UV CD spectra also clearly show isodichroic point at 206 nm, suggesting a two-state process. The intrinsic fluorescence spectra also show enhancement of emission intensity at 330 nm as well as a blue shift of the emission maximum to 330 nm (Fig. 21A). The fluorescence emission spectra also show isobestic point at 360 nm, which is not apparent in the presence of glycerol. The emission intensity at 330 nm plotted against sorbitol concentration clearly demonstrates a sigmoidal pattern, where the transition reaches nearly completion at around 2.5 M. From the ANS fluorescence data in the presence of sorbitol, even at 3.0 M sorbitol substantial ANS binding can be seen suggesting the presence of exposed hydrophobic patches even at high sorbitol concentrations. Though the near UV CD spectrum in the presence of 3.0 M sorbitol is similar to that of the native state, intrinsic fluorescence shows only about 50% recovery relative to the native state. This could probably be due to solvent quenching, or due to the presence of exposed hydrophobic regions as shown by ANS fluorescence (Fig. 23C).

The fluorescence intensity of hexokinase A in the presence high concentrations (7.5 M) of glycerol is much higher and close to that of the native state as compared to that in sorbitol at 3.0 M. It appears that there are two related phenomena occurring simultaneously, one the compaction of the molten globule during which the chromophores are getting buried resulting in a blue shift in emission maximum and second, the rearrangements of tertiary interactions around the chromophores. The difference in the effects of glycerol and sorbitol suggests a differing mechanism of their action.

Figs. 18 A, B, 22 A, B and 23D presents the data on the effect of substrate glucose on the pH 2.5 state of hexokinase A. At low concentrations, glucose is almost ineffective in inducing structural changes in the enzyme. However, at high concentrations it promotes the formation of structure to similar extent as by other linear polyols. This is evident from data generated using various structural probes in the study.

Glucose and sorbitol are both 6 carbon compounds, while the former is cyclic the latter is linear. It is known that cyclic compounds are more effective in stabilizing the structure of proteins (Back et al., 1979). 2.0 M glucose induced the formation of secondary structure comparable to that of the native state, whereas 3.0 M sorbitol concentration was required to achieve nearly the same amount of secondary structure (Figs. 17,18). Since glucose is a substrate of hexokinase, apart from solvent-mediated effects by which sugars and linear polyols are expected to act, glucose is expected to cause specific changes in and around the active site of the protein owing to its binding. It is not clear at the moment why the magnitude of negative ellipticity observed in the near UV CD spectra (Fig. 18B) is more than native state

(pH 5.0) at high concentrations of glucose, whereas there is not much change in the positive ellipticity. Binding of glucose causes large conformational changes in the native protein leading to the closure of the cleft between the domains as seen in the X-ray structure of glucose bound hexokinase A (TA Steitz, personal communication). This would, thus, change tertiary interactions and hence the nature of the near UV CD spectrum. From Fig. 22B it can be seen that glucose-induced structural transition is comparable to that in sorbitol. However, the transition is completed at 1.5 M in the case of glucose whereas for sorbitol this concentration is nearly 2.5 M (Fig. 21B). Interestingly, ANS intensity did not show any change up to 1.5 M glucose. Above this concentration there was a steep fall in the intensity of ANS fluorescence compared to any other cosolvent used. The contrasting behavior of hexokinase in the presence of glucose at high concentrations as observed by near UV CD and ANS fluorescence suggests that the glucose bound form might have different conformation from the unbound form of the protein in the presence of other polyols.

DISCUSSION
Molten globule and thermal denaturation

In the previous chapter we have shown that at pH 2.5 yeast hexokinase A exists as an intermediate with about 62% of native helicity as shown by far UV CD and largely disrupted tertiary interactions. The emission maximum for the native protein is 330 nm and for the unfolded protein in 8.0 M urea is 353 nm, whereas at pH 2.5 the emission maximum shifts to 340 nm suggesting that the tryptophans are partially solvent exposed. These results together with ANS fluorescence data suggest the existence of a molten globule like intermediate in the pH 2.5 state of hexokinase A.

Molten globule state of various proteins is considered to consist of a wide range of structures varying from relatively disordered to highly ordered ones (Ptitsyn, 1995; Fink *et al.*, 1998; Kamiyama, 1999). Molten globule states of some proteins show cooperative transition upon heating (Arai and Kuwajima, 2000) while for others no transition is observed. To qualify for being a true thermodynamic state, a macroscopic system has to show first order phase transition, i.e., it has to show discontinuity in the first derivatives of the thermodynamic potential such as enthalpy, entropy, and volume, etc. while a second order phase transition shows difference in the second derivatives such as heat capacity and compressibility, etc. (Privalov, 1996). Calorimetry is the direct method to confirm the presence of first order phase transitions (Gittis *et al.*, 1993). There have been mixed observations about the cooperative nature of the molten globule (Gittis, 1993; Ptitsyn and Uversky, 1994; Privalov, 1996; Pande and Rokhsar, 1998; Pfeil, 1998). Thorough investigation of the conformational stability of the

molten globule state by Griko (2000) has shown that the molten globule is stabilized by negative entropy and enthalpy of hydration over the positive conformational entropy and enthalpy of internal interactions. DSC melting of yeast hexokinase A at pH 2.5 (Fig. 1) did not show any enthalpy change and the excess heat capacity was observed to be a continuous function of temperature with a positive slope, suggesting the gradual exposure of non polar groups to the solvent upon heating (Spolar *et al.*, 1992; Makhatadze and Privalov, 1995).

Molten globule states have been found to show cold denaturation supporting the view that they are stabilized by considerable hydrophobic interactions (Nishii *et al.*, 1994; Lassalle *et al*, 2003).

The melting of the secondary structure of the pH 2.5 state of hexokinase A monitored by CD at 222 nm also does not show any cooperative transition, whereas unfolding by urea shows a cooperative transition, which appears to be slightly biphasic possibly due the different stabilities of the two domains in the protein. The superimposibility of the transitions monitored by CD at 222 nm and ANS fluorescence suggests that the overall transition could be approximated by two two-state transitions. Yeast hexokinase A has three helices in the small domain while the large domain is rich in α-helices in the native state. Thus, the apparently biphasic nature observed in urea unfolding at pH 2.5 could be indicative of differences in the domain stabilities.

