Effect of solvent environment on stability, folding and aggregation of Human γD Crystallin

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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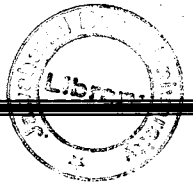

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CERTIFICATE

This is to certify that the present work entitled '**Effect of solvent environment on stability, folding and aggregation of Human γD Crystallin**' submitted to Jawaharlal Nehru University, New Delhi, in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy, embodies original research work carried out in the School of Biotechnology, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full, for any other degree or diploma of any other university.

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Dedicated to

my parents

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Saurabh Singh

Symbols and Abbreviations

kcal/ mol	kilo calories per mole
Asn	asparagine
Ala	alanine
kJ/mol	kilo joules per mole
Δ	change in a property
G	free energy change
G^0	free energy under standard temperature and pressure conditions
G_{tr}	free energy of transfer
H_2O	water
D_2O	deuterated water
N	native
D	denatured
R	gas constant
T	temperature in Kelvin
K	equilibrium constant
P	pressure
x	cosolvent
C_x	concentration of x
S	entropy
H	enthalpy
V	volume
β_T	derivative of volume with respect to pressure
α^*	derivative of volume with respect to temperature
C_p	specific heat capacity
T_m	temperature representing mid point of thermal denaturation transition
F	folded
U	unfolded
pI	isoelectric point
Ka	ionisation constant
FRET	Forster's resonance energy transfer
Φ	phi values
rhG-CSF	recobinant human- colony stimulating factor
w	water
μ	chemical potential
p	protein
c	activity coefficient
Cpx	preferential hydration parameter of protein
SAS	solvent accessible surface area
α	fractional exposure of an amino acid residue in protein
K_m	Michaelis-Menten constant
k_{cat}	catalytic constant
DHFR	dihydrofolate redutase

kDa	kilo Dalton
PDB	Protein Data Bank
TB	Terrific Broth
LB	Luria-Bertani Broth
O. D.	optical density
IPTG	isopropyl thio-galactoside
M	Molar
m	milli
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel elctrophoresis
FPLC	fast performance liquid chromatography
SP	sulphho-propyl
n	nano
a.u.	arbitrary units
R.F.I.	relative fluorescence intensity
GdmCl	guanidinium chloride
CD	circular dichroism
DTE	dithio erythritol
^0C	degree celcius
λ	wavelength
exci	excitation
emi	emission
SAXS	small angle X-ray scattering
PEG	polyethylene glycol
TMAO	trimethylamine oxide
DMG	dimethyl glycine
EG	ethylene glycol
%	percent
Cm	concentration of a chemical denaturant representing mid point of equlibrium protein denaturation curve
bp	base pairs
g	grams
μ	micro
dd	double distilled
TE	Tris-EDTA
DTT	dithiothreitol
lb	pounds
PMSF	Phenyl methyl sulphonyl fluoride
w/v	weight by volume
v/v	volume by volume
r.pm.	revolutions per minute
UV	ultra vilolet
Θ	ellipticity
M_0	mean residue weight of the protein
lit	liter
min.	minutes

hrs	hours
PBS	phosphate buffered saline
PCI	phenol:chloroform:isoamyl alcohol
DNase	deoxyribonuclease
RNase	ribonuclease

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Chapter 1

Introduction

Introduction

Proteins are major biomacromolecules necessary for carrying out various physiological functions. They are the polypeptides with characteristic sequence of amino acid residues which fold to acquire a unique three dimensional conformation to perform a given function. For proteins to be physiologically functional they must be properly folded to attain this unique conformation called native conformation which must be stable enough in the given condition to discriminate it thermodynamically from other possible conformations. The kinetically controlled former process is called protein folding and the latter is referred as protein stability. Both protein folding and stability is due to an interplay of weak non-covalent interactions to first bring the unfolded polypeptide chain to folded native conformation and then to maintain it's nativity in variable conditions in order for the protein to remain functional. A slight alteration in these weak interactions may lead to the unfolded polypeptide getting misfolded to a non native conformation. On the other hand, altered interactions may not interfere with critical interactions for protein folding and instead might only interfere with interactions necessary to keep protein kinetically stable. Thus the folded protein though is able to attain its almost native structure but is not stable enough and is prone to undesirable changes like truncation or aggregation.

Forces determining protein stability

Free energy of stabilization of globular proteins in solution is only 5 to 10 kcal/mol which is equivalent to only a few weak intermolecular interactions (Dill, 1990). Thus, the folded conformations are only marginally stable than unfolded, biologically inactive conformations under physiological conditions. This small net conformational stability is the result of much larger contributions from several important forces. The major destabilizing force is the conformational entropy (Pace, 1990). The entropy of an unfolded protein is large because rotation around the bonds in the polypeptide backbone and the side chains is less restricted than in a folded protein. Other major contributing forces are hydrogen bonding and the hydrophobic effect.

a) Hydrogen bonds

Hydrogen bonds form between an electronegative atom with a covalently bound hydrogen, DH (the donor), and another electronegative atom, A (the acceptor). When DH and A form a hydrogen bond, their electronic structure is perturbed very little compared to the changes that accompany covalent bond formation. In proteins, the electronegative atoms of most importance are 0 and N; the hydrogen bond of most importance is that between the amide hydrogen and the carbonyl oxygen in a peptide group. These hydrogen bonds make up 68% of the hydrogen bonds that occur in globular proteins (Stickle et al., 1992) art from it there also exist backbone-side-chain, side-chain-backbone, and side-chain-side-chain donor-acceptor contributions. Out of this side-chain-side-chain hydrogen bonding is believed to play a major role in stabilizing proteins. (Ragone, 2001). The driving force in folding was initially thought to be this intramolecular hydrogen bonding (Pauling et al., 1951), then the hydrophobic effect (Kauzmann, 1959). In recent times, it has been argued that intramolecular hydrogen bonding is destabilizing (Dill, 1990; Honig & Yang, 1995), partially stabilizing and destabilizing (Honig, 1999) and once again, an important driving force (Takano et al., 2003; Pace et al., 2004). During the past two decades, the advent of protein engineering brought a hope that the newfound ability to introduce site directed mutants at will would provide ready answers to such unresolved questions (Oxender and Fox, 1987). The goal of studying hydrogen bonding mutants is to learn how much stability is gained when a polar group that is hydrogen-bonded to water in the unfolded state is dehydrated to form a specific intramolecular hydrogen bond in the folded state. The most common approach has been to replace a side chain involved in a hydrogen bond, such as Asn, with a side chain that cannot form a hydrogen bond, such as Ala (Serrano et al., 1992; Shirley et al., 1992; Green et al., 1992). These studies point out that a folded protein loses stability by burying polar groups but gains it back by forming hydrogen bonds. However, despite thousands of mutational experiments, disagreement about the energetic role of hydrogen bonding still remains.

Studies investigating influence of hydrogen bonds on packing density suggested that hydrogen bonds may contribute to protein stability, in part, by increasing packing density in the protein interior, and thereby increasing the contribution of van der Waals

interactions to protein stability (Schell et al., 2006). Molecular dynamics simulation studies dealing with thermodynamics of hydrogen bond breaking and formation in solutions of alcohol show that free energy of hydrogen bond formation is essentially independent of the environment (around 5 kJ/mol), suggesting that buried hydrogen bonds (e.g., in proteins) do not contribute significantly to protein stability (Spoel et al., 2006). On the other hand studies exploring the contribution of hydrogen bond reveal that H-bond strength varies from one protein to another stabilizing in one and destabising in the other and presumably at different sites within the same protein (Shi et al., 2002). These studies suggest that contribution that polar groups make to protein stability depends strongly on their environment (Takano et al., 2003).

b) Electrostatic interactions

Specific interactions in proteins are largely electrostatic and are important in protein folding, stability, flexibility, and function. Different conformations adopted by protein in varying conditions can have electrostatic interactions contributing favorably or nonfavorably (Kumar & Nussinov, 2002; Matousek et al., 2007). Electrostatic interactions in the Denatured states can include specific non-native interactions that can even persist in the transition state for protein folding. These electrostatic interactions can be energetically significant and their modulation either by mutation or by varying solution conditions can have a major impact upon protein stability (Cho et al., 2008). There exist both close-range electrostatic interactions (salt bridges) and the long range electrostatic interactions in proteins. Salt bridges are formed by spatially proximal pairs of oppositely charged residues in native protein structures. A single salt bridge can contribute up to 3–5 kcal/mol to the free energy of protein folding (Anderson et al., 1990). Often salt-bridging residues are also close in the protein sequence and fall in the same secondary structural element. Salt bridges are rarely found across protein parts which are joined by flexible hinges, a fact suggesting that salt bridges constrain flexibility and motion. Recent computational and experimental evidence shows that salt bridges can be stabilizing (Permyakov et al., 2005; Ibarra-Molero et al., 2004) or destabilizing (Hendsch and Tidor 1994). Salt bridges are also reported to effect folding kinetics in a context dependent way (Ibarra-Molero et al., 2004). Structural and thermodynamic

comparisons of thermophilic and mesophilic proteins indicate that salt bridges contribute to reduced heat capacity change of unfolding (Lee et al., 2005). They can thus contribute significantly towards the thermophilic-mesophilic protein stability differential (Kumar & Nussinov, 2002; Nakamura, 1996) as salt bridge networks could accommodate stochastically the disorder of increased thermal motion to produce thermal stability (Missimer et al., 2007).

Long range electrostatic interactions can be both repulsive and attractive in nature also play important role in determining protein stability (Pace et al., 2000; Grimsley et al., 1999). Favorable long-range electrostatic interactions when present in denatured state lead to compaction of unfolded polypeptide chain thus reducing the net contribution of electrostatic interactions to protein stability (Funahashi et al., 2003) and may bring down the overall stability of the protein owing to the changes in the long-range electrostatic interactions. Long range interactions may also be sometimes insignificant in determining protein stability (Sun et al., 1991) while can be very critical in determining the protein dynamics (Fadrna et al., 2005) and meanwhile can also influence protein-protein interactions (Ramirez-Carrozzi & Kerppola, 2001).

c) Hydrophobic effect

The burial of hydrophobic residues is considered to be the major driving force for protein folding and stability (Dill, 1990; Rose et al., 1985; Nakai et al., 1988; Shortle & Meeker, 1986; Kellis et al., 1988, 1989; Alber and Matthews, 1987; Yutani et al., 1987; Matsumura et al., 1988; Shortle et al., 1990; Sandberg & Terwilliger, 1991). First one is to calculate the hydrophobicity scale of individual residues based on their transfer free energy from water to organic solvents like n-octanol (Herrmann et al., 1995), N-methylacetamide (Roseman, 1988) and cyclohexane (Lomize et al., 2002) assuming the solvent to mimic the folded protein's interior. Studies with octanol provide reasonable transfer free energy ΔGtr values for the peptide groups and amino acid side chains to estimate the contribution of the groups buried in folding to protein stability. The other method uses site-directed mutagenesis as a tool to investigate the forces that stabilize proteins focused on the hydrophobic effect. The approach has been to replace one hydrophobic side chain with another and measure $\Delta(\Delta G)$. To minimize the contribution

of steric strain, mutants are created to have a smaller side chain replacing the larger one. Many such studies on proteins like tryptophan synthase (Yutani et al., 1987), T4 lysozyme, barnase (Golovonov et al., 2000), staphyloccocal nuclease (Chen et al., 2004), gene 5 protein from bacteriophage Φ (Zhang et al., 1996) and chymotrypsin inhibitor 2 (Ahmed et al., 2008) have been reported. Other studies deal with core packing and strain to assess the effect of adding larger hydrophobic residues to the interior of globular proteins (Lim et al., 1994). $\Delta(\Delta G)$ values for hydrophobic mutants increase with the amount of nonpolar surface area buried, as expected. In order to compare the hydrophobic substitutions at the same accessibility the measured $\Delta(\Delta G)$ values have been divided by the fraction buried for the side chain in the wild-type protein so that the $\Delta(\Delta G)$ values are compared at the same accessibility, namely, 100% buried. In reality burial doesn't happen to be 100% and cavities are formed due to non sufficient van der Walls interactions to fill the cavity formed completely which leads to an overestimated $\Delta(\Delta G)$ values (Yamada et al., 1994) by ~0.11 kcal/mol (Ratnaparkhi & Varadarajan, 2000) Moreover recent studies on protein stability in H_2O and D_2O (Efimova et al., 2007) establishes the importance of hydrophobic effect as D_2O is a poorer solvent for non polar amino acids than H_2O implying that larger and efficient packing of the hydrophobic core in D_2O leads to enhanced stability.

Thermodynamics of protein stability

The conformational stability of a protein is defined as the free energy change, ΔG, for the reaction folded to unfolded under physiological conditions. The equilibrium between the two states can be represented as

$$N \leftrightarrow D$$

and conformational stability of a protein can be described as

$$\Delta G = \Delta G_D - \Delta G_N = -RT \ln K = -RT \ln [D]/[N]$$

where [D] and [N] represent the concentrations of denatured (D) and native (N) states, ΔG_D and ΔG_N represent the free energies of D and N, and K and ΔG are the equilibrium constant and standard free energy change, respectively. The folding–denaturing transition in proteins is a highly cooperative process. In certain cases, as a rule for smaller proteins, it suffices to describe this transition within a 2-state approach involving the native state N and the denatured state D, only.

Since there are multiple perturbation modes to induce the transition, ΔG is a multidimensional function of all the parameters which can independently be varied in an experiment, e.g. temperature T, pressure P, cosolvent x, concentration of cosolvent Cx, pH, etc.:

$$\Delta G = \Delta G^0 + f\,(T, P, Cx, pH, \ldots)$$

ΔG^0 depends on the reference state only, hence, is, for a transformation under fixed conditions, a constant. The function f contains all the system parameters which characterize the denaturing transition of a protein in a given solvent, e.g. the changes in entropy, volume compressibility, specific heat, thermal expansion, etc. Keeping all solvent associated parameters constant and considering variation in P and T only, since the transition is characterized by a latent heat, it is of 1st order, and, consequently, is governed by the Clausius–Clapeyron equation

$$dP/dT = \Delta S/\Delta V$$

ΔS and ΔV are the entropy and volume changes associated with the transition. Both quantities depend on the actual pressure P and temperature T where the transition takes place. The boundaries of the stability phase diagram, i.e. the area in a pressure–temperature plane where the protein is stable in its native state, can then be determined from a solution of this equation. The first derivatives of the volume with respect to pressure and temperature, namely

$$\beta_T = (\delta V/\delta P)_T \text{ and } \alpha^* = (\delta V/\delta T)_P,$$

the absolute changes of the volume with pressure and temperature, are closely related to the compressibility and the thermal expansion of the protein, respectively. Likewise, the derivatives of the entropy with respect to pressure and temperature are associated with the thermal expansion and with the specific heat capacity $Cp=T(\delta S/\delta T)_P$, respectively. These are system parameters which are assumed to be roughly independent on pressure and temperature and therefore δS and δV depend only linearly on T and P, and, hence, the equation can easily be integrated. The result is a general 2nd order curve in P and T whose shape may be elliptic, parabolic or hyperbolic:

$$aP^2 + bT^2 + 2cPT + 2f\,P + 2gT + const = 0$$

thus derived coefficients a, b, c, etc. are related to the changes of the system parameters along the transition (Scharnagl et al., 2005), namely to $\Delta\beta_T$, $\Delta\alpha$, ΔC_P, and to ΔS_0 and ΔV_0, the entropy and volume change at the reference pressure and temperature (P0, T0).