The above observations raise the question that whether the molten globule represents a universal thermodynamic state and whether its unfolding could be approximated by a two state transition (Privalov, 1996). The results obtained by us suggest that yeast hexokinase A at pH 2.5 does appear to have residual tertiary interactions, partially formed hydrophobic core and an overall topology reminiscent of the native protein. Native-like tertiary fold, i.e., spatial organization of secondary structure elements (α-helices and β-strands) has been observed in α-lactalbumin (Peng and Kim, 1994) and interluekin-4 (Redfield *et al.*, 1994). Mizuguchi *et al.*, (2000) studied the long range interactions in molten globule state of α-lactalbumin by constructing chimeric domains between bovine and human lactalbumins, and observed long range interactions between residues 1-34 and 86-123 of human lactalbumin, and the apparent cooperativity was closely associated to the stability of the molten globule.

In the presence of polyols, except EG, yeast hexokinase A at pH 2.5 shows an endotherm in DSC experiment and the minimum concentration required for the same was about 27% w/v for linear polyols and 1.0 M for glucose and trehalose. The $\Delta H_{cal}/\Delta H_v$ ratio is less than unity and approaches unity with increasing cosolvent concentration suggesting that low calorimetric enthalpy could be due to the existence of a mixed population. The addition of polyols could overcome the repulsive charge-charge interactions and converting the non-

cooperative molten globule form to the cooperative folded state. The near and far UV CD and intrinsic fluorescence data suggest that the cooperative folded state is more native like. Though near UV CD shows almost native like spectrum in the presence of 6.0 M glycerol or 3.0 M sorbitol but under these conditions intrinsic fluorescence spectrum is far from the native state and shows binding to the ANS. These results, put together, suggest that the final state observed might be devoid of some tertiary interactions.

DSC scans especially at lower polyol and sugar concentrations, reveal a small and broad peak immediately after the first transition. This type of less cooperative transition has also been observed for the MG states of SNase mutant in the presence of salt (Carra et al., 1994). Due to low enthalpy and absence of corresponding cooperative changes in the CD and UV melting curves, this transition may be arising due to changes in the hydration of non-polar groups or melting of the residual structure. It appears that cosolvents stabilize the molten globule state and convert it to a more native-like cooperativly folded intermediate state.

Diversity in the effect of polyols

Systematic studies carried out on amino acid solubilities, and preferential solvent interactions of proteins and their thermal stabilities in aqueous polyol solutions have demonstrated that the main driving force for protein stabilization by polyols is by strengthening of the hydrophobic interactions (Gekko, 1981; Gekko and Timasheff, 1981a, b; Kataoka et al., 1995) and the transfer free energy of most of the non-polar amino acid side chains from water to aqueous polyols is positive, the magnitude of which increases with an increase in concentration and the number of OH groups in the polyol (Gekko, 1981). Polyols have been found to stabilize proteins by preferential hydration mechanism (Xie and Timasheff, 1997a, b). They have also been observed to induce molten globule state for the acid unfolded state of proteins (Davis-searles et al., 1998; Kamiyama et al., 1999). Polyols are likely to exert negligible effects on electrostatic interactions since the dielectric constant of water is not altered much in the presence of even high polyol concentrations (Akerlof, 1932). In the present study, all polyols except EG have been stabilizing (and inducing structure) the molten globule state and leading to a cooperative transition in the DSC measurements. This suggests that these neutral osmolytes can overcome the unfavorable electrostatic interactions that are present in the proteins at low pH and salt concentration. EG is known to be a destabilizer of native state and the free energy of transfer values of non-polar amino acid side chains are negative for the transfer to aqueous solutions of ethylene glycol (Nozaki and Tanford, 1965; Back et al., 1979; Davis-Searles et al., 1998). The large positive value for the transfer free energy of peptide group from water to polyols (Gekko, 1982) suggests that

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increase in secondary structure content could be attributed to strengthening of peptide-peptide hydrogen bonds. The T_m values of several proteins observed in the presence of polyols are linearly dependent on polyol concentration for a given cosolvent, thus suggesting the additivity of the thermodynamic mechanism (Gekko and Koga, 1983).

Molten globule like states have 50% increase in volume and exposed non-polar surface area compared to the native state (Ptitsyn, 1995). This situation would be unfavorable in the presence of polyols and would drive the equilibrium towards native like state with diminished protein-solvent interface. Far UV CD is the indicative of the residual structure; in the presence of sorbitol CD melting curves at 222 nm show more helicity than pH 2.5 state, both before and after transition. Xie and Timasheff (1997a) have observed greater preferential hydration of RNase A in sorbitol in the temperature denatured state compared to the native state. The denatured state being thus energetically unfavorable, helps in pushing the structure towards native state. It has also been suggested that compaction during protein folding leads to secondary structure formation (Dill, 1990; Chan and Dill, 1991; Yee et al., 1994). Polyols have been known to decrease the partial specific volume and adiabatic compressibility of several native proteins, suggesting that they become more compact in the presence of these cosolvents (Priev et al., 1996; Almagor et al., 1998). EG, which is known to be a destabilizer (Back et al., 1979) also induces the formation of secondary structure (Fig. 15A) and an increase in the fluorescence intensity with out blue shift of the emission maximum (Fig 19A) suggesting that there is strengthening of secondary structure but the tertiary structure is still loose. Comparison of spectroscopic data of conformational changes induced by EG and other polyols with thermal denaturation in the presence of cosolvents show a positive correlation of appearance of thermal transition and blue shift of emission maximum and positive ellipticity at 292 nm. CD melting curves at 222 nm show distinguishable differences between the effects of sorbitol and glycerol. While sorbitol increases the compactness of both the native and the denatured states, which is evident from the higher ellipticity values at 222 nm glycerol has negligible effect on the denatured state. Sorbitol, xylitol and glucose have been found to increase the surface tension of water (Kaushik and Bhat, 1998), while glycerol has been observed to decrease the surface tension of water. Glycerol interacts favorably with the protein surface polar groups and gets repelled from the exposed non-polar surfaces. In order to keep the non-polar surface minimum, it favors the formation of a compact native state by what is known as the solvophobic mechanism (Gekko and Timasheff, 1981a, b). Since molten globule like states have different extents of their surface exposed to the solvent relative to either native or denatured states, glycerol is expected to interact differently with the denatured state compared to the molten globule state because of high density of non-polar clusters on its

surface. Thermal denaturation in the presence of glycerol at pH 2.5 shows high enthalpy and low T_m (Fig. 9) compared to all other polyols used, which could be due to the presence of less residual structure as observed by CD melting curves at 222 nm compared to sorbitol (Fig. 10). On the other hand, glucose being substrate of hexokinase is effective even at lower concentrations of 1.0 M and results in a cooperative thermal transition of the protein. The stabilization by glucose appears to be originating both from specific binding effect as well as general solvent mediated effect.

There is an absence of sigmoidal transition for the thermal denaturation of secondary structure at low cosolvent concentrations (1.5 M sorbitol, 4.0 M glycerol) monitored by CD at 222 nm (Fig. 10) while under the same conditions (even at 3.0 M for glycerol) tertiary structure breakdown shows a cooperative transition monitored by UV-absorbance (Fig. 13). This suggests that different regions in the protein respond differently to temperature, while some interactions melt cooperatively, others probably undergo a second order phase transition. Similar trend has been observed with lysozyme dissolved in 99% glycerol, where the breakdown of tertiary interactions takes place in a cooperative manner while the secondary structure does not show any transition (Knubovets et al., 1999). In contrast to the absence of sigmoidal transition at 1.5 M sorbitol, increasing the concentration of sorbitol to 2.5 M results in the appearance of sigmoidal transition (Fig. 10) suggesting that at sufficiently high cosolvent concentration, constituent structural elements are interacting and producing single cooperative unit. It appears that with increasing cosolvent concentration, there is an increase in the concentration of protein molecules that unfold cooperatively with extensive heat absorption and a decrease in the concentration of the protein molecules that unfold gradually without any excess heat absorption as well as an increase in intra molecular cooperativity (i.e. the appearance of transition corresponding to the secondary structure melting). The order of cosolvents in terms of increasing the T_m is: trehalose>glucose>sorbitol>xylitol >glycerol.

To sum up the results presented in this chapter, it has been observed that the unique conformation of proteins is the consequence of subtle balance of stabilizing and destabilizing interactions. Addition of stabilizing cosolvents alters this balance to favor the native state over the denatured state. For an intermediate state like the molten globule, this will be governed by the interactions of the amino acid side chains with the varying solvent conditions and would dependent on the nature of the cosolvent molecule as well as its concentration.

In the present work, we demonstrate the strategy of using cosolvents that are available in a series (destabilizing to stabilizing action), to study the conformational changes in proteins. Our results show the varying effects of polyols on the molten globule like intermediate state of yeast hexokinase A in which the lower member of the polyol, ethylene

glycol populates a non-native like intermediate state while all other cosolvents convert the non cooperative molten globule state to a cooperative intermediate which is structurally very close to the native state. This study also demonstrates the ability of polyols and sugars, except ethylene glycol to compact the hydrophobic core, the effect becoming stronger with increasing concentration and the number of OH groups in the polyol molecule. Unlike polyols, glucose stabilizes the molten globule, probably by binding to the active site in addition to exerting a common solvent-mediated stabilizing effect just as polyols.

Refolding of Yeast Hexokinase A

INTRODUCTION

The folding of proteins to its functional state, from the information encoded in its amino acid sequence, is a key feature of the conversion of genetic information into biological activity. In recent years studies on the refolding of small globular proteins, which represent two-state systems, have begun to provide insight into the fundamental nature of the complex folding process (Jackson and Fersht, 1991; Capaldi and Radford, 1998; Jackson, 1998; Kuhlman et al., 1998; Myers and Oas, 2001; Grantcharova et al., 2001). However, there is little information available about the development of higher levels of organization in large proteins, which are composed of well-defined structural domains. These proteins require specific interdomain interactions to maintain the native state. However, little is known about the roles of these interactions during each stage of folding (Parker et al., 1996). Also, refolding of large proteins is complicated by greater propensities of side reactions such as aggregation of the partly folded intermediates (Jaenicke, 1999; Goldberg et al., 1991), cis-trans isomerization of prolines (Kamen and Woody, 2002a) and the reactivity of the thiol groups (Horowitz and Hua, 1995; Estape et al., 1998). Proper refolding of such systems requires helper protein molecules known as chaperones (Ellis, 2001; Hartl and Hayer, 2002). It would, thus, be of interest to study the folding of a protein comprising of a few domains, under conditions where folding is reversible and aggregation is minimal. Hexokinase A from yeast could provide an ideal system for such a study.

Thermal denaturation of yeast hexokinase A has been found to be a partially to completely irreversible process depending on the pH. The pI of yeast hexokinase A is 5.1 and close to this pH thermal denaturation results in visible precipitation. At pH 8.5, thermal denaturation has been shown to be merely reversible when the solution is heated just a little above the completion of the thermal transition (Tiwari, 1999). As mentioned in previous chapters, yeast hexokinase A is an α/β protein, consisting of two unequal sized domains. The active site cleft lies between the two domains and the kinetics of its organization, therefore, can provide information about the process by which the two domains dock to form the native structure.

For any protein refolding study, reversibility is an obligatory prerequisite that allows for a mechanistic interpretation of the folding kinetics. In order to make folding studies feasible it is, therefore, necessary to optimize the conditions for spontaneous refolding. The present study focuses on optimizing the conditions for refolding of hexokinase A from either GdmCl, urea or an acid denatured state which represents a range from the completely unfolded to the partially unfolded molten globule like state. Under optimum reversible conditions, we have made an attempt for an initial characterization of the kinetic aspects of

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refolding. The folding kinetics of hexokinase A has been monitored by catalytic activity, Intrinsic and ANS fluorescence.

RESULTS

The fluorescence emission of native and unfolded hexokinase A was studied by simultaneous excitation of tryptophan and tyrosine fluorescence at 280 nm. Fluorescence of hexokinase A is dominated by tryptophan with emission λ_{max} of 330 nm, which is characteristic of apolar environment of the tryptophan residues. Unfolding of hexokinase A by urea and GdmCl is accompanied by a drastic reduction in the fluorescence intensity at 330 nm as well as a shift in λ_{max} to 353 nm, reflecting the exposure of tryptophans to polar environment. Acid denaturation at pH 2.5 led to the formation of a molten globule like intermediate, with a λ_{max} of 340 nm and with a considerable amount of native secondary structure as evident from the far UV CD data (Figs.1a, b).
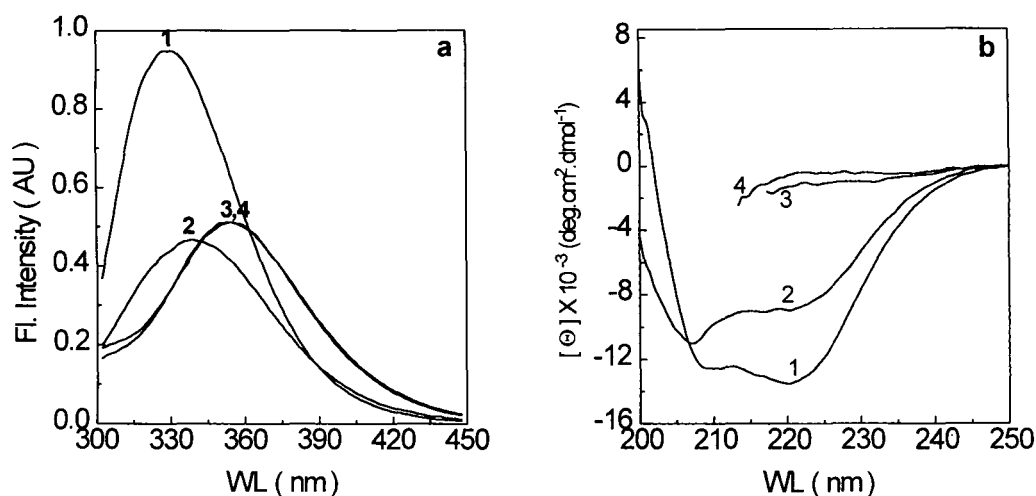