Factors affecting protein stability

a) Effect of temperature

High temperature induces denaturing transition accompanying the conversion of structured protein to random coil form with solvation of hydrophobic amino acids. As a consequence, water solvating hydrophobic molecules forms locally ordered structures which have low entropy and low enthalpy due to well aligned hydrogen bonds in them (Kim et al., 2005;). As melting of these structures requires energy, temperature induced protein denaturation is accompanied by an increase in specific heat. Accordingly, the change of the specific heat, ΔC_P associated with the temperature induced transition

$$\Delta C_P = Cp_D - Cp_N,$$

is generally positive (Griko and Privalov, 1992). As long as ΔC_P remains positive the complete solvation of hydrophobic core of the protein interior does not happen due to still

remaining structure and therefore the difference in enthalpy, ΔH, between the native and the denatured state keep on increasing as the temperature is raised, according to

$$\Delta H\,(T) = H\,(T1) + \Delta C_P\,[T\text{-}T1]$$

Meanwhile the respective difference in entropy, ΔS, also increases, since the conformational ordering melts away with the increasing temperature. At some critical temperature $T = T_m$, the enthalpic term, ΔH, and the entropic term, -TΔS, cancel, rendering a free energy change ΔG of zero. At this temperature Tm, energetically more favorable denatured state the transition to the denatured state takes place because it is energetically more favorable (Trefethen et al., 2005; Schoeffler, 2004)

Protein stability is optimum at a certain temperature and increasing or decreasing from this temperature leads to reduced stability and thus like high temperature induced denaturation proteins re also subjected towards cold denaturation i.e. at lower temperatures. Lowering the temperature decreases the enthalpy term so that it eventually becomes negative and may compensate the entropy term, TΔS, which is positive due to decreasing entropy. The actual transition temperatures into the denatured state depend of course on pressure: High pressure at low temperature may destabilize the locally ordered structures (Cai et al., 2005) because it counteracts an optimum alignment of the hydrogen bonds. However, in the low pressure, high temperature regime, pressure may stabilize the respective structures to some extent (Marques et al., 2003)

b) Effect of pressure

It has been shown in numerous studies that hydrostatic pressure may lead to disruption of the intermolecular forces maintaining native protein structure, which is accompanied by a decrease in the volume of the protein–water system, and simultaneous unfolding. In part, this is explained by the fact that more water molecules can fill in the protein's void volumes when they become accessible to solvent upon dissociation. This results in a total volume contraction and is favoured under high pressure (Gross & Jaenicke 1994; Mozhaev et al., 1996; Silva et al., 1992; Royer 2002). Moreover, solvent-exposure of charged groups that have been involved in stabilization of protein assemblies

(as salt bridges) also contributes to the overall volume reduction through the so-called electrostriction effect, which consists in a tightly ordered arrangement of the solvent dipolar molecules around charged solutes. Another important, yet still debated (Boonyaratanakornkit et al., 2002), factor contributing to the pressure effect on polypeptide assemblies stems from the hydration of hydrophobic residues under high pressure (e.g. hydration volumes of model hydrocarbons such as benzene or methane are negative (Gross & Jaenicke 1994). Pressure denaturation studies provide a fundamental thermodynamic parameter for protein unfolding, the ΔV^{o}, in addition to being an alternative method for perturbing the folded state, and thus elucidating its stability. Denaturation of proteins is usually studied at atmospheric pressure using high temperature, guanidinium hydrochloride or urea as denaturants. Interpretation of the results obtained using such methods may be complicated by the following: (i) varying the temperature changes both the volume and the thermal energy of the system at the same time and (ii) the thermodynamic parameters of denaturation by guanidinium chloride or urea are influenced by the binding of these molecules to proteins. The use of pressure is also advantageous from the fact that it is a rather mild denaturing agent and from methodological points of view: the transition to native conditions (renaturation) is achieved simply by releasing the pressure. Furthermore, the effects of pressure on proteins are generally found to be reversible, and seldom are they accompanied by aggregation or changes in covalent structure.

Also with respect to the kinetics of the folding reaction, pressure studies are of particular use, as they allow to evaluate the volume profile during the folding process and to characterize the nature of the barrier to folding or unfolding and the corresponding transition state. Moreover, pressure studies present an important advantage due to the positive activation volume for folding, the result of which is to slow-down folding substantially, in turn allowing for relatively straightforward measurements of structural order parameters characteristic for folding intermediate states, which are difficult or even impossible to quantify on much faster time-scales corresponding to ambient pressure conditions.

$\Delta G=\Delta G_D-\Delta G_N$, the free energy change associated with protein denaturation, becomes lower as pressure is increased, at least above some threshold pressure. $\Delta G\ (T) =$

ΔG (T1) + ΔV [P-P1]. $\Delta V = V_D - V_N$ is the volume change in going from the native to the denatured state. As a rule, ΔV is negative because the structure of the native state has voids, for instance in the protein pockets, which are squeezed away in the denatured state so that its volume is smaller and, hence, the transition into the denatured state becomes favored under high pressure. In the low pressure, high temperature regime ΔV can also be positive. Increasing the pressure up to some critical level $P=P_0$, the protein may eventually cross the boundary $\Delta G=0$, and the transition to the denatured state takes place. The respective transition at low pressure is less straightforward to understand. First of all, we note that, in a large temperature range, the low pressure denaturation regime would require negative pressure, a condition which has, so far, not been realized experimentally. Accordingly, low pressure denaturation can experimentally be investigated in a rather limited temperature range only, for instance at high temperature. There is indeed a temperature range in which high pressure leads to a stabilization of the native state, and, consequently, low pressure to a destabilization associated with denaturation. High pressure protein stabilization takes place in a range where the change $\Delta\beta_T = -(\delta\Delta V / \Delta P)T$ is negative meaning that the denatured state is less compressed than the native state. If so, $\Delta V = V_D - V_N$ will be positive and, since higher pressure stabilizes the smaller volume, the native state is favored. The question, however, what determines the temperature–pressure range with a negative $\Delta\beta_T$, remains to be answered. At rather high pressure (i.e. outside this range) the denatured state is far from being a random coil state. It is plausible that unfolding to a random coil against high pressure is severely hindered. Instead, the high pressure denatured state is still kind of a globular state where the voids in the protein are squeezed to a high degree so that $V_D < V_N$. On the other hand, in the lower pressure range and at sufficiently high temperature, unfolding to a random coil like state is still possible. Accordingly, the protein acquires a larger surface and, concomitantly, a larger volume. In addition, compression is much harder than in the native state because the compressible voids have vanished and the hydration shell is harder to compress than bulk water due to the ordered structures induced by the hydrophobic amino acids.

c) Effect of pH

Variation in pH causes changes in ionization status of titratable groups altering electrostatic interaction. The pH dependence of the thermodynamic stability (ΔG (F-U)) of a protein arises as a consequence of differential pK(a) values between folded (F) and unfolded states(U). A significant component of the free energy difference between native and denatured states is due to a small number of amino acids whose pKas are shifted anomalously in the native protein (Pace et al., 1990; Yang and Honig, 1993; Yang et al., 1992, Hu et al., 1992). General shifts of side-chain pK_a values in unfolded states to lower than standard pK_a values (by 0.3–0.4 pH units on average) have also been predicted, pointing toward the presence of stabilizing electrostatic interactions within unfolded states of proteins under non-denaturing conditions (Elcock, 1999; Tan et al., 1995) Other than this changes in pH also changes the overall net charge on the protein. When proteins possess both positively and negatively charged groups (e.g., at pH values close to the pI), anisotropic charge distribution on the protein surface could give rise to dipoles. In such cases, protein–protein interactions could be highly attractive, making assembly processes such as aggregation energetically favorable. Thus pH plays an essential role in determining protein stability.

The unfolding free energy, ΔG_{FU}, can be calculated at any pH (Tollinger et al., 2003) as

$$\Delta G_{FU} = -RT \ln\left(\sum_{m=0}^{i}[UH_m] \Big/ \sum_{m=0}^{i}[FH_m]\right)$$

where $[UH_m]$ and $[FH_m]$ represent sums of concentrations corresponding to the binding of m protons to i binding sites for protons (ionizable groups) in the unfolded and folded states, respectively, R is the universal gas constant, and T is the temperature. ΔG_{FU} can be separated into a pH-independent term, ΔG^{0}_{FU} (representing non-electrostatic contributions to ΔG_{FU}, as well as electrostatic contributions at a pH where all ionizable sites i are protonated in both states), and terms related to protonation/deprotonation equilibria involving individual ionizable groups, $\Delta G^{pH}_{FU}(i)$ (representing the pH dependence of the

contribution of proton binding at site i to the overall ΔG_{FU}) as

$$\Delta G_{FU} = \Delta G^{0}_{FU} + \sum_{i} \Delta G^{pH}_{FU}(i).$$

Assuming an equilibrium between four species (folded and unfolded state, protonated, and deprotonated) at each site i, individual, pH-dependent values of $\Delta G^{pH}_{FU}(i)$ can be calculated for each group as

$$\Delta G^{pH}_{FU}(i) = -RT\, ln\left(\frac{[H^+]+K^U_a(i)}{[H^+]+K^F_a(i)}\right),$$

where $K^U_a(i)$ and $K^F_a(i)$ are the ionization constants for group i in the unfolded and folded state, respectively. The maximum of $\Delta G^{pH}_{FU}(i)$ between very high pH where both states are fully deprotonated and very low pH where both states are fully protonated is given by $\Delta G^{pH}_{FU,max}(i) = 2.303RT \cdot \{pK^U_a(i) - pK^F_a(i)\}$. Relative values of the pH dependence of the thermodynamic stability for a system containing multiple (i) ionizable groups can be calculated by summation as $\sum_i \Delta G^{pH}_{FU}(i)$. The calculations above provide a simple means to dissect the pH dependence of ΔG_{FU} into additive contributions due to individual ionizable groups.

Above calculations do not include conformational adaptations in response to changes in the ionization state which tend to complicate the calculations of changes in stability as a function of pH and therefore this kind of changes must be taken into account before proceeding with the calculations.

d) Effect of ionic strength

Ionic strength of the solutions determines protein stability by multiple mechanisms e.g., the Hofmeister effect (Toth et al., 2008; Zhou, 2005; Cacace et al., 1997), preferential hydration (Hong et al., 2004; Wright et al., 2002, Arakawa and Timasheff, 1984), electrostatic effects and weak ion binding (Ramos and Baldwin, 2002; Maldonado et al., 2002; Nishimura et al., 2001). Combination of all these effects complicates the interpretation of salt effects. Ions in some cases may stabilize proteins by

high affinity binding to specific sites. While the ligand induced stabilization is ion-specific and usually observed in the low salt concentration range, bulk ionic strength effects play a role in screening surface charge–charge interactions. Hofmeister effects, which occur at still higher salt concentrations, may strengthen the hydrophobic force by increasing the surface tension of the solvent, or stabilize peptide dipoles through specific ionic interactions. Recent results suggest that the efficiency of different salts to screen charge-charge interactions correlates with their denaturing strength and with the position of the constituent ions in the Hofmeister rankings (Perez-Jimenez et al., 2004). In the absence of site-specific ion binding, differential salt effects in reflect primarily differences in Coulombic interactions, e.g. between natural or designed variants of a protein. The Hofmeister effects that occur in parallel are usually insensitive to local changes in amino acid sequence. All these various mechanisms can affect the stability of both the native state and the unfolded state (Pradeep and Udgaonkar, 2004).

Charges can be shielded by counter ions which are present in solvent surrounding and thus ionic cosolvents can regulate the electrostatic interactions on the protein surface. But the effect by these ions are highly context dependent as surface charges which are involved in thermodynamically stabilizing the protein shielding of which can lead to destabilization. On the contrary shielding of surface charges which are repulsive to other surface charges in the closer vicinity can lead to further stabilization of the protein. Also at high temperatures dielectric constant of water decreases which gives higher impact to electrostatic interactions. Thus at higher temperatures, high solvent ionic strength acts against repulsive surface interactions helping in protein stability while further destabilize proteins if the surface charges are attractive and protein stabilizing like in salt bridges. Because pH determines the type, total, and distribution of charges in a protein, salt-binding effects may be strongly pH dependent. High ionic strength can also facilitate charge shielding of repulsive interactions at pH<<<pI and at pH>>>pI but would otherwise counter attractive salt bridging at pH=pI.

At low concentrations, the predominant effect of ions in solution results from charge shielding, which reduces electrostatic interactions. However, at high concentrations of certain salts, in addition to charge-shielding effects, preferential binding of ions to the protein surface can result in a decrease in thermodynamic stability of the

native conformation and an increase in equilibrium solubility (Mertz and Leikin, 2004; Arakawa and Timasheff, 1984). Other salts that are preferentially excluded from protein surface show stabilizing or salting-out effects (Zhou, 2005).

With the addition of salt, the electrostatic interactions will be shielded and the pKa values can show shift from their normal expected values (Lindman et al., 2007). These shifts together with other salt mediated effects can even account for concentration dependent differential and anomalous salt effects as the net effect on protein stability is determined by alteration in intensity of different protein-salt interactions (Spencer et al., 2005). Thus salts affect protein stability by modifying the ionic strength of the solution, which overall can be slightly stabilizing or destabilizing depending on the nature of the specific charge distribution within the protein.

e) Effect of solvent environment

Since the stability of proteins results from a large number of counteracting enthalpic and entropic contributions which favor the folded state as compared to the unfolded state only marginally, it is clear that changes in the interactions of the protein with the solvent, for instance by adding a cosolvent, may have a severe influence on protein stability. The nature and the magnitude of the individual contributions of the protein solvent interactions to the free energies G_D and G_N of the respective states of the protein (denatured, D or native, N) are highly dependent on the solvent environment. Since water is the environment in which proteins exist and operate, the structure and dynamics of the hydration water is directly linked to protein flexibility and stability (Cioni et al., 2005; Soares et al., 2003). Consequently, chemical denaturants and cosolvents which change the properties of the hydration water can readily alter the equilibrium between different conformational ensembles. Free energy of transfer of amino acids from vacuum to water varies from that from vacuum to a different solvent. Thus free energy of individual amino acids differs in varying solvent conditions and along changes the thermodynamic stability of proteins comprising these amino acid residues (Pace et al., 2004). Accordingly, changing the chemical potential of the solvent by changing the concentration of cosolvents and/or denaturants provides a valuable tool for probing protein stability and protein–water interaction. For example, the addition of

glycerol to a solution of a native state protein leads to changes in structure and dynamics as reflected, for instance, in changes of volumes and compressibilities (Almagor et al., 1998; Priev et al., 1996).