Fig. 1. a: Fluorescence emission spectra of hexokinase A excited at 280 nm at 18 °C. Native (pH 7.8) (1); acid denatured (pH 2.5) (2); in 8.0 M urea (3), and 6.0 M GdmCl (4). b: Far-UV CD spectra of hexokinase A. Native (pH 5.0) (1); acid denatured (pH 2.5) (2); in 8 M urea (3), and 6.0 M GdmCl (4).

Fig. 2 shows the equilibrium transition curve for GdmCl-induced denaturation monitored by fluorescence emission intensity at 330 nm. From the plot it appears that the transition is biphasic in nature, which could be due to the differential stability of the two domains in the protein as evidenced by the calorimetric transition as well (Chapter 3). The GdmCl-induced transition curve is also similar to the thermal denaturation monitored by intrinsic fluorescence (Fig. 2; Chapter 3), which suggests that the drastic reduction in the emission intensity at low GdmCl concentration could be due to the opening up of the smaller domain, which has three out of the four tryptophans buried inside. The second transition

appearing between 1.0 and 3.0 M GdmCl could be due to the unfolding of the larger domain that has only one partially exposed tryptophan. The smaller domain appears to be highly sensitive to very low concentrations of GdmCl. Due to the absence of a clear pretransition baseline the 3-state fitting was poor, and hence we were able to determine the transition zone only. From the transition curve it is apparent that hexokinase A is completely unfolded above 3.0 M GdmCl.
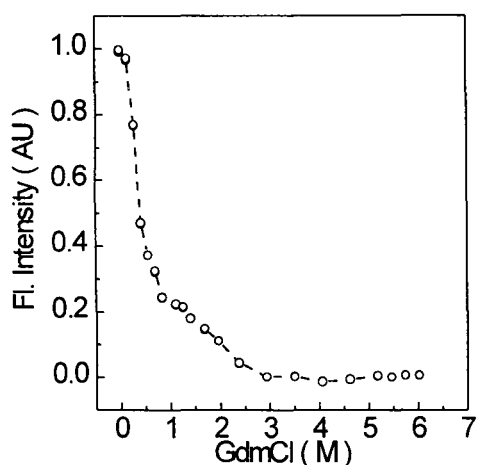
Fig. 2: GdmCl denaturation curve of hexokinase A monitored by intrinsic fluorescence at 20°C. The protein solution was excited at 280 nm and the emission intensity at 330 nm was monitored as a function of GdmCl concentration in 100 mM Tris, pH 7.8. Protein concentration was 20 µg/ml.

Fig. 3 shows the refolding of hexokinase A, denatured by 6.0 M GdmCl, 8.0 M urea, and acid (pH 2.5) as a function of time carried out by manual mixing with the refolding buffer. The results demonstrate that the cysteins have to be in the reduced form for the successful refolding of the protein. In the absence of the reducing agent dithioerythritol (DTE), denaturation by 6.0 M GdmCl or 8.0 M urea or acidic pH resulted in smaller refolding yields, more so with the case of GdmCl and urea denatured states; the order of refolding yields being: acid (pH 2.5)>8.0 M urea>6.0 M GdmCl. The presence of the reducing agent, both during denaturation and refolding resulted in greater than 80% reversibility for all the denaturants used. From these results it appears that keeping the cysteins in the reduced form during refolding is a prerequisite for spontaneous refolding of hexokinase A. Hexokinase A has four cysteins but no disulphide bond and in the absence of a reducing agent, the cysteins can get paired to form non-native disulphide bonds leading to a reduction in the biological activity. The pKa of cystein is 9.1 in model peptides (Fersht, 1998) suggesting that at pH 2.5 the thiols would be well protonated. The higher yields obtained for the acid denatured protein even in the absence of DTE is, therefore, due to the cysteins being protonated at the low pH. This also suggests that oxidation of cysteins is taking place in the denatured state rather than during the initial collapse and subsequent rearrangement of the protein structure. If disulphide bond formation predominantly occurrs during refolding, then the acid denatured protein would result in more or less similar refolding yields as obtained from the 8.0 M urea

denatured protein. The addition of DTE further increases the refolding yield to about 85-90% suggesting that in its absence the lower yields are likely to arise due to some oxidation reaction of the thiol groups either in unfolded state or during refolding leading to the non-native structures. The final refolding yields obtained in the absence of the reducing agent from 8.0 M urea denatured protein is about 30% compared to 15% from the 6.0 M GdmCl denatured protein. This difference could be due to the masking of charges on the ionized cystein by GdmCl as it is a salt, thus favoring the disulphide bond formation (Creighton, 1992). The presence of reducing agent not only increases the refolding yields but also accelerates the folding rate by about 7 times compared to the situation where it is absent (Table 1).
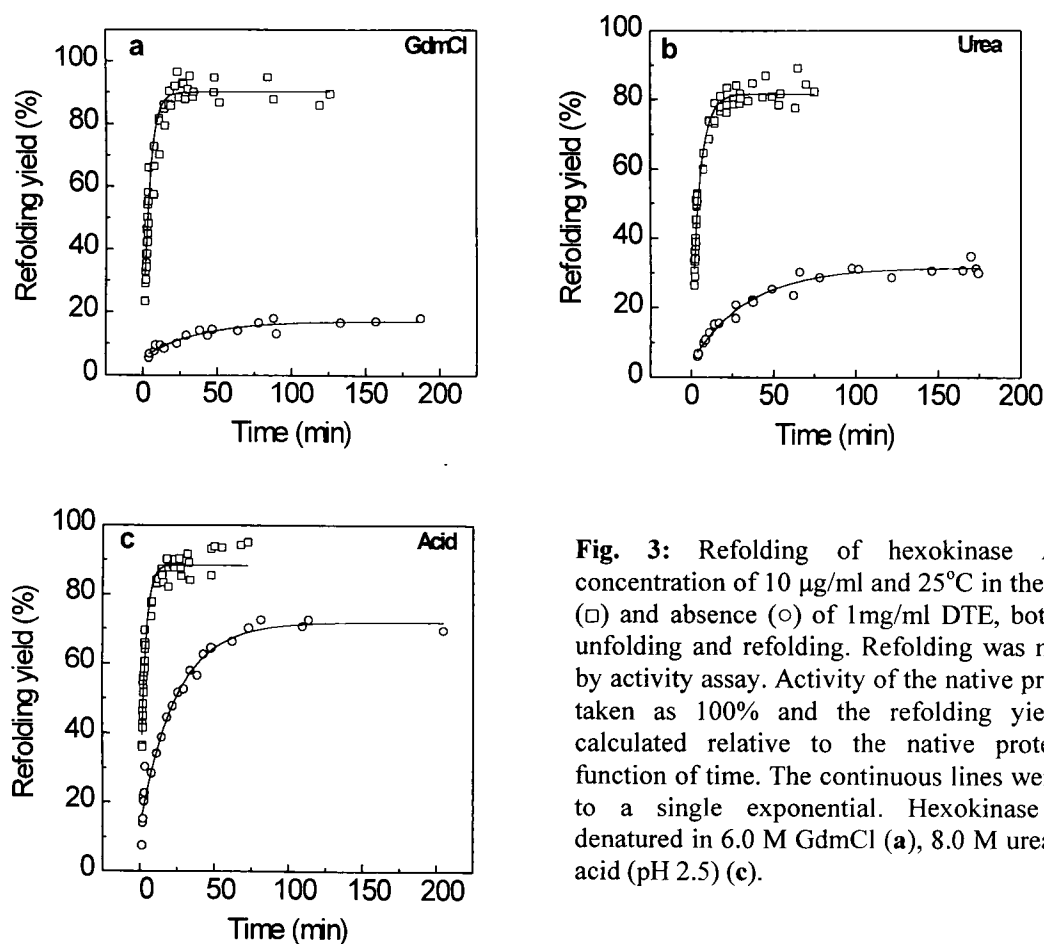

Fig. 3: Refolding of hexokinase A at a concentration of 10 µg/ml and 25°C in the presence (□) and absence (○) of 1mg/ml DTE, both during unfolding and refolding. Refolding was monitored by activity assay. Activity of the native protein was taken as 100% and the refolding yields were calculated relative to the native protein as a function of time. The continuous lines were best fit to a single exponential. Hexokinase A was denatured in 6.0 M GdmCl (a), 8.0 M urea (b), and acid (pH 2.5) (c).

Table 1: Apparent rate constants and amplitudes of hexokinase A folding from urea, GdmCl and acid denatured state in the absence (-) or presence (+) of 1mg/ml DTE at 25°C. Refolding kinetics were monitored by the recovery of activity as a function of time.

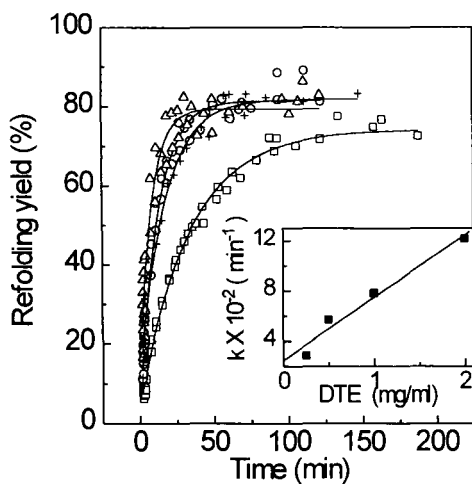| Denaturant	k (min⁻¹)	Amplitude
Urea (+)	0.193±0.016	68.0±4.0
Urea (-)	0.029±0.007	27.18±4.0
GdmCl (+)	0.198±0.023	80.5±5.0
GdmCl (-)	0.026±0.008	11.78±4.5
Acid (+)	0.313±0.034	84.8±4.0
Acid (-)	0.041±0.005	60.0±3.0

Fig. 4: Refolding kinetics of hexokinase A at 10 μg/ml as a function of the concentration of DTE included only in the refolding buffer: 0.25(□), 0.5(+), 1.0(o), and 2.0(Δ) mg/ml. Refolding of the 6.0 M GdmCl, 100 mM Tris, pH 7.8 denatured protein was monitored by activity assay at 25 °C. Insert shows dependence of the rate constant on DTE concentration.
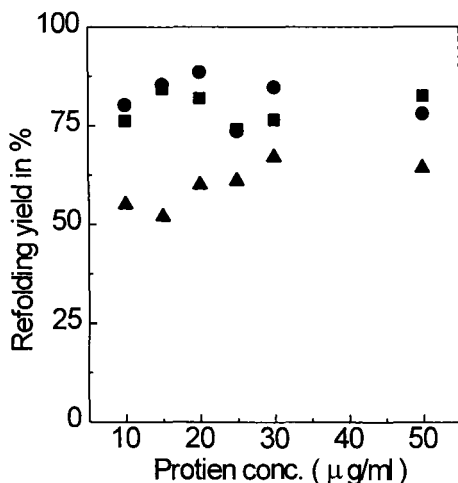

Fig. 5: Refolding of hexokinase A as a function of protein concentration. The protein was denatured either by 8.0 M urea (■, ●) or 6.0 M GdmCl (▲). The residual concentration of the denaturant in the refolding buffer was 0.4 M for urea and 0.3 M for GdmCl. The protein was refolded and incubated at 25 °C for 2 hours (●, ▲) or 5 hours (■) and assayed for activity and the refolding yield was calculated relative to that of the native state.

Fig. 4 shows the progress curves for refolding as a function of the concentration of DTE, included only in the refolding buffer. The results suggest that DTE is capable of improving the refolding yields, even though the protein was denatured in the absence of DTE indicating the possible accessibility of the cross linked regions to the reducing agent during refolding. In comparison to the results presented in Figs. 3a-c, wherein the reducing agent was included both during unfolding and subsequent refolding, inclusion of the reducing agent (DTE) only in the refolding buffer shows about <5% fast folding species within the dead time of manual mixing (about 1.0 min). A refolding yield of about 20% for urea and GdmCl denatured protein and 35% for acid (pH 2.5) denatured protein has been obtained during the dead time of manual mixing of 1.0 min, when the reducing agent was included both during denaturation as well as during refolding (Figs. 3a-c). These results demonstrate the dual effect of DTE on enhancing the refolding yield as well as the rate. Within the concentration range used, the refolding rates are linearly dependent on the DTE concentration (Fig. 4 inset). Since these experiments have been carried out using activity assay as a probe to monitor the formation of the native state, and some of the cysteins are in proximity to the active site, the refolding rates obtained may not truly represent the conformational refolding rates. This

presumption is based on the work of Otieno *et al.*, (1977) who have shown that the derivatization of a single thiol group present at the active site of enzyme leads to the loss of its activity. Nonetheless, the results obtained by us suggest that disulphide bond formation is the major obstacle for spontaneous refolding of hexokinase A the formation of which needs to be avoided.