The mechanisms of the cosolvent–protein interaction include following possibilities: (1) direct contact interaction of cosolvent molecules with the protein; (2) indirect effects via the perturbation of the hydration layer; (3) combination of (1) and (2), a disruption of the water structure in the hydration shell, so that water molecules are released and enable a direct interaction of cosolvent molecules with protein groups. Thus compatible cosolvents are those which do not disturb protein functionality, while noncompatible cosolvents, e.g. denaturants like urea, guanidinium hydrochloride, alcohols, etc. tend to induce a disruption of protein structure. There can be another category of molecules which tend to compensate and counter action of non-compatible cosolutes and stabilize the folded form against denaturation under external stress (examples are sugars, polyols, monomeric amino acids and methylamines). These are the osmolytes which are able to stabilize cells in vivo against dehydrating stress (e.g. salinity) and/or volume changes by maintaining an osmotic equilibrium. Some of the cosolvents called chaotrops are also known for disordering the water structure while some others known as kosmpotrops are known to induce disorder in the water structure whereas the so-called kosmotrops induce order. Due to the close relation between the structure of water and protein stability the coordination of water molecules by cosolvent molecules does have a significant influence on protein stability.

Combined effect of all these factors of solvent interaction however is also dependent strongly on the surface topology of the protein and the structure of the hydration shell as well as on the concentration of the cosolvent.

Protein folding

The protein folding problem is widely recognized as a challenge to investigators engaged in biological research at molecular level for previous many decades (Basharov, 2003). It is formulated simply as either how does a polypeptide chain fold to the native protein, or how does the amino-acid sequence specify unique three dimensional structure of the protein. A number of phenomenological models is proposed for the mechanism of

protein folding, such as the framework (Lin and Chang, 2007; White et al., 2005, Santra et al., 2004), diffusion-collision (Zhang et al., 2005; Myers and Oas, 2001), nucleated collapse (Djikaev, 2007; Kuo et al., 2005; Guo and Thirumalai et al., 1997; Dagget et al., 1996; Moult and Unger, 1991), hierarchic (Compiani et al., 2004; Tiana et al., 2003; Chakraborthy and Peng, 2000), and so on models (Zhou and Karplus, 1999;). Different theories and several course grained (Lu and Liu, 2008; Cecconi at al., 2008; Cho et al., 2008) and lattice models (Wang and Klimov, 2008; Cellmer et al., 2005, Skolnick and Kolinski, 1991) have been proposed based on the considerations of statistical physics and mathematical statistics to describe protein folding process.

The protein folding problem is three different problems: the folding code—the thermodynamic question of how a native structure results from the interatomic forces acting on an amino acid sequence; protein structure prediction — the computational problem of how to predict the native structure of a protein from its amino acid sequence and folding speed (Levinthal's paradox) - the kinetic question of how a protein can fold so fast.

The hydrophobic interaction was believed to be a dominant component to the folding code that the folding code is distributed both locally and non-locally in the sequence, and that native secondary structures are more a consequence than a cause of folding forces. Much of the current trends in deciphering the folding code include use of purely physics-based methods, without knowledge derived from databases (such as statistical energy functions or secondary structure predictors), to explore native structures and folding processes. Advantages of these physics based approaches would be the ability to predict conformational changes, such as induced fit, a common and important unsolved problem in computational drug discovery; the ability to understand protein mechanisms, motions, folding processes, conformational transitions etc. However physics-based methods are currently limited by some inaccuracies in the force-fields and by huge computational requirements. Continuous experimental supplementation therefore is needed to upgrade these theoretical inaccuracies in order to improve predictions. The question of folding mechanism has driven major advances in folding experiments. Key advances include ability to measure folding events on timescales faster than a few milliseconds and to monitor individual chain monomers during folding. All these

developments were complemented by fast laser temperature-jump methods (Nguyen et al., 2003); mutational methods that give quantities called Φ values, which can identify those amino acids that control the folding speed; FRET methods that can watch the formation of particular contacts (Haas, 2005, Deniz et al., 2000); hydrogen exchange methods that see structural folding events (Zhou et al., 2006; Maity et al., 2004, Gorski et al., 2004); and extensive studies on model proteins, including cytochrome c, chymotrypsin inhibitor 2, barnase, apomyoglobin, src, fyn SH3 domains, proteins L and G, WW domains etc. These studies have led to the revelation that protein folding speeds, which vary over more than eight orders of magnitude, correlate with the topology of the native protein. Fast folders have mostly local structures like helices and tight turns, whereas slow folders, though not always, usually have more non-local structure, such as β sheets (Silva et al., 2005; Shea et al., 2002).

Protein destabilization, misfolding and aggregation

Interest in the problem of protein destabilization or misfolding and aggregation has increased in recent years due to sharp rise in the number and volume of therapeutic proteins produced commercially and the recognition of the central role of protein aggregates in degenerative diseases.

Nonnative protein aggregation describes the assembly from initially native, folded proteins of aggregates containing nonnative protein structures (Chi et al., 2003). Aggregation is often irreversible. Protein aggregation behaviors such as onset, aggregation rate, and the final morphology of the aggregated state (i.e., amorphous precipitates or fibrils) have been found to depend strongly on the properties of a protein's solution environment, such as temperature, pH, salt type, salt concentration, cosolutes, preservatives, and surfactants (Campioni et al., 2008; Nielsen et al., 2007; Sasahara et al., 2007; Chi et al., 2003; Manno et al., 2004) as well as the relative intrinsic thermodynamic stability of the native state (Galka et al., 2008; Scharnagl et al., 2005; Hill et al., 2005, Minton, 2000).

The reaction order for the rate-limiting step determines the apparent order of the aggregation reaction (Chi et al., 2003). A number of proteins have been found to follow first order aggregation kinetics (Golub et al., 2008; Hoiberg-Nielsen et al., 2006; Weijers,

2003), suggesting that the rate-limiting step is unimolecular (e.g., a conformational change) but some of the molecules like rhG-CSF show bimolecular reaction following a second-order reaction limited by collision frequency which shows that protein conformation alone cannot explain the aggregation behaviors. The aggregation transition states of some proteins have been identified as a structurally expanded species within the protein native state ensemble (Clark, 2005; Chi et al., 2003).

Protein molecules also assemble to form higher order aggregates. Molecular assembly processes occur as a result of attractive intermolecular interactions (Chi et al., 2003). Thus, an understanding of protein aggregation also requires information about the nature and magnitude of these interactions. The osmotic second virial coefficient (B22) is a thermodynamic solution parameter that directly quantifies overall protein– protein interactions on the molecular level. Positive B22 values indicate the overall dominance of repulsive forces between protein molecules, where protein–solvent interactions are favored over protein– protein interactions. Negative B22 values reflect overall attractive forces between proteins, with protein–protein interactions being favored over protein—solvent interactions (Sinibaldi et al., 2008; Winzor et al., 2007). Morphology of the solid phases formed are predominantly determined by the mechanisms of molecular approach, reorientation, and incorporation of native proteins, which are governed by the strength and range of protein colloidal interactions (Hoyer et al., 2004). Protein stability and aggregation is therefore controlled by both conformational stability and colloidal stability, and, depending on the solution conditions, either could be rate limiting. To successfully stabilize protein against aggregation, solution conditions need to be chosen not only to stabilize the protein native conformation but also to stabilize protein against attractive intermolecular forces.

Proteins are only marginally stable and are highly susceptible to both chemical and physical degradation. Chemical degradation refers to modifications involving covalent bonds, such as deamidation oxidation, and disulfide bond shuffling (Chi et al., 2003; Hau et al., 2002 Reubsaet et al., 1998). Physical degradation includes protein unfolding, undesirable adsorption to surfaces, and aggregation (Bolanos-Garcia, 2008 ; Schmitt et al., 2007). Nonnative aggregation is particularly problematic because it is encountered routinely during refolding, purification, sterilization, shipping, and storage

processes. Aggregation can occur even under solution conditions where the protein native state is highly thermodynamically favored and in the absence of stresses. Partially unfolded states however adopt a collapsed conformation that is more compact than the unfolded state and has substantial secondary structure and little tertiary structure, have large patches of contiguous surface hydrophobicity and are much more prone to aggregation therefore than both native and completely unfolded conformations (Fink, 1995). There exists an ensemble of native substates with a distribution of structural expansion and compaction. Kendrick et al. showed that the aggregation of rhIFN-γ proceeds through a transiently expanded conformational species within the native state ensemble (Kendrick et al., 1998).

Outstanding progress has been made in the development of therapeutic strategies targeting these diseases. Three promising approaches (Rochet, 2007) include: (1) inhibiting protein aggregation with peptides or small molecules identified via structure-based drug design or high-throughput screening; (2) interfering with post-translational modifications that stimulate protein misfolding and aggregation; and (3) up regulating molecular chaperones or aggregate-clearance mechanisms. Ultimately, drug combinations that capitalise on more than one therapeutic strategy will constitute the most effective treatment for patients with these devastating illnesses.

How do cosolvents affect protein stability, dynamics, folding and aggregation ?

Effect of cosolvents on protein stability

Thermodynamic description for a ternary solution can be described with components water (w), cosolvent (x) and protein (p). The corresponding concentrations of the solutes are n_x and n_p, respectively (Scharnagl et al., 2005). Each component is characterized by the respective chemical potential $\mu_i = \mu_i^0+RTln(c_in_i)$ (i =w, x, p) with activity coefficient c_i and standard chemical potential μ_i^0. The protein is present at infinite dilution, hence, $c_p=1$. Nonideal behavior arises due to the dependence of the standard states of cosolvent and protein on the concentration of the cosolvent.

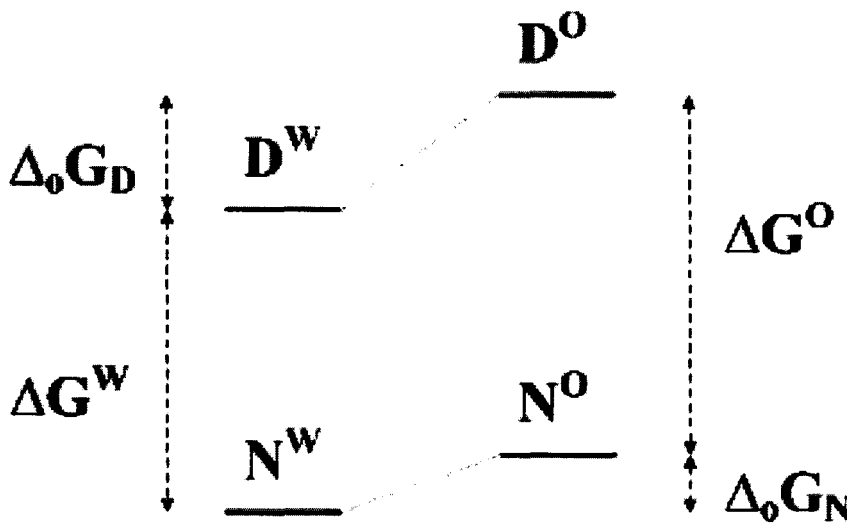

Fig. 1. Relative Gibbs energy (ΔG) diagram for native (N) and denatured (D) states, from water (W) to osmolyte (O) solution.

Cosolvent-induced effects on protein stability manifest themselves through changes in the free energy of the solvated protein. The change in protein stability can be related to the changes in the concentration of the components of the solvent around the protein. A change in the number of water and cosolvent molecules in the protein phase results from the interaction with the protein (either direct or indirect). This may lead to an excess population of one sort of molecules in the vicinity of the protein compared to the reference phase. Possible interaction mechanisms are (i) association of cosolvent molecules with the protein eventually in competition with water; (ii) inaccessibility of the protein to a specific cosolvent molecule due to steric reasons; (iii) solvent reorganization. Thereby, the range of influence of the protein on the local solvent composition does not only include directly bound molecules. Due to long-range interactions, the influence extends over several layers of waters. Cosolvent can penetrate into the solvation shell and exchange with water and hence, C_{px} has contributions from both water and cosolvent affinities. The addition of cosolvent therefore creates the possibility to probe the role of hydration water for the stability of proteins. The influence of the cosolvent on protein stability is directly related to the change in preferential binding coefficient of the cosolvent (or, equivalently, the change of hydration) upon denaturation. A change in one

parameter implies as a direct consequence, changes in all other parameters. A requirement for the stabilization of the native structure by a cosolvent is a negative value of ΔC_{px}, suggesting that the proteins are preferentially hydrated or the cosolvent is preferentially excluded from the local domain of the protein. Therefore, in order to stabilize the native state, the value of $C_{px}(D)$ must be more negative than $C_{px}(N)$, a condition tantamount with the finding that the cosolvent is more strongly excluded from the denatured protein than from the native one.

On a microscopic scale, the stabilization or denaturation of a protein due to the interaction with a cosolvent can be understood in terms of the interactions of the cosolvent molecules with those residues which come into contact with water upon denaturation. The formation of excess or deficit population in the vicinity of the protein with respect to a certain component of the solvent has contributions from (i) nonspecific steric exclusion of cosolvent or solvent molecules on the basis of excluded volume as an entropic consequence of a higher population of one component, and (ii) specific binding in competition with water molecules. The relative contributions from the two processes depend on the nature of the cosolvent, i.e. on its size, flexibility, charge, polarity, etc. and on the surface topology of the protein. The interpretation of cosolvent-induced stabilization of a protein in its native state on the basis of excluded volume effects relates stabilization to the increase of the steric– repulsive interactions in the water–cosolvent mixture relative to the pure water solvent. The stabilization or destabilization due to this effect depends on the geometrical structure of the denatured state in relation to the native state as well as on the average size of cosolvent molecules compared to water. If cosolvent molecules are significantly larger than water molecules, cosolvent is excluded from a certain volume shell around the protein, whereas water is not. Since excluded volume is proportional to the solvent-accessible surface area (SAS) of the protein. The exclusion of cosolvent due to steric reasons therefore tends to stabilize protein conformations with lower SAS, a condition found mostly in compact proteins.

Since the denatured state usually is characterized by a more open structure with larger SAS, excluded volume will stabilize the native state. In the surface tension model (Jackson and Sternberg, 1994; Lee and Timasheff, 1981), the stabilization of the native state is attributed to the increasing surface tension of water upon the addition of cosolvent

which increases the free energy requirement to accommodate the increased surface area of more open denatured states. On the other hand, preferential binding can enhance or attenuate the stability. Le Chatelier's principle implies that if a cosolvent binds preferentially to the native protein, the native state will be stabilized and denaturation becomes less favorable as the cosolvent concentration increases. Conversely, cosolvents that bind preferentially to the denatured protein will destabilize the native state. Another model which is employed to analyze cosolvent induced stability changes is the transfer free energy model (Auton et al., 2008; Alonso and Dill, 1991). Transfer energies of individual amino acids are used to predict the transfer free energy of the whole protein. In this model, steric effects and binding contributions are simultaneously included. The energy transfer data indicate that the osmophobic theory explains protein stabilization. The transfer Gibbs energy values estimated from the ΔG_{tr} values were used for the analysis of protein stability measurements in osmolytes. In such analysis, both denatured structure information and native structure information are needed. The transfer Gibbs energy values, from water to an osmolyte, of the denatured and native states were calculated using Eq.

$$\Delta G^{o}_{D} - \Delta G^{o}_{N} = \Sigma\, \alpha\, \Delta G_{tr}$$

Here, α represents the fractional exposure of an amino acid residue in the native or denatured state against each amino acid, and ΔG_{tr} represents the transfer Gibbs energy of the amino acid. The α value for the native state (α_N) is obtained from the accessible surface areas (ASAs) of the amino acid residues in the native structure and the amino acids. In the case of the denatured state, we have to prepare a denatured structure for such analysis. $\alpha_N = \Sigma\, ASA_N/ASA_{amino}$, $\alpha_D = \Sigma\, ASA_D/ASA_{amino}$ (3) Here, ASA_{amino}, ASA_N, and ASA_D are the ASAs of an amino acid, the native state, and the denatured state, respectively.

Various thermodynamic models for the cosolvent effect on protein stability as well as the volumetric analysis can be combined in the rigorous statistical mechanics frame of the Kirkwood–Buff theory (Schellman, 2005), which allows for relating the excess number of cosolvent molecules around the protein to their radial distribution function, which is available from experimental as well as from computer simulation data.

The pair correlations of proteins with water and osmolytes are the determining structural factors for the proteins' response to the presence of osmolytes (Rosgen et al. 2007). Only very recently, however, has effort been devoted to calculate thermodynamic solvation of proteins from experimental data (Shimizu et al., 2006; Rosgen et al., 2005; Schurr et al., 2005; Shulgin and Ruckenstein, 2005; Shimizu, 2004; Shimizu and Boon 2004). Solvation effects of water and osmolyte as well as their impact on protein stability is correlated. Hydration is found to be more sensitive to osmolyte size than osmolyte type (excluded volume effect of hydration). Hydration effects are of minor importance for the unfolding energetics of proteins in different osmolyte solutions and for the energetics of the bulk osmolyte. Water self-correlations are largely unaffected by osmolyte concentration and type. The energetic contribution of water self-hydration turns out to be insignificant to both the energetics of osmolytes and proteins in osmolyte solutions. This is consistent with a finding that the water– water correlation in the vicinity of the chaotrope (urea) and the cosmotrope (TMAO, trimethyl-amine-N-oxide) are very similar with regard to both angles and distances between water molecules (Gallagher and Sharp, 2003). Differences between osmolytes stem mostly from osmolyte self-solvation. Energetically, neither bulk water nor protein hydration is the main player in osmolyte concentration-dependent effects on protein stability and osmolyte energetics. Very predictable and monotonic solvation properties might be prerequisite for a molecule to be useful as a biological osmolyte. The very simple activity coefficient (Rosgen et al., 2004 a, b) and solvation behavior (Rosgen et al., 2005; 2007) seems to be a general property of osmolytes. Fine-regulation of the chemical activities of cellular components have to rely on the constant solvation behavior without sudden switches.

Thus, the combined effect of factors like solvophobicity, surface tension, excluded volume, water structure changes and electrostatic repulsion are all responsible for preferential exclusion and the effects exclusion has on protein properties (Auton et al., 2006). The effects lead to significant decrease in protein conformational entropy in native and denatured states in native conditions contributing to the mechanism of protein stabilization by stabilizing cosolvents like naturally occurring osmolytes (Chen et al., 2006).

Thus, the protein stability has thermodynamic as well as kinetic aspects. Thermodynamic stability, which is a positive value for the unfolding free energy at physiological temperature does not guarantee that the protein will remain in the native state during a given time scale, since irreversible protein alterations (even if they occur from lowly populated unfolded or partially unfolded states) may deplete the native state in a time-dependent manner. A strong scanning-rate dependence of the thermal denaturation transitions therefore is a signature of kinetic control for many protein systems (Remmele et al., 2005; Jayaraman et al., 2005; Sanchez-Ruiz et al., 1988). Many proteins therefore are likely to be naturally selected to have significant kinetic stability which include examples like α-lytic protease, Cu/Zn superoxide dismutase, the viral capsid protein SHP11 and human low-density lipoprotein. The interest of understanding protein kinetic stability is emphasized by the fact that some emerging molecular approaches to the inhibition of amyloidogenesis focus on the increase of the kinetic stability of the protein native state. Moreover, kinetic stability may be of considerable biotechnological importance as the proteins and the solvent conditions employed in technological applications often imply irreversible denaturation and kinetic control of the stability. Recent work exploring the kinetic stability of lipase (Rodriguez-Larrea et al., 2006) suggests that a solvation barrier arising from the asynchrony between breaking of internal contacts and water penetration may contribute to the kinetic stability. Thus general existence of water-solvation barriers that contribute to protein kinetic stability seems to be consistent with the fact that the dehydrated and lyophilized enzymes denature irreversibly at very high temperatures, indicating very high kinetic stability. Thus enhancing protein stability for biotechnological applications may in many cases mean enhancing protein kinetic stability and solvent mediated approach could be adopted to improve the kinetic aspects of the stability too.

Effect of cosolvents on protein dynamics

Stabilizing forces that protect proteins from denaturation may or may not be distinct from those forces that rigidify the protein. Crystallographic studies have identified thermophilic proteins as possessing smaller Debye-Waller factors than their mesophilic counterparts, suggesting a link and inverse correlation between thermal

stability and protein dynamics (Vihinen, 1987). In addition, there have been a number of studies, both experimental and computational, that provide a correlation for (Wagner and Wuthrich, 1979; Lazaridis et al., 1997; Zavodszky et al., 1998; Svingor et al., 2001; Tsai et al., 2001) or show no evidence for (Colombo et al., 2008; Fitter and Heberle, 2000; Hernandez et al., 2000; Fitter et al., 2001; Grottesi et al., 2002) a link between dynamics and stability.

Proteins do not exist in a unique conformation, but can exist in a very large number of somewhat different structures called conformational substates. So an instantaneous structure is characterized by the positions of all N atoms in the protein, the hydration shell, and some part of the bulk solvent. An instantaneous structure is a point in the conformation space and the protein motions are transitions among these points. This establishes that the solvent is the master and the protein the slave, as far as large-scale motions (which involve the largest amplitude motions that proteins make so that the fluctuating solvent should constrain these motions) are concerned (Frauenfelder et al., 2006).

The studies demonstrate that stabilizing osmolytes restrict the increase in conformational space in the presence of chemical denaturants, causing more restricted, native-like protein fluctuations (Doan-Nguyen and Loria, 2007). This could possibly limit access to higher energy conformational substates that would ultimately lead to protein denaturation. Meanwhile hydrogen exchange studies on proteins (Wang et al., 1995; Idiyatullin et al. 2003) have revealed that rate of exchange of slow exchanging amide protons are further slowed down in presence of stabilizing osmolytes. Because stabilizing osmolytes oppose an increase in protein surface area exposure, slowing of the hydrogen exchange rates strongly indicates that the cosolvents affect the dynamics of the compact unfolded state ensemble of the protein where the exchange was protected to a significant extent. The fact that other components (intermediate and fast) of the hydrogen exchange rates could not be affected in a significant way suggested that the compatibility of stabilization of the osmolytes is decided by the unperturbed dynamics of the functionally critical flexible and dynamic regions of the native state ensembles. Thus naturally occurring stabilizing cosolvents seem to be evolutionary selected on their ability to discriminate between the dynamics of various conformational ensembles.

The hydration water dynamics and their dynamical coupling with the protein are essential for protein dynamics and biological function. Inelastic neutron scattering experiments on Staphylococcal nuclease (Nakagawa H et al., 2008) looking into the effect of hydration on protein dynamics at differing hydration levels have revealed that the partial hydration is sufficient to affect the harmonic nature of protein dynamics, and that there is a threshold hydration level to activate the anharmonic motions. Thus, hydration water has been found to control both the harmonic and anharmonic protein dynamics and the hydration effects are strongly dependent on both temperature and hydration. These results indicate that to understand protein dynamics the hydration water dynamics should be revealed.

Cosolvents and enzyme activity

Stabilizing osmolytes are preferentially excluded from the protein domain and therefore no direct interaction between the osmolyte and the protein is expected to bring a change in K_m and k_{cat} values. At the same time compatible osmolytes might affect the association of substrate with enzyme through solvation effects on substrates or enzyme active sites, or by means of effects on the thermodynamic activity of substrates or enzyme (Abel et al., 2008; Agarwal, 2005; Chang et al., 2008). Polyols are observed to bring no significant effect on K_m and k_{cat} of enzymes like lysozyme and RNase-A (Haque et al., 2005). The lack of effect on activity parameters could be due to that polyols have little or no effect on the solvation properties of substrates and enzyme active sites. Similar effects are also observed in case of prolines (Diamant et al., 2001). Compatible osmolytes must therefore be able to discriminate between the solvation characteristics of structurally and functionally critical domains of the protein.

Effect of cosolvents on protein folding

Solvation has a dramatic effect on the energy landscape of the proteins as the concept of amino acids having helical and beta sheet forming propensity incorporates the environmental solvent effects (Levy et al., 2001). This means that kinetic studies looking into transition from unfolded state to folded state can provide insights on how solvent could possibly alter these accumulations of various conformational ensembles depending on their respective stabilization/ destabilization under defined solvent conditions. This

should however also depend on whether solvent mediated equilibrium effects reflect native state stabilization or unfolded state destabilization.

Studies of unfolding rates of some proteins like DHFR with varying solvent ionic strength have revealed that slower unfolding rates are followed at high ionic strength conditions without altering unfolding rates at native conditions suggesting kinetic stabilization of the protein predominantly from destabilization of the unfolded states as observed for some thermophilic enzymes (Gloss et al., 2008). This indicates that haloadaptation harnesses the effects of increased salt concentrations on the properties of the aqueous solvent to enhance protein stability. Also, simulation studies looking into the effects of incorporating water molecules while observing peptide folding *in silico*, have revealed that peptide conformers with high solvent-accessible hydrophobic surface area have low hydration density around hydrophobic residues, whereas a concomitant higher hydration density around hydrophilic residues was observed. The dewetting effect was found to stabilize the fully folded state. The results suggested that dehydration-driven solvent exposure of hydrophobic surfaces may be a significant factor determining peptide conformational equilibria and could be a potential driving force in peptide folding (Daidone et al., 2007). This enhances the possibility that hydrating cosolvent molecules could significantly alter the rates of folding depending on their respective solvation effects varying conformational entities accumulating during protein folding. This is further evidenced by studies on cutinase exploring the effect of trehalose on its folding which is found to favor an intermediate off-folding pathway and might even decelerate the distinct folding event (Melo et al., 2003). On the other hand, pressure-jump-induced kinetics on RNase A has also implicated a two-dimensional energy surface containing a pressure- and temperature-dependent barrier between two isomeric unfolded states (Font et al., 2006). Analysis of the activation volume of the kinetic phases revealed a temperature-dependent shift of the unfolding transition state to a larger volume. However, the observed effect could be compensated by glycerol. This indicated towards the hydration dependent folding/unfolding mechanism of ribonuclease.

The fact that the folding reaction is significantly influenced by the nature of the bulk solvent (Parker et al., 1997) is evidenced by solvent viscosity effects on the folding rates. Viscogenic agents however are known to affect folding rates not only by increasing

solvent viscosity but also by increasing protein stability (Jacob et al., 1999; Pradeep and Udgaonkar, 2007; Mishra et al., 2007). Studies with both protein and solvent confined within a space limit has shown that solvent-mediated affect could lead to destabilization of the native state and unfolding happened to a relatively compact form of the unfolded state (Lucent et al., 2007). Thus, the confinement of solvent has a significant impact on protein kinetics and thermodynamics.

Effect of cosolvents on protein aggregation

Presences of various kinds of additives have been found to prevent protein aggregation (Dong et al., 2004). Ligands and cosolutes that alter protein conformational stability also influence the rate of formation of non-native aggregates (Chi et al., 2003). For example, in the presence of polyanions, aggregation of acidic fibroblast growth factor (Fan et al., 2007) and native recombinant keratinocyte growth factor (Derrik et al., 2007) is greatly inhibited. It has also been shown that the addition of weakly interacting preferentially excluded solutes can reduce the rate of protein aggregation. For example, sucrose has been shown to inhibit aggregation of hemoglobin (Kerwin et al., 1998), rhIFN-γ (Webb et al., 2001), keratinocyte growth factor (Chen and Arakawa, 1996), immunoglobulin light chains (Auffray and Rougeon, 1980), and rhGCSF (Thirumangalathu et al., 2006).

Protein folding aids can be categorized into two groups. Molecules like acetamide, acetone, thiourea and L-arginine stabilize unfolded protein or folding intermediates. The presence of these additives decrease the folding rate with increase in their concentration and can minimize the aggregation at a particular concentration. The other group include molecules like glycerol which act as protein stabilizers (Baynes and Trout, 2004; Mishra et al., 2007). In the presence of these kinds of folding aids, both the refolding rate and yield were enhanced by increasing their concentration to a proper value. So the cooperative application of the two kinds of folding aids could result in favorable refolding rate and better protein yields. Some of the aggregation suppressors like ammonium sulphate salts have also been correlated to their surface tension property to be described as one among the factors to prevent thermal aggregation (Hirano et al., 2007). Cosolvents as folding aids or aggregation suppressers could also act indirectly by

facilitating molecular chaperones enhancing their ability to assist refolding and minimize aggregation (Lange et al., 2005).