One of the reasons for the irreversibility or low refolding yields of many large proteins is the aggregation during refolding. During refolding the partially refolded species that have exposed hydrophobic patches can interact noncovalently in an intermolecular fashion resulting in the formation of aggregated or misfolded non-native species (Chiti *et al.*, 2002). In such cases the refolding yield decreases with increasing protein concentration. Fig. 5 shows the refolding yield dependence on the protein concentration during refolding for 8.0 M urea and 6.0 M GdmCl denatured protein at an identical residual concentration of respective denaturant. The absence of any trend of a decrease in the refolding yield with increasing protein concentration at least up to 50 μg/ml suggests that aggregation is not a prominent factor for refolding under the conditions used. However, refolding at higher protein concentrations needs to be carried out to rule it out completely. It was not possible to carry out refolding at higher protein concentrations due to technical reason since there would be much higher residual concentration of the denaturant during refolding which is likely to reduce the refolding yields due to their destabilizing action at higher concentration. The residual concentration of urea and GdmCl in these experiments was 0.4 M and 0.3 M, respectively during refolding. GdmCl being more potent denaturant than urea, results in lower refolding yields compared to the urea denatured protein at the above residual denaturant concentration.

Refolding kinetics of hexokinase A monitored by intrinsic as well as ANS fluorescence at 20°C has been shown in Fig. 6. The experimental kinetic data for intrinsic fluorescence fits well to a single exponential fit with about 50% missing amplitude within the mixing dead time of about 10sec. The fluorescence intensity of the native state has been observed to decrease slightly with time, while there is no change for the denatured state. The intrinsic fluorescence intensity value for the refolded protein matches with that of the native state in about 40 min after refolding. Yeast hexokinase A is known to lose activity at alkaline pH at very low protein concentration (Williams and Jones, 1976). The observed decrease in intensity could thus be related to its inactivation as result of perturbed tryptophan environment. These changes are very small relative to the changes observed for refolding. The enhanced fluorescence of ANS when it binds to exposed hydrophobic regions of partially folded protein molecule has been used extensively to detect intermediates in protein folding (Ptitsyn *et al.*, 1990; Semisotnov *et al.*, 1991). The time dependent desorption of ANS during

the folding of hexokinase A was measured by inclusion of the dye in the refolding buffer (Fig. 6, inset). During the dead time of the experiment, a large increase in fluorescence enhancement was observed relative to that in the presence of the fully unfolded protein suggesting that a molten globule like intermediate with exposed non-polar surface is formed during the dead time of mixing. The kinetic trace for the refolding monitored by intrinsic fluorescence fits well to a single exponential with a rate constant of 0.20 min^{-1}, whereas a sum of two exponential fit is required for ANS fluorescence data yielding rate constants of 1.203 min^{-1} and 0.107 min^{-1}. None of the rate constants obtained for ANS fluorescence match with those observed for intrinsic fluorescence. Fitting to single exponential yields similar rate constants for both the intrinsic as well as the ANS fluorescence monitored refolding. Yeast hexokinase A has three tryptophans buried in the small domain, and one partially exposed tryptophan in the large domain. Thus, most of the intrinsic fluorescence signal should arise from the folding of the small domain, whereas ANS fluorescence being global in nature indicates the differential desorption in different regions of the protein while the protein is refolding.
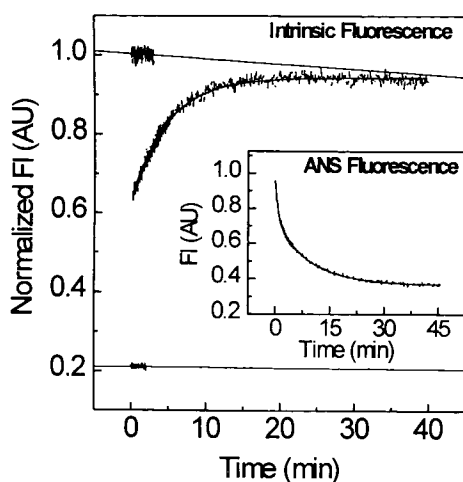

Fig. 6: Fluorescence detected refolding of hexokinase A. The protein was denatured by 6.0 M GdmCl containing 1mg/ml DTE and refolded in 100 mM Tris pH 7.8 containing 1mg/ml DTE at 20°C. The protein concentration was 1mg/ml during denaturation and 20 μg/ml during refolding. For intrinsic fluorescence, protein was excited at 280 nm and the emission intensity at 330 nm was monitored as a function of time. The upper baseline is for the native protein and the lower baseline for the 6.0 M GdmCl denatured protein. For ANS fluorescence (inset), excitation wavelength of 400 nm was used while the emission was monitored at 480 nm. The data were fit to a single phase exponential for intrinsic fluorescence and a sum of two exponentials for the ANS fluorescence monitored refolding.

Interpretation of ANS binding results is complicated by the uncertainty in the nature of the binding interactions as well as the possibility of perturbations of the folding pathway by the hydrophobic nature of the probe molecule. By binding to a non-native state, the stabilization of such structures may be enhanced artificially by the presence of ANS (Miranker and Dobson, 1996). Nonetheless, the slow refolding observed by intrinsic fluorescence and the ability of refolding species to bind to ANS suggests that the unfolded

protein initially collapses rapidly to an intermediate state and that the rearrangement of the intermediate to attain the native state is the rate limiting step. This is quite evident from the slow phase kinetics observed during protein folding (Fig. 6).
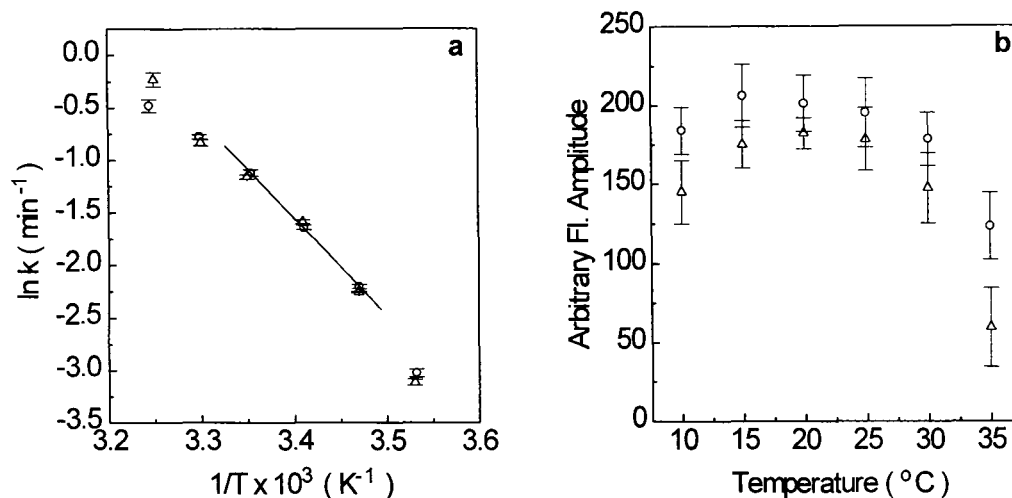

Fig. 7: Dependence of ln k (a) and arbitrary fluorescence amplitudes (b) on the refolding temperature. The protein was either denatured by 6.0 M GdmCl (Δ) or acid (pH 2.5) (o). The refolding rate constants were obtained by single exponential fit to the intrinsic fluorescence refolding kinetic data.