Outline of the current research problem

Proteins are the major biomacromolecules involved in variety of physiological functions. They are the polypeptides which fold to acquire a particular three dimensional conformation so as to carry out the given function. As discussed earlier that any alteration in the specific conformation of proteins may result in variety of physiological disorders. To avoid any such alterations, polypeptide chains have to first get properly folded from the unfolded state and the folded state has to be stable enough under varying conditions to carry out the required function. Thus either misfolding or destabilizations of the proteins lead to serious disorders.

Cataract is one such disease where the constituent proteins of the lens gets aggregated that interfere with its transparency subsequently leading to blindness. Mammalian lenses are made of long fiber cells, enclosing a major cytoplasmic component lens–specific proteins, the crystallins. α-crystallins (molecular weight ~7,50,000-1,200,000) and β-crystallins (β_h~180,000;β_l~60,000) are heterogenous oligomeric proteins, while γ-crystallins are monomers (molecular weight~ 20,000) (Bloemendal and de Jong, 1991). These constitute an estimated 35% of the net weight of the lens. Using a mixture of these different sized protein assemblies to fill the lens fibre cells ensures polydispersity and prevents crystallization. In order to fulfill their optical function, crystallins have to be first and foremost soluble. Since they have to last longer in relation to the whole life span of the organism they must also be stable. Crystallins contribute to the transparency and refractive power of the lens by short-range interactions among themselves and cytoskeletal elements in a highly concentrated matrix (Bloemendal and de Jong, 1991; Delaye and Tardieu, 1983). A disruption in this interactive order (either through alteration in solubility or in stability) may be caused by different types of protein condensates, such as aggregates, coexisting liquid phases or crystals. Any such disruption results in increased light scattering and lens opacity or cataract.

Cataractous crystallin proteins may be divided into two categories: α-crystallins

and β γ - crystallins. α-crystallin is a member of small heat shock proteins acting as molecular chaperone(Raman et al., 1997), is thought to bind to unfolded polypeptide chains during the times of stress and is thus crucial for preventing aggregation. It forms polydisperse multimers having molecular masses ranging from 300 to 1200 kDa, depending on the solvent conditions and other variables. It is a predominantly β-sheet with less than 20 % helix content.

β γ -crystallins are however small (20-30 kDa) proteins primarily composed of antiparallel β-sheets. β-crystallins and γ -crystallins are structurally similar. They both comprise four Greek key motifs separated into two domains. β-crystallins form domain-swapped dimers in solution owing to their flexible sequence. Truncation and deamidation of βB1 leads to altered conformation. These alterations happen extensively in ageing human lenses and may be important for age onset cataract formation.

The γ -crystallins are monomeric in solution. In addition, γ -crystallins are the only known crystallins having attractive forces between molecules and are therefore more likely to form cataractogenous aggregates (Tardieu et al., 1992). Also, as with age, chaperone action of α-crystallin gets reduced due to destabilization, therefore it becomes necessary to set aggregation prone γ-crystallins as an alternative target to design drugs for cataract. γ -crystallins differ from the α- and β-crystallins in one important aspect: the interactions between the γ -crystallins are attractive (Tardieu et al., 1992). This feature reduces the osmotic pressure in the lens but it also makes γ-crystallins more susceptible to aggregation and phase separation, a phenomenon that diminishes the homogeneity of the lens and causes cataract. Also α-crystallin, in order to show its chaperone behavior, forms complexes with β- and γ -crystallins. *In vitro* studies on these complexes have revealed that α-β-crystallin complexes remain soluble for a long time in contrast to α- γ - crystallin complexes that form precipitates as a function of time. Such differential behavior *in vitro* provides a strong indication that γ-crystallins pose a more severe cataract risk than β-crystallin. Human γD crystallin is the model of our choice as this is the most abundant among the expressed γ-crystallins in the lens.

However, Human γD crystallin as the model for solvent mediated protein stability

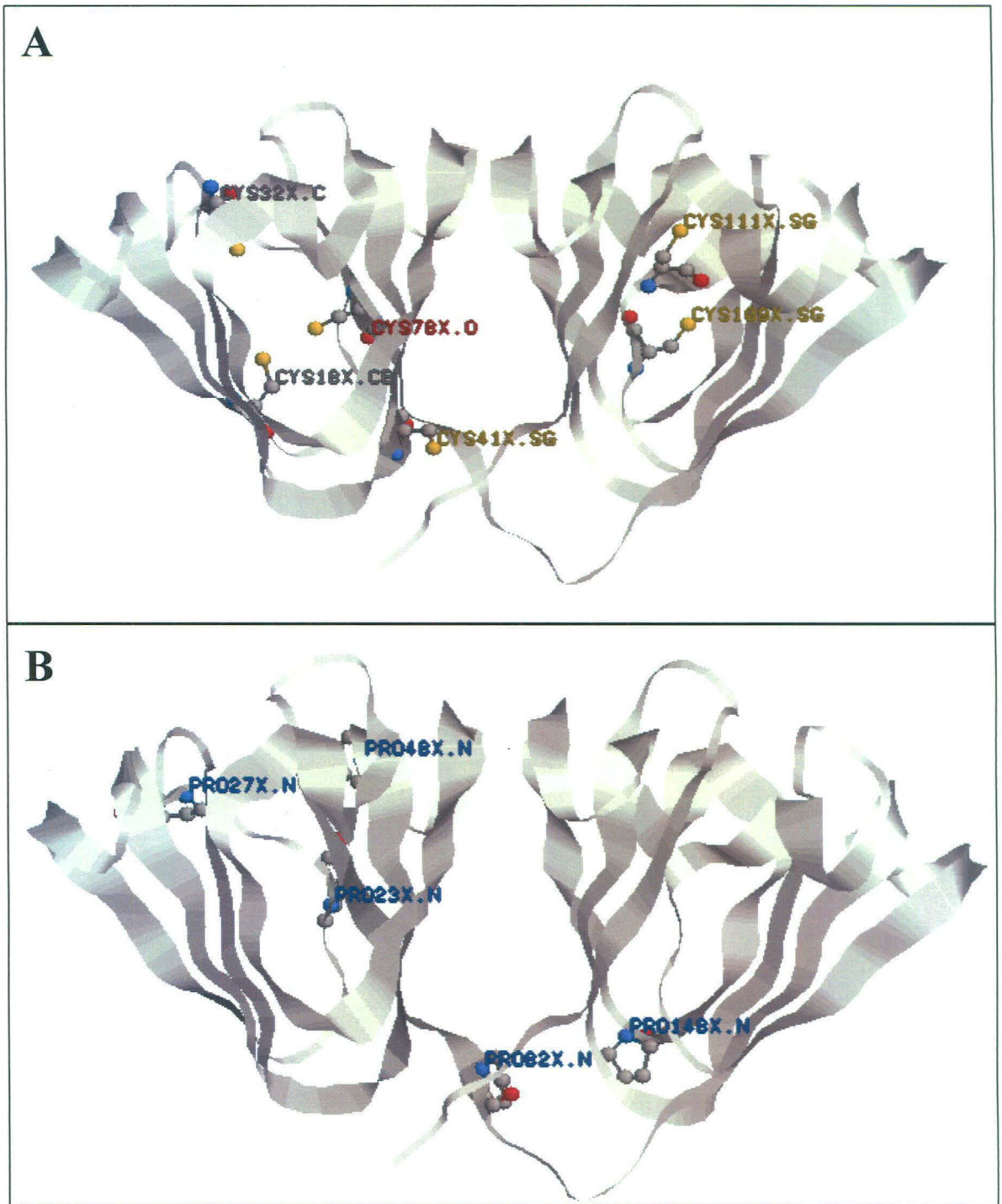

Figure 2: Conformational constraints in Human γD crystallin showing two β greek key domains joined by a hinge with A. buried cysteines and B. prolines. (PDB code:1HK0)

studies has been selected on the basis of some additional background studies:

A) Proteins tend to compromise between rigidity (for stability) and flexibility (for folding, function and degradation) as a result of which the free energy of stabilization of globular proteins in solution is equivalent to only a few weak intermolecular interactions. Stabilizing cosolvents or ligands therefore can bring about additional increments in this free energy of denaturation. These can play their role in both ways either by bringing in the enthalpic effects or through entropy by reducing the flexibility (cystine bridges, increased proline content etc.), or by water release from residues buried upon folding and association. Protein stability and function can also be maintained by increased ion binding and glutamic acid content (as in halophiles) allowing it to compete for water during high salt conditions. Similarly, proteins facing the outside extremes of pH adopt to the conditions by possessing anomalously high contents of ionizable amino acids in order to have buffering ability. Thus, the basic mechanisms of molecular adaptation include changes in packing density, charge distribution, hydrophobic surface area and in the ratio of polar:non-polar or acidic:basic residues (Jaenike 2000). Human γD crystallin seems to have all these adaptations as it possesses high packing density, distribution of acidic and basic residues, calcium binding property (to possibly provide the protein with ion buffering ability), distribution of surface charges (to facilitate protein-protein attractive order) and surface salt bridges (to overcome the solubility concerns under high protein concentration).

B) Proteins can exist in concentrated or crowded solutions (Minton, 2001; Hall and Minton, 2003; Rivas and Minton, 2004) where the concentration of which might not be high in a solution, is forced to exist in a considerably reduced volume fraction of the total solution volume due to the presence of an inert solute at high concentration. Human γD crystallin is different in a way that they exist in crowded conditions but crowding is not solute mediated, instead the protein itself existing at very high concentration create the protein-protein mediated crowding. Concentrated and crowded solutions are often encountered in pharmaceutical milieu as well as in physiological environment. The behavior of a protein molecule in such an environment is significantly affected by the presence of other molecules. The primary consequence is the alteration of the activity or effective concentration of protein in solution, which further results in change in protein

structure, function and its stability (Minton, 2005; Saluja and Kalonia, 2008). In physiological systems, the consequences of high protein concentration coupled with rather minor structural alteration and sequence mutations are expressed in the form of various diseases and disorders due to protein assembly processes (Hardy and Gwinn-Hardy, 1998; Koo et al., 1999 and Lansbury, 1999). Examples include cataract (Stradner et al., 2004), neurodegenerative diseases including Alzheimer's and Parkinson's disease (Meehan et al., 2004), systemic amyloidosis (Harper and Lansbury, 1997), polyglutamine disorders like Huntington's disease (Koo et al., 1999), etc. From a pharmaceutical perspective, high protein concentrations in solutions pose formulation challenges originating from protein solubility, manufacturing challenges due to high viscosity of some of these solutions and often result in compromised stability of the protein in solutions with regard to self-association and aggregation (Shire et al., 2004).

C) Using small molecules to bring in desired conformational changes or to inhibit protein misfolding in recent times has helped develop potential therapeutic strategies against misfolding diseases (Rochet, 2007). Small molecules like members of polyol series (glycerol, erythritol, xylitol and Sorbitol) and amino acids and their methyl derivatives (glycine, sarcosine, betaine, etc.) are well known to occur in nature as protein stabilizers (Arakawa and Timasheff, 1982a and b, 1983, 1985). These osmolytes stabilize proteins through preferential hydration and have been found to enhance thermal stability of several proteins (Ahmad, 1999; Radha et al., 1998; Kaushik and Bhat, 1999; Kaushik and Bhat, 2003). Studies on the effect of osmolytes on protein folding have also been reported (Mukaiyama, 2008). Refolding studies on Citrate synthase (Mishra and Bhat, 2005) and on Carbonic anhydrase (Yoshimoto et al., 2003) have revealed that by carefully manipulating the solvent environment one could enhance the refolding of the proteins considerably. Meanwhile osmolytes are also reported to influence aggregation and amyloid fibril formation in different ways (Yang et al., 1999; Ignatova and Gierasch, 2006) All this information opens tremendous scope for utilization of these molecules straight away or molecules derived from these as potential therapeutic molecules against many protein destabilization or misfolding related disorders (Tanaka et al., 2004, 2005).

In light of these background studies, we have carried out detailed conformational studies of the wild type Human γD crystallin to understand how its physico-chemical

cataract in particular and the subjects of protein-solvent interactions, protein stability, solubility, folding and aggregation in general.

In this context therefore studies on solvent mediated effects on stability, folding and aggregation were carried out with following objectives:

- To study the conformational properties of Human γD-crystallins extensively and alteration in these properties when subjected to varying solvent conditions in terms of pH, ionic strength, presence of naturally occurring osmolytes of polyols, Polyethylene Glycols, amino acids and their derivatives etc., so as to optimize and define proper conditions for maximum stability and least aggregation.

- To characterize the aggregation prone intermediate on the folding pathway and to specify alteration in its population/physico-chemical properties under varying solvent conditions so as to obtain maximum refolding yield, in turn defining the best stabilizing conditions.

- To further explore the role of non–specific attractive interactions among the γD-crystallins and the possible solvent mediated factors involved therein.

Chapter 2

Materials and Methods

Materials and methods for expression and purification of Human γD crystallin

Materials and methods for expression of Human γD crystallin

Materials

Bacterial strains used in this study

E. coli strains	Applications
DH5-α	Cloning with plasmid vectors. Gives high efficiency transformants.
BL21(DE3)	Carries genome-integrated T7 RNA polymerase gene. Used for high-level gene expression with T7 promoters.

Clone of γD crystallin

A clone of γD crystalline, expressible in *E.coli* BL21(DE3) cells was made available by Dr. N. H. Lubsen, Netherland. The clone consisted of plasmid pET3a which carried a gene of γD crystalline (~522bp) along with downstream T7 promoter. The plasmid was amplified in *E.coli* (DH5) cells by growing the transformed cells overnight in LB medium. The plasmid was isolated from this overnight grown culture by using mini prep and was stored at – 20°C. Glycerol stocks were also prepared of the culture of DH5α cells carrying plasmid and kept at -80 °C for storage.

Media used for cultivation of *E.coli*

LB medium (Luria Bertani medium)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

20 g of LB powder (Hi Media) was dissolved in double distilled water (ddH_2O) to make up the total volume to 1 litre. The media was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

LB Agar plate

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g

35 g of LB agar powder (Hi Media) was dissolved in ddH_2O to make up the total volume to 1 litre. The media was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in. Appropriate antibiotics were added as needed and poured in petri dishes. All plates were stored at 4°C

TB (Terrific Broth)

Tryptone	12 g
Yeast Extract	24 g
Glycerol	4 ml
KH_2PO_4	2.31 g
K_2HPO_4	12.54 g

Phosphate salts were autoclaved separately and the total volume made up to 1L after mixing.