The slow kinetic phase observed for many proteins during folding has been shown to be the result of cis-trans isomerization of the X-Pro peptide bonds (Brandts et al., 1975; Kiefhaber et al., 1990; Wedemeyer et al., 2002). In model peptides, this isomerization occurs with a relaxation time of 10-100 sec near room temperature (Brandts et al., 1975). In the unfolded proteins the trans isomer of proline is more favorable than the cis, but in a folded protein structural constraints can result in the stabilization of the cis isomer. The equilibrium for the isomerization occurs relatively slowly due to the high energetic barrier of about 20 kcal/mol. The activation energy for the isomerization can be calculated from the dependence of ln k on temperature where k is the rate constant for the isomerization reaction. Fig. 7a shows the temperature dependence of ln k for refolding of the protein from GdmCl and acid denatured (pH 2.5) states. Interestingly, both follow the same kinetics with activation energy of 18.2 kcal/mol suggesting that the observed slow phase could arise from proline isomerization. The fluorescence detected amplitudes were observed to decrease with increasing temperature (Fig. 7b) but the relative amplitudes with reference to the native state at that temperature remain the same. The Arrhenius plot (Fig. 7a) appears to be non-linear. Many proteins show non-linear Arrhenius kinetics wherein the non-linearity has been attributed to the heat capacity difference between the unfolded state and the transition state for the folding reaction (Segawa and Sugihara, 1984). For more complex reactions, the stability

of metastable intermediates decreases at high temperatures leading to the non-linear behavior (Creighton, 1993). The downward curvature of Arrhenius plots can be caused by the change in the rate-limiting step (Oliveberg *et al.*, 1995). For simplicity, the linear portion of the data in Fig. 7a has been considered for the calculation of the activation energy.

DISCUSSION

As a first step toward elucidating the folding pathway the conditions for the complete reversibility have been optimized. Sensitive probes like the activity assay have been used to monitor the native state formation from the GdmCl, urea, and acid (pH 2.5) denatured protein. It has been clearly shown that the presence of the reducing agent DTE during folding is necessary for increasing both the refolding yield as well as the refolding rate, suggesting that the formation of disulphide bonds is the major obstacle for complete reversibility. Williams and Jones (1976) have observed that hexokinase A rapidly loses activity at 35 °C in two kinetic phases and that the presence of a reducing agent prevents the inactivation of the second kinetic phase suggesting the hyper reactive nature of cysteins. Hyper reactive cysteins were also observed in human basic fibroblast growth factor (hFGF-2) and rhodanese (Estape *et al.*, 1998; Horowitz and Hua, 1995). Non-reducing SDS-PAGE results obtained by us shows that disulphide bond formation is predominantly intramolecular rather than intermolecular (data not shown).

Yeast hexokinase A has four cysteins located in the large domain, all being in the reduced form in the native protein. However, crystal structure shows that none of these 4 cysteins are in sufficiently close proximity to form a disulphide bond in the native state. Cys 268, Cys 398, and Cys 404 are 10-12 A° apart and lie close to the interdomain region. Domain movements and increased flexibility of this region in the presence of a denaturant might be resulting in disulphide bond formation under in-vitro conditions of alkaline pH and an oxidizing environment.

An understanding of the protein folding in terms of the description of the events that occur during folding require a combination of several experimental techniques. By comparing the time resolved activity of acylphosphatase, Chiti *et al.*, (1999) detected an intermediate, which was spectroscopically silent. In the present case, such a comparison (Fig. 8) reveals that the rate of regain of activity is slower (k (activity): 0.23 ± 0.05 min^{-1} and k (Trp fluorescence): 0.30 ± 0.02 min^{-1}) than that of fluorescence signal and the amplitude is also about 1/3 (20% for activity and 60% for Trp fluorescence) that of the spectroscopic signal after 1.0 min of initiation of refolding. The non-coincidence of the two signals suggests that the protein is undergoing some conformational changes before attaining the catalytically active native state,

which is otherwise indistinguishable by fluorescence. This could be explained from the knowledge of the distribution of tryptophans with three out of the four tryptophans in hexokinase A are localized in the small domain while the large domain has only one partially exposed tryptophan. The suspected conformational changes might involve either rearrangements in the large domain whose fluorescence should be weak or the docking of the two domains in the right orientation.
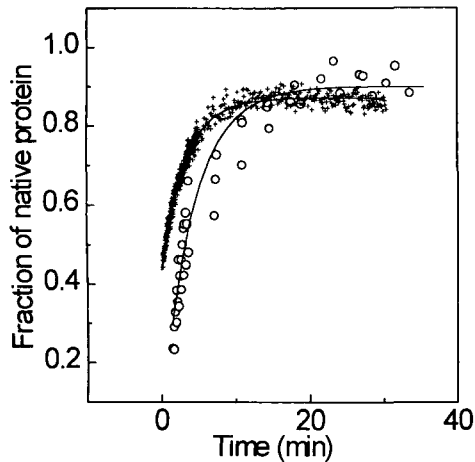

Fig. 8: Comparison of the refolding kinetics of hexokinase A monitored by activity assay (o) and by tryptophan fluorescence (+) carried out under identical refolding conditions except for protein concentration. The protein concentration during refolding was 20 µg/ml for tryptophan fluorescence and 10 µg/ml for catalytic activity based refolding. The protein was denatured by 6.0 M GdmCl and refolded in the presence of 1mg/ml DTE in 100 mM Tris, pH 7.8 at 25°C.