Antibiotics

Ampicillin

The dry sodium salt of ampicillin was dissolved in sterile ddH_2O to make the stock solution of 100 mg/ml. The stock solution was sterilised by filtration through a 0.22 micron disposable filter and stored at -20°C. The working concentration of the antibiotic was 100μg/ml for broth and plates.

Reagents

$T_{10}E_1$ buffer, pH 8.0

Tris-Cl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

The above components were mixed and the pH adjusted to 8.0 with dilute HCl. Finally, the buffer was sterilised by autoclaving.

2 N NaOH

8 g of Sodium hydroxide was dissolved in 45 ml of ddH_2O. Finally, volume was made up to the 100 ml with ddH_2O. Solution was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

10% Sodium dodecyl sulphate (SDS)

100 g of SDS (electrophoresis grade) was dissolved in 700 ml of sterile ddH_2O and was heated at 55°C for 2 minutes. The final volume was made up to 1 litre with sterile ddH_2O.

3 M Sodium acetate (pH 5.2)

408 g of Sodium acetate was dissolved in water. pH was adjusted to the desired value with Acetic acid. ddH_2O was added to make 1 litre total volume. Solution was sterilised by autoclaving for 15 minutes at 121°C/15 lb/sq in.

DNase free RNase A

Pancreatic RNase A was dissolved at a concentration of 10 mg/ml in 0.01 M Sodium acetate, pH 5.2, Boiled at 100°C for 15 minutes. Allowed to cool slowly at room temperature. pH was adjusted by adding 0.1 volumes of 1 M Tris HCl (pH 7.4). Dispensed into aliquots and stored at -20°C.

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ powder was dissolved in 700 ml of water. EDTA does not dissolve completely until the pH of the solution reaches 8.0. pH was adjusted to 8.0 with 10 M NaOH. Finally ddH_2O was added to 1 litre and solution was autoclaved.

1 M $CaCl_2$

147 g of Calcium chloride ($CaCl_2.2H_2O$) was dissolved in 1 litre of ddH_2O and solution was sterilised by filtration with a 0.22- micron filter membrane.

1 M Tris (pH 6.8, 7.0, 7.2, 7.4, 7.5, 8.0, 8.8)

121.1 g Tris base was dissolved in 800 ml of ddH_2O. pH was adjusted to desired value by adding concentrated HCl. Final volume was made up to 1 litre with ddH_2O and sterilised by autoclaving.

50 % Glycerol

50 ml of pure glycerol was added to 50 ml of ddH_2O and mixed thoroughly. Finally solution was autoclaved for 15 minutes at 121°C/15 lb/sq in.

70 % Ethanol

70 ml of pure ethanol was mixed with 30 ml of sterile ddH_2O to make up the total volume to 100 ml and stored at 4°C.

1 M DTT

3.09 g of Dithiothreitol was dissolve in 20 ml of 0.01 M sodium acetate (pH 5.2) and stored at –20°C after filter sterilization with 0.22-micron filter.

30% Acrylamide (29:1)

Acrylamide	29 g
N, N'-methylenebisacrylamide	1 g

ddH_2O was added to make up the total volume of 100 ml. The solution was filtered and kept at 4°C.

10% Ammonium persulphate

10 g ammonium persulphate powder was added in 100 ml of sterile ddH_2O, mixed and solution was kept at 4°C.

Ethidium bromide

100 mg ethidium bromide tablet was dissolved in 10 ml of water. Left overnight on 37°C shaker to dissolve the tablet completely and stored in dark at room temperature.

10 M Ammonium acetate

385.4 g of ammonium acetate was dissolved in 150 ml of ddH_2O. Volume was made up to 500 ml. Solution was sterilized by autoclaving for 15 minutes at 121°C/15 lb/sq in.

1M IPTG solution

2.73 g of IPTG powder was dissolved in 8 ml of sterile ddH_2O. Volume was made up to 10 ml with ddH_2O. The solution was sterilised by filtration through a 0.22 - micron disposable filter and stored into small aliquots at -20°C.

Phenol: Chloroform: Isoamyl alcohol

25 parts (v/v) Phenol (previously equilibrated in 150 mM NaCl / 50 mM Tris HCl, pH 7.5 and 1 mM EDTA) was mixed with 24 parts (v/v) Chloroform and 1 part (v/v) of Isoamyl alcohol. This solution was stored in a dark coloured glass bottle at 4°C.

5 M NaCl

292.2 g of sodium chloride was dissolved in 800 ml water and made up the total volume to 1 litre with water. The solution was sterilised by autoclaving.

100 mM Phenyl methyl sulphonyl fluoride (PMSF)

174 mg of PMSF powder was dissolved in 10 ml of isopropanol. The solution was divided in aliquots and stored at -20°C.

50× TAE buffer

Tris base	242 g
Glacial acetic acid	57.1 ml
$Na_2EDTA.2H_2O$	37.2 g

Sterile ddH_2O was added to make up the total volume to 1 litre.

5 × Tris glycine buffer

Tris base	15.1 g
Glycine	72.0
SDS	5.0 g

ddH_2O was added to make up the total volume to 1 litre.

6 × Gel loading buffer

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Ficoll (Type 400)	15% (w/v)

ddH_2O was added to make up the total volume of 10 ml. Stored at room temperature.

Alternatively, the DNA loading dye was made with following components.

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30% (v/v)

ddH_2O was added to make up the total volume of 10 ml. Stored at 4°C.

PBS (Phosphate Buffered Saline)

NaCl	100 mM
KCl	4.5 mM
Na_2HPO_4	7 mM
KH_2PO_4	3 mM

Water was added to make up the total volume and sterilised by autoclaving. The buffer was stored at 4°C.

10X D (20% Dextrose)

20 g of dextrose (D-glucose) was dissolved in 100 ml of water and autoclaved for 15 minutes

1 M potassium phosphate buffer, pH 6.0

132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 were mixed and the pH adjusted to 6.0 (with phosphoric acid or KOH). It was then sterilized by autoclaving and stored at room temperature.

Recipes for Separating gel and Stacking gel

12% Separating gel	**20.0 ml**
30% Acrylamide mix (29% Acrylamide & 1% Bis-acrylamide)	8.0 ml
1.5M Tris-Cl, pH 8.8	5.0 ml
10% SDS	0.2 ml
10% APS	0.2 ml
TEMED	0.008 ml
Distilled water	6.6 ml

Stacking Gel -	**5.0ml**
30% Acrylamide mix	0.83 ml
1.0 M Tris-Cl, pH 6.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml
Distilled water	3.4 ml

2 X Sample buffer	**100ml**
Trizma base	1.52 gm
Glycerol	20.0 ml
SDS	2.0 gm
2-Mercaptoethanol	2.0 ml (for reducing condition)
Bromophenol blue	0.002%

Electrophoresis buffer	**1000 ml**
Tris base	3.02 gm
Glycine	14.4 gm
SDS	1.0 gm
pH adjusted to 8.3.	

Methods

Plasmid isolation (mini preparations)

Mini preparation was done to obtain plasmid DNA in reasonable amounts for purification. For this a total of 5 ml of the overnight grown culture was pelleted in turns in microfuge tubes at 12,000 x 'g' for 2 minutes. The supernatant was discarded and 250 µl of solution I (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0) was added to the cell pellet and resuspended by vigorous vortexing. 400 µl of freshly prepared solution II (0.2 N NaOH, 1 % SDS) was added and mixed gently by inverting the contents of the tube. Finally 350 µl of ice-cold solution III (5M potassium acetate, glacial acetic acid) was added, mixed well and stored on ice for 10 minutes. The microfuge tube was centrifuged at 12,000 x 'g' for 10 minutes at 4°C and the supernatant was transferred to a fresh tube. Equal volumes of P:C:I mix was added to the supernatant and mixed by vortexing. The mixture was centrifuged again for 10 minutes at 12,000 x 'g' and the upper aqueous phase was pipetted out into a new microfuge tube and 0.7 volumes of isopropanol was added at room temperature and mixed well. The mixture was centrifuged again at 12,000 x 'g' for 30 minutes at room temperature. The pellet was washed twice 100 µl 70% ethanol and dried in a dry bath. The dried pellet was finally suspended in 75 µl of $T_{10}E_1$ and treated with RNase A.

DNA quantitation

The concentration of DNA was determined by spectrophotometry in UV range. Optical density (OD) of DNA solution was measured at 260 nm and 280 nm with appropriate blank of the solvent in which DNA was dissolved, using quartz cuvettes. An OD at 260_{nm} = 1 was considered equivalent to concentrations 50 µl g/ml for double stranded DNA and 20 µl g/ml for single stranded oligonucleotides. The ratio of OD_{260} /OD_{280} was determined to check the purity of the DNA preparation. The ratio of protein free pure DNA should be 1.8 to 2.0.

Preparation of competent *E. coli* cells

The competent cells were prepared with a slight modification in the standard protocol (Sambrook et al. 1989). A glycerol stock of *E. coli* cells was streaked on LB agar plate using four-flame method. A single colony was picked and inoculated in 5 ml

of LB broth and incubated overnight at 37°C with shaking. After 16 hours, 500 µl of the culture was used as inoculum (1% final concentration) for a 50 ml LB broth. Cells were grown to an OD_{600} of 0.3-0.5. The cells were chilled on ice and then transferred to a pre chilled sterile oakridge tube under aseptic conditions. The cells were centrifuged at 2,500 x 'g' for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 25 ml of chilled 100 mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were centrifuged again at 2,500 x 'g' for 5 minutes at 4°C. The pellet was resuspended in 5 ml of 100 mM $CaCl_2$ and 50% glycerol was added to it to a final concentration of 15%. The cells were kept on ice for 2-3 hours and were finally stored at – 70°C as 200 □l aliquots. Next day one aliquot was used to transform with 10 ng DNA of a standard plasmid in order to check the efficiency of the competent cells as number of transformants per microgram of plasmid DNA.

Transformation of *E. coli* cells

A 200 µl aliquot of competent cells was thawed on ice. ~10 ng of plasmid DNA was added to the thawed cells and incubated on ice for 30 minutes. Then cells treated with heat shock by keeping the cells in water bath set at 42°C for 90 seconds. The cells were immediately transferred onto ice and 800 µl of autoclaved LB broth was added to it. The cells were kept on incubator shaker set at 37°C, for 1 hour. Out of 1 ml culture, 100 µl of cells were plated on a LB agar plate containing an appropriate antibiotic for selection of transformants. The LB plate was incubated at 37°C for 12-16 hours for the emergence of colonies.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel apparatus with 1X TAE as electrophoresis buffer. As per requirement, 1.0 to 1.5% agarose was dissolved in 1X TAE buffer by heating it in a boiling water bath. After allowing it to cool to 40 - 45°C, ethidium bromide was added to it to a final concentration of 1µg/ml. The gel was set by pouring it into a casting tray in which a comb of desired tooth size was inserted at one end to form wells. After the gel had set, the comb was removed and the gel was transferred to the gel tank filled with the electrophoresis buffer. Samples mixed with

loading buffer were loaded into the wells. The gel was run at a constant voltage of 4-5 V/cm. The DNA bands were visualised under 260 nm UV light on a trans-illuminator.

Growth media and conditions for expression studies

For expression of γD crystallin primary cultures were grown overnight in LB medium (10 ml media in 100 ml flasks) with ampicillin (100 □g/ml) at 37°C with constant shaking at 220 rpm. Next day, 5 ml of the overnight grown culture was used to inoculate 500 ml media (Lauria broth (LB) and/or Terrific Broth (TB) media) in 2 L flasks with 100 μg/ml ampicillin and the culture was grown in shaking incubator at 37°C while continuously shaking at 220 rpm. Cells were grown to an OD_{600} of 2.0 - 2.5 and induced with 1mM IPTG (final concentration). The cells were harvested after 8 hours post induction by centrifugation at 4000 x 'g' for 10 minutes.

Preparation of protein samples for SDS- PAGE

The cell pellets were resuspended in PBS buffer and 5X standard protein loading dye was added to make final concentration of 1X in each sample. Samples were boiled for 5 minutes in water bath, and centrifuged at 12,000 x 'g' for 10 minutes at room temperature. The supernatant was loaded onto a 12% SDS-PAGE for analysis.

Purification of Human γD crystallin

Human γD pET3a recombinants were transformed into E. coli BL21 cells. Clones were grown overnight in LB medium containing ampicillin. The fresh overnight culture was used to inoculate 1 lit. of LB medium with ampicillin. Expression of recombinant protein was induced at O. $D._{600}$ 0.6 by adding IPTG to a final concentration of 1mM after an additional 8 hours at 37^0C, cells were harvested by centrifugation and resuspended in 30 ml 25mM tris HCl, 5mM EDTA, 50mM Glucose pH 8 .0 containing protease inhibitor cocktail. Cells were lysed by French press, DNase I was then added to 50 μg/ml concentration and the lysate was incubated for 2 hrs at room temperature. The lysate was then cleared by centrifugation for 45 min at 36000 rpm. The supernatant was stored at 4^0C. Supernatant obtained after centrifugation was subjected gel filtration chromatography on a Sephadex G-75 column. 5ml sample was loaded on a 2.5cm X 50cm sephadex G-75 column. Gel filtered purified protein was further purified by cation-exchange chromatography on sulphopropyl Sephadex C-50 column.

A) Gel filtration chromatography

The cell lysate was further subjected to gel filtration chromatography. For this, a Sephadex-G-75 column (2.5 cm X 50 cm in dimension) was equilibrated with filtered and degassed 100mM sodium phosphate buffer with final chloride concentration of 2.3mM and 5ml of the total cell lysate of 1liter culture (suspended in ~10ml lysis buffer) was loaded into the column and eluted at a flow rate of 0.5 ml per minute. Gel filtration marker proteins of known molecular weight were applied to the column for molecular mass estimation of the recombinant protein.

B) Cation exchange chromatography

For a cation exchange chromatography, 50 ml of dialysed protein was loaded on a 5 ml SP-Sephadex C-50 column equilibrated with 0.275 M acetate buffer, pH 4.8. Then, the column was extensively washed with the same buffer. The bound protein was eluted with 50 ml of linear gradient of 0.275 M Acetate buffer + 1 M NaCl covering the range from 0.1 M - 0.5 M NaCl. Protein gets eluted around 0.35 M NaCl. After purification column is washed extensively by 2 M NaCl to wash out any bound fraction of the impurities. Column is either stored in 20% ethanol or again equilibrated with acetate buffer for next cycle of purification. Fractions of interest were pooled and the pooled fractions were further concentrated by using 3kDa centricon filter and spun at 5000 rpm till a desirable amount was reached. Total protein concentration was estimated by spectrophotometer with protein extinction coefficient, $E_{280}^{1mg/ml}$ =2.9.

Protein gel electrophoresis

Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method described by Laemmli (1970). The stacking gel containing 4% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8) were mixed and polymerized. The separating gel had 12% or 15% acrylamide depending on the case and 0.1% SDS. Running buffer consisted of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 containing 0.1% SDS. The protein samples were prepared in sample buffer [0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and 5% β-mercaptoethanol (Laemmli, 1970)] and immersed in a boiling

waterbath for 3-5 minutes. Standard marker protein mixture (Boehringer, Bangalore Genei) was run simultaneously to calculate the subunit molecular size of the proteins.

Coomassie Brilliant Blue staining

SDS-Polyacrylamide gels containing more than 200 ng protein concentration were visualised by standard Coomassie Brilliant Blue R 250 (CBB R 250) staining solution {0.1% (w/v) CBB dissolved in 25% (v/v) methanol and 10% (v/v) acetic acid in water}, followed by de-staining in 25% (v/v) methanol and 10% (v/v) acetic acid in water.

Cell Biomass measurement

The biomass concentration was monitored by measuring the optical cell density (OD_{600}) at 600 nm in triplicate and average has been reported.

Materials and methods for stability, folding and aggregation studies on Human γD crystallin

Materials

Polyols like sorbitol, xylitol, erythritol and chemicals like guanidinium chloride (GdmCl), dithioerythritol (DTE), TMAO, DMG, Sarcosine, Betaine used in these experiments were procured from Sigma Chemical Co. St. Louis, USA. PEG200, PEG 400, PEG 1000, PEG 3350, PEG 8000, Glycine, Glycerol, ethylene glycol, tris, sodium dihydrogen phosphate and disodium hydrogen phosphate were from Merck, India., Arginine, Proline, acetic acid, sodium acetate, calcium chloride and NaCl were from Sisco Research Laboratories Pvt. Ltd. India. The chemicals used were of the highest purity grade and were used without any further purification. Millipore water purification system prepared water was used to make all the solutions and buffers. The pH of solutions was adjusted using Radiometer pH meter model PHM 220, Lab meter, by adding sodium hydroxide or hydrochloric acid solutions accordingly. The pH standards used for calibrating the pH meter were from either Radiometer or Sigma Chemical Co. were procured from Sigma Chemical Co., USA.

Methods

Preparation of protein and buffer solution

The protein solution was mixed with either 10 mM Sodium phosphate buffer pH 7.0 or water and then loaded in Centricon concentrators (Amicon Inc., USA) to concentrate and to remove any minor impurities left after purification by centrifugation at 5,000×g. The protein concentration was calculated based on the extinction coefficient (ε_{280} 2.9 mg^{-1} ml^{-1}) by using Cecil 8080 and Hitachi U-2000 UV–visible spectrophotometers. Protein solutions were centrifuged for 10 min at 30,000×g in a Sorvall RC 5C plus centrifuge to remove any suspended impurities.

20mM sodium phosphate, pH 7.0 buffers were used for the making Human γD crystallin protein solutions. The following solutions were prepared for the pH dependent studies: 20 mM glycine-HCl or NaOH, for pH 2.5, 3.0, 3.5. 10.5, 11.0; 20 mM acetate for pH 4.0, 4.5, and 5.0; 20 mM Tris for pH 8.5 and 9.0. All the cosolvents at given concentration were dissolved in buffers the desired pH was obtained HCl or NaOH. DTE was added in each case to 5mM final concentration to provide reducing conditions. 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.

Circular Dichroism studies

CD spectra were recorded on a Jasco J-815 model spectropolarimeter equipped with a peltier based temperature controller and a 0.1-cm path length cuvette. The instrument was calibrated with (+)-10-camphor sulfonic acid. In general, an average 5 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 were acquired at 25 °C. 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. 1cm path length cuvets were used in the far UV regions. The protein concentrations for the far UV spectra acquisition were 100 μg/ml. The data were normalized using the equation

$$[\Theta]=M_0\,\Theta/10lc$$

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 21.6 kDa and the total number of 173 residues for Human γD crystallin were taken to calculate mean residue weight.

Fluorescence studies to measure the changes in tertiary structure

Any perturbation in the tertiary structure of the protein due to the changes in the local environment was measured by studying a change in the fluorescence spectrum of tryptophan residue i.e. change in the intensity (Quantum yield) and the emission maxima. Tryptophan fluorescence studies were carried out to characterize the stability and folding characteristics of Human γD crytstallin. 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 10 μg/ml for the intrinsic fluorescence was used. For intrinsic fluorescence, excitation wavelength of 295 nm was used, with an excitation slit width of 10 nm and an emission slit width of 10 nm. The emission was recorded from 300 to 400 nm. All the spectra were measured at 25 °C.

Thermal denaturation of Human γD crystallin

The wavelengths 320 nm corresponding to native state and 360 nm corresponding to denatured state were used for measuring the thermal transition of the protein using fluorescence spectroscopy. Ratio of intensities at 360nm and at 320nm was plotted and the resulting data was normalized to give final thermal denaturation plot. The apparent mid transition temperature (Tm) of γD crystallin has been calculated using 10 μg/ml protein concentration for each set of experiments.

Equilibrium renaturation-denaturation

For the refolding samples, 100 μg/ml Human γD crystallin was first denatured in 6 M GdmCl containing 5mM DTE for 6 hrs and the protein was then refolded by diluting to a final concentration of 10 μg/ml in 20 mM phosphate buffer pH 7.0 containing 5mM DTE. For denaturation samples10 μg/ml protein was mixed in different concentration sof GdmCl. The protein was equilibrated for 36 hrs before recording their fluorescence signals at 360 nm and 320 nm when excited at 295 nm. pH dependent changes in teriary structure of the protein were monitored by recording the fluorescence spectra of the

samples incubated in desired pH condition for 5hrs.

Folding/unfolding kinetic studies

For the refolding kinetics, 100 μg/ml Human γD crystallin was first denatured in 5.5 M GdmCl containing 5mM DTE for 6 hrs and the protein was then refolded by diluting to a final concentration of 10 μg/ml protein and 1.5 M GdmCl concentration in 20 mM phosphate buffer pH 7.0 containing 5mM DTE. For denaturation samples, 10 μg/ml protein was mixed in 5.5 M GdmCl. Both refolding and unfolding kinetics were monitored by fluorescence spectroscopy by recording signals at 360 nm and 320nm when excited at 295 nm. The stop-flow kinetic studies were performed on a JASCO-815 CD polarimeter provided with attached Biologic stop-flow accessory and an extra channel for fluorescence detection. For monitoring early events of protein unfolding kinetics in presence of NaCl, all three solutions (NaCl, GdmCl and denatured protein in 5.5M GdmCl) were programmed to mix to reach a final concentration of 5.5M GdmCl and 10 μg/ml protein concentration in a 80 μl cuvette

pH induced unfolding kinetic studies

For acid induced denaturation kinetic studies, 10 μg/ml protein was dissolved and mixed in 20 mM glycine pH 2, 2.5 and 3 containing 5mM DTE and the changing tertiary structure of the protein was monitored at 360 nm and 320nm when excited at 295 nm.

Heat induced aggregation kinetics

Aggregation kinetics with variable pH was monitored at 80^0C and 25^0C by monitoring light scattering by fluorescence spectroscopy with excitation and emission wavelength at 360nm with excitation and emission slit widths of 5 nm each. 10 μg/ml protein was subjected to different pH conditions at given temperature with separate 20mM buffers for each case. (Phosphate at pH 2 and 2.5, Gly-HCl at pH3 and 3.5, Acetate from 4-5.5, phosphate from 6-8, Tris at 8.5, Gly- NaOH from 9-10.5 and carbonate at 11) and 20mM DTE. Same procedure was adopted for studying thermal aggregation in presence of varying additives of polyols, PEGs, NaCl, glycine and their methyl derivatives, TMAO, Arg, Proline etc.

Aggregation kinetics while refolding from chemically denatured Human γD crystallin

100 μg/ml protein was denatured in 5.5M GdmCl, 5mM DTE for 6 hrs and then rapidly diluted to 5 μg/ml final concentration in varying renaturation buffers. Aggregation kinetics were observed in 20mM phosphate buffer, 5mM DTE, pH7 at 25^0C by monitoring light scattering by fluorescence spectroscopy with excitation and emission wavelength set at 360nm with excitation and emission slit widths of 2.5nm each.

Results and Discussion

Chapter 3

Expression, purification and characterization of Human γD crystallin

Protein expression and purification studies were carried out essentially according to already published work with slight modification (Flaugh et al., 2005 and reference there in).

Expression of Human γD crystallin

Studies of protein expression have routinely been carried out with *E. coli* BL21(λDE3)pLys strains as this strain is known to prevent the leaky expression of the protein from the T7 promoter and facilitates the lysis of the cells to release the expressed protein (Kruse et al., 1996). But the strain puts an extra pressure on cell growth due to the presence of ampicillin and chloramphenicol as double selection markers. Human γD crystallin was expressed in both *E. coli* BL21(λDE3) and its pLys strain as hosts so as to distinguish between the expression profile of the two strains. Both the hosts were found to express the protein equally well with an added advantage of fast and larger biomass in case of BL21(λDE3) strain, further expressions were proceeded with *E. coli* BL21(λDE3) strains(figure 3.1). Analysis of the protein revealed a prominent band at ~20 kDa (lane 2 and 4 in figure 3.1) in the induced bacterial culture in comparison to the uninduced cell lysates (lane 1 and 3 in figure 3.1).

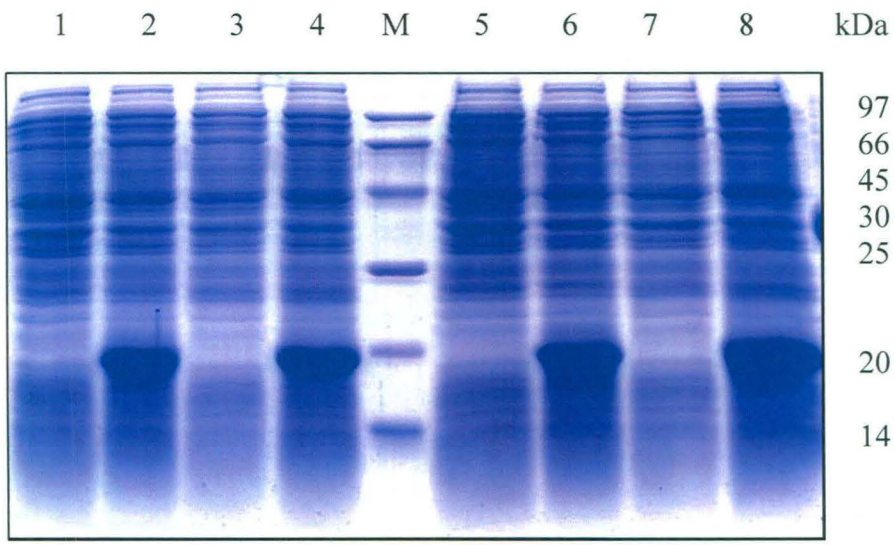

Figure 3.1: Expression of Human γD Crystallin in *E. coli*: lane 1 and 2- BL21(λDE3) uninduced and induced, lane 3 and 4 BL21(λDE3) pLys uninduced and induced, M-molecular weight marker, lane 5 and 6- expression in LB medium (uninduced and induced), lane 7 and 8-expression in TB medium (uninduced and induced).

Media composition is crucial for recombinant protein production as it affects the expression of recombinant protein in several ways. An ideal medium should be able to support cellular growth to maintain high specific growth rates and finally give high recombinant protein yields. As specific growth rate is a function of availability of substrates, hence the availability of nutrients especially during the induction phase of the cells is critical for recombinant yield (Covalt *et al.*, 2005). The composition of media can affect the specific growth rate of cells as well as the biomass yield. Thus it becomes an important factor for recombinant protein production. Various strategies are reported where media optimization improved production of recombinant protein (Niccolai *et al*, 2003, Tong *et al*, 2001, Li *et al.*, 1994). We therefore studied the expression of γD crystallin in different complex and highly enriched TB medium which usually promised higher biomass in culture.

The culture was grown in TB medium untill it reaches an OD_{600} of 4 and thereafter was induced by adding 1 mM IPTG. The cells were harvested 4hrs post induction and samples were analyzed on SDS PAGE for the expression of γD crystallin. The expression levels were also compared with those observed in LB medium. The results showed that there was no significant change in the expression levels of the protein in both the media (lane 6 and 8 in figure 3.1). Although the specific yield of the cells did not change with media but the volumetric product yield obtained in TB medium higher than that in LB medium. This increase in volumetric product yield was because of the higher biomass achieved in TB medium. We therefore decided to use TB medium for the production of recombinant Human γD crystallin for the purpose of conducting aggregation, folding and stability studies.

Purification of Human γD crystallin

Expressed cells were centrifuged and resuspended in lysis buffer containing protease inhibitor cocktails, DNase I and lysozyme. The mixture was then subjected to sonication/French press to lyse the cells leading to release of expressed protein in soluble form. Thus obtained protein mixture was then centrifuged again to get rid of cell debris and other high molecular weight impurities. The 90% of the protein was recovered in the supernatant.

Gel filtration chromatography

Supernatant was collected and subjected to gel filtration chromatography on a

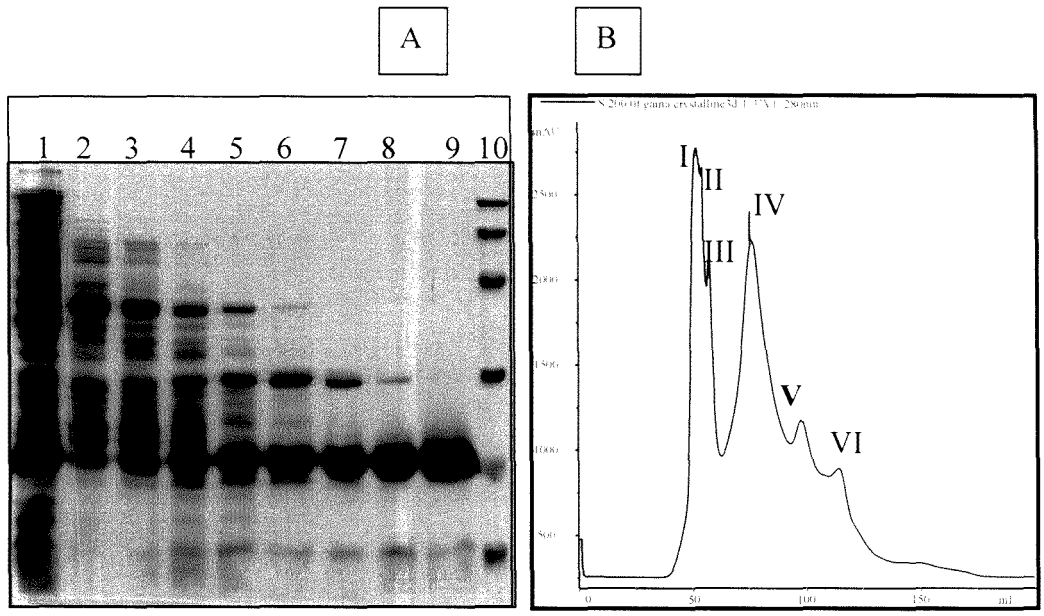

Figure 3.2: A. 12% SDS-PAGE analysis of fractions containing Human γD crystallin. lane1. Sonicated cell lysate. lane 2-9. gel filtration eluted fractions from peak I to peak V of elution profile. Lane 10. molecular weight markers (as in fig. 3.1). B. Elution profile of gel filtration on superdex 200HR column. The protein in the peak 5 of the gel filatration chromatogram is depicted in lane 9.

superdex 200HR FPLC column. Eluted protein fractions were analysed on the SDS-PAGE for the presence of Human γD Crystallin. Intense bands of crystallin could be observed along with few faint bands of high molecular weight contaminating proteins indicating partial purity of the protein. Fractions enriched with relatively pure protein were collected and used for further purification steps.