Interestingly, refolding from either the U↔N or I_E (acid denatured) ↔N (Fig. 7) follows similar kinetics suggesting that the observed kinetic phase is a late event during folding and possibly involves structural rearrangements to form the correct tertiary interactions. This can be understood in the framework of the hierarchic protein folding model as described by Arai and Kuwajima (2000) according to which in the first stage of folding the protein partially folds into a compact but structurally diverse state, while the specific tertiary interactions occur in the second stage. The large time constants (of the order of mins) observed in the present case suggest that the formation of the specific tertiary interactions during the I_E↔N transition involve a kinetic trap and that a large free energy barrier has to be overcome to attain the native state.

The sequence and structural analysis of yeast hexokinase A has revealed that it has 23 prolines, all of them being in the trans form. The calculated activation energy of 18.2 kcal/mol suggests that the slow folding phase could arise due to cis-trans isomerization of the prolines. However, preliminary studies carried out using double jump (interrupted unfolding) folding (N→U→N) experiment wherein the native protein was unfolded for varied periods of time prior to being refolded, did not show any change in the fluorescence amplitude of the slow phase when the denaturation time was varied between 30 sec to 1 hour at 1°C (data not

shown). If cis-trans isomerization is the rate-limiting step for refolding then refolding amplitudes of the slow phase increase exponentially with the time spent in the denatured state (Kiefhaber, 1995). Another test to identify whether the slow phase is due to cis-trans isomerization of prolines is by using the cyclophilin (peptidyl-prolyl isomerase, PPI) catalyzed refolding.
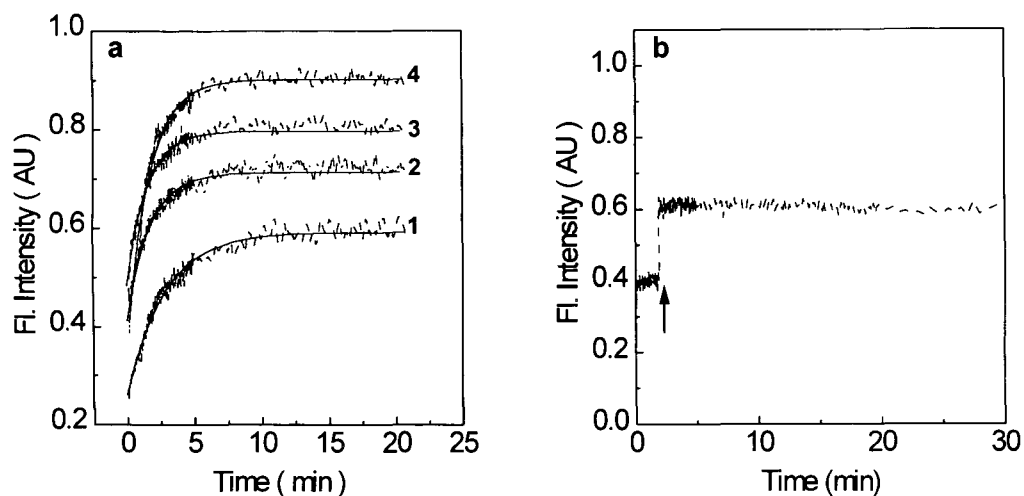

Fig 9: Peptydyl prolyl isomerase (PPI) catalyzed refolding of hexokinase A. a: Hexokinase A was refolded in the presence of 1:0 (1), 1:0.225 (2), 1:0.337 (3), and 1:0.450 (4) molar ratio of protein and PPI. Data fit to single exponential yielded the rate constants (min^{-1}) of 0.35±0.02 (1), 0.55±0.03 (2), 0.59±0.04 (3), and 0.60±0.04 (4). b: Enhancement of the emission intensity of native hexokinase A in the presence of 1:0.225 molar ratio of hexokinase A and PPI. The arrow indicates the time point of PPI addition.

The major limitation of this approach is the accessibility of the protein backbone to the active site of PPI (Eyles and Gierasch, 2000). Our results show nearly doubling of the refolding rate at 1:0.1 molar ratio of protein and PPI compared to the control without PPI (Fig. 9a). Further increase in the PPI concentration up to 1:0.4 molar ratio of the protein and PPI do not show any enhancement in the refolding rate. But surprisingly, the emission intensities at the end of the folding reaction increase linearly with increase in the concentration of PPI (exceeding the emission intensity value for the native protein), although the magnitude of the amplitudes remain the same suggesting that the increase in the final emission intensity was taking place by increase in the magnitude of fast folding species within the mixing dead time. Interestingly, the addition of PPI to the native state at 1: 0.1 molar ratio resulted in a significant enhancement of the fluorescence emission intensity (Fig. 9b). This suggests that some X-Pro peptide bonds are in equilibrium between the cis and the trans forms in the native state itself and that the addition of PPI catalyses this isomerization. During unassisted refolding also, the final folded state must be equilibrating between cis and trans forms, which was evident from equivalent emission intensity values for the native and the refolded protein

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at sufficiently long time, when most of the protein is refolded (Fig. 6). This result also suggests that cis-trans isomerization is observed in the native state which is probably getting accelerated during unfolding. This could be the reason why a characteristic exponential pattern for refolding amplitudes of the slow kinetic phase is absent in the double jump experiments i.e., cis-trans isomerization rate constants and the unfolding rate constants probably have similar values or both the processes are coupled.

Prolyl isomerization has been shown to act as a regulatory switch between different protein conformations (Biernat *et al.*, 1992; Schutkowski *et al.*, 1998). In some proteins, both the isomers can be tolerated in the native state, giving rise to an alternative native state (Reimer and Fischer, 2002). This native state heterogeneity caused by X-Pro peptide bond has been shown to have biological function (Reimer and Fischer, 2002; Stukenberg and Kirschner, 2002). Recently Pappenberger *et al.*, (2003) have unraveled the kinetic mechanism of native state prolyl isomerization reaction in tendamistat. They have shown that the alternative native states differ by 9.5 kJ/mol in free energies and their unfolding rates differ by 10 times. Unlike tendamistat, in which the native state prolyl isomerization is spectroscopically silent, this isomerization in hexokinase A can be monitored by fluorescence, and hexokinase A could prove to be a good model for further studies in this direction to understand the functional significance of native state cis-trans isomerization of prolines.

In conclusion, in our preliminary work on the refolding of hexokinase A, we have succeeded in optimizing conditions for refolding and have carried out refolding kinetic studies from GdmCl, urea and pH 2.5 denatured states. Yeast hexokinase A is a large protein containing 468 residues and its folding in vitro seems to be complicated by the presence of cysteins and a large number of prolines. Keeping cysteins in the reduced form is essential for efficient folding. Further rigorous experiments are required to unravel the complexity of hexokinase A refolding. A reasonable approach would be to use protein engineering to eliminate cysteins and selectively studying the domain folding by introducing or eliminating the fluorophores. These studies in conjunction with stopped-flow techniques would throw further light on the early folding events of hexokinase A.

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