Cation exchange chromatography

Human γD crystallin has a pI of ~5.7 and has a network of charged residues on its surface and therefore at acidic pH, it is sufficiently charged. Charged status of the protein at acidic pH (pH 4.8) was therefore exploited further to purify and concentrate the protein by cation exchange chromatography on SP sephadex C-50. A linear NaCl gradient was applied on the column to purify the protein against remaining impurities (figure 3.3 A).

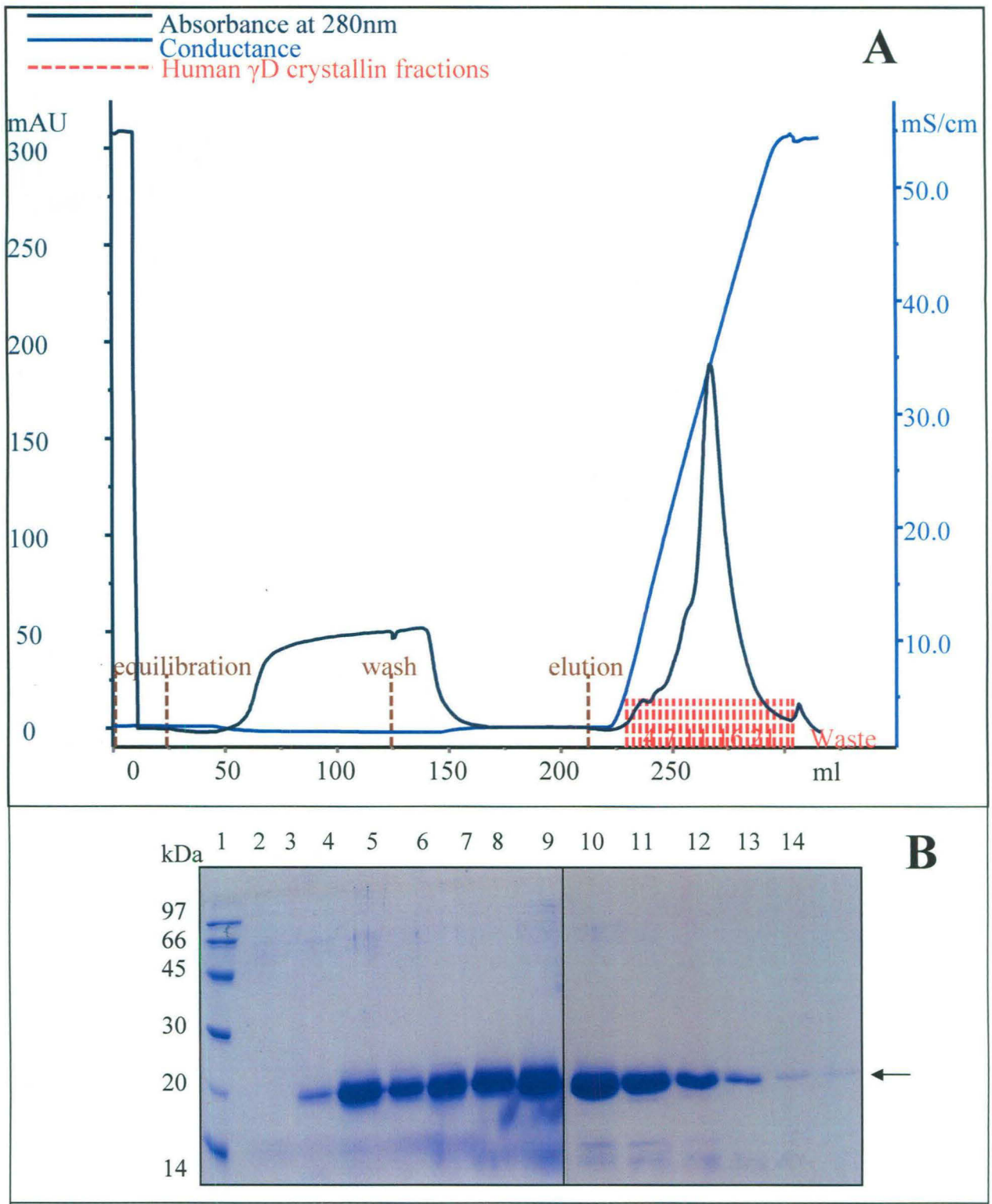

Figure 3.3: Cation exchange chromatography of partially purified protein on SP- Sephadex C-50 column obtained after gel filtration chroamtography. A. Elution profile and B. SDS-PAGE analysis of Human γD crystallin protein fractions after a gradient of increasing NaCl concentration was applied. Lane 1. molecular weight markers. Lanes 2-14 are the samples containing crystallin at ~0.35M NaCl.

Purified fractions of the protein (lane 12 in figure 3.3 B) were then dialysed against 20 mM sodium phosphate buffer, pH 7, concentrated and quantified spectrophotometrically. The protein stock was stored at 4°C and was ready for further characterization.

Spectroscopic characterization of the purified Human γD crystallin

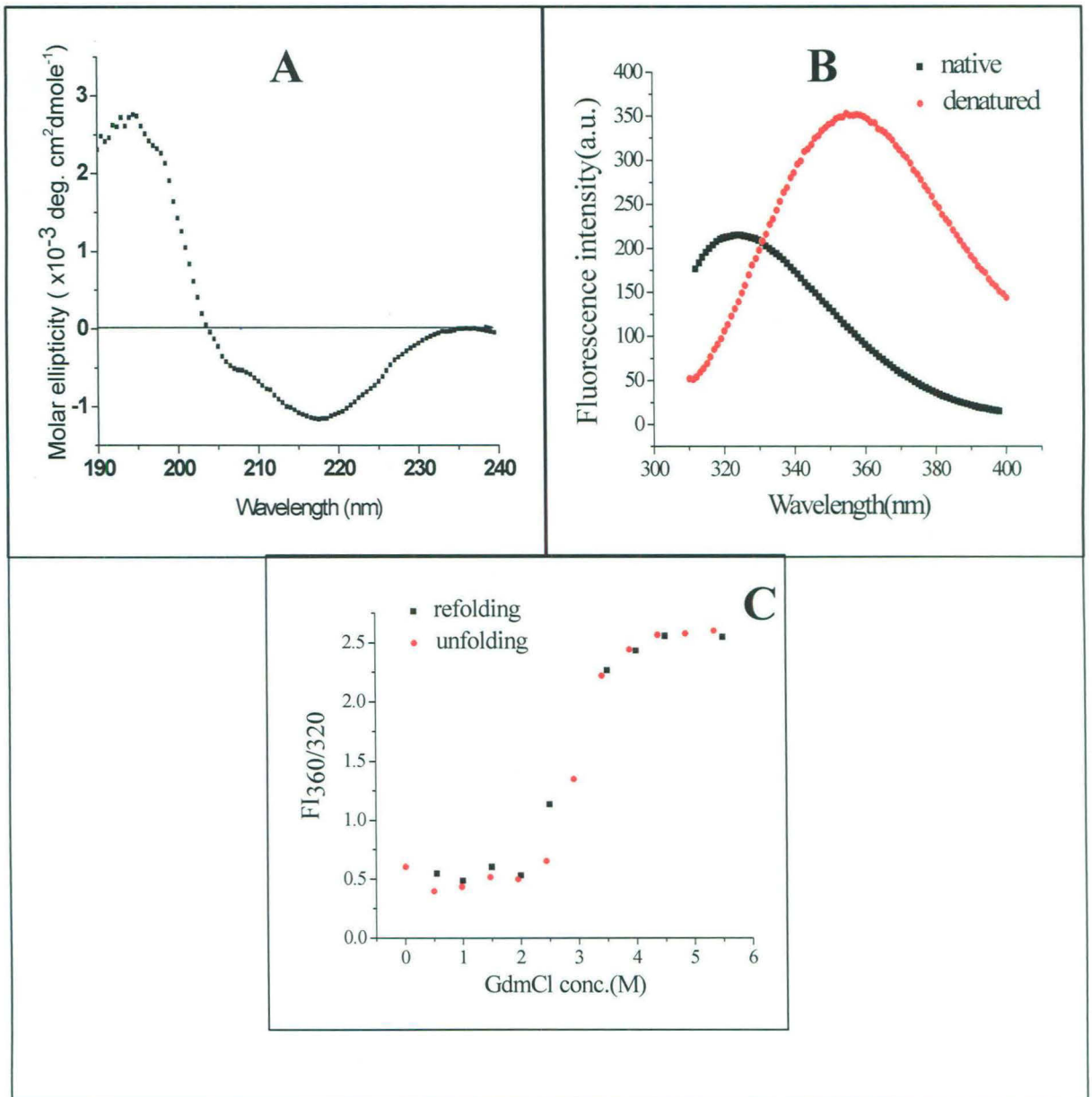

Figure 3.4: Characterization of Human γD crystallin: A. Far UV-CD spectrum B. fluorescence spectrum of Native protein in 10mM phosphate buffer and denatured fluorescence spectra in 6 M GdmCl in phosphate buffer, pH 7, 25 °C. equilibrium denaturation-renaturation curves in presence of GdmCl, pH 7, 25 °C.

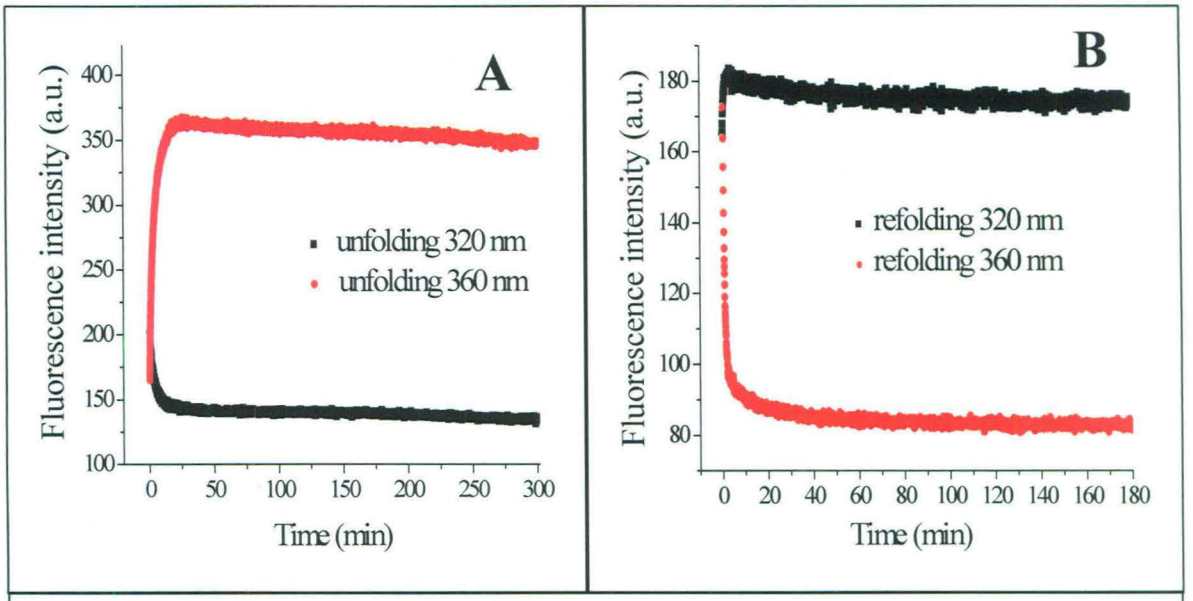

Figure 3.5: A. Human γD crystallin Unfolding kinetics in 5.5 M GdmCl and B. Refolding kinetics in 1.5 M GdmCl, pH 7, 25 °C at wavelengths corresponding to native (320 nm) and denatured state (360 nm).

Properties of purified protein were characterized and confirmed by CD and fluorescence spectroscopy. Predominantly β sheet characteristics of the protein were confirmed in far UV CD spectrum of the protein where a clear minimum at 218nm could be observed (figure 3.4 A) (Pande et al., 2000). Since the protein did not have any molecular signature for fluorescence, therefore a combination of fluorescence spectral characteristics for both native and chemically denatured state was carried out to verify the tertiary structural content of the protein. Nativity of the tertiary content was confirmed by the emission maximum at 325nm in the fluorescence spectrum which corresponded to quenched fluorescence characteristics of buried tryptophans (Kosinski-Collins et al., 2004, Chen et al., 2006) which when completely exposed in chemically denatured protein show its fluorescence spectrum red shifted with emission maximum at 355nm (figure 3.4 B). The reliability and the nativity of the protein was further supported by equilibrium denaturation/renaturation profile (figure 3.4C), unfolding kinetics in 5.5 M GdmCl (figure 3.5 A) and refolding kinetics of chemically denatured protein in 1.5 M GdmCl (figure 3.5 B). All the spectroscopic characteristics were found similar to already

published literature on Human γD crystallin thereby confirming the purity of recombinant Human γD crystallin (Kosinski-Collins and King, 2003).

Chapter 4

Effect of pH and NaCl on Human γD crystallin denaturation and aggregation

Thermal stability of Human γD crystallin

Human γD crystallin is a protein with a tightly packed hydrophobic core and network of salt bridges on the protein surface to provide high thermodynamic stability and greek key structure to provide kinetic stability against denaturation stress. When the protein was subjected to thermal denaturation, onset of denaturation transition was found to be at ~75°C (figure 4.1) suggesting a very high free energy barrier for protein unfolding.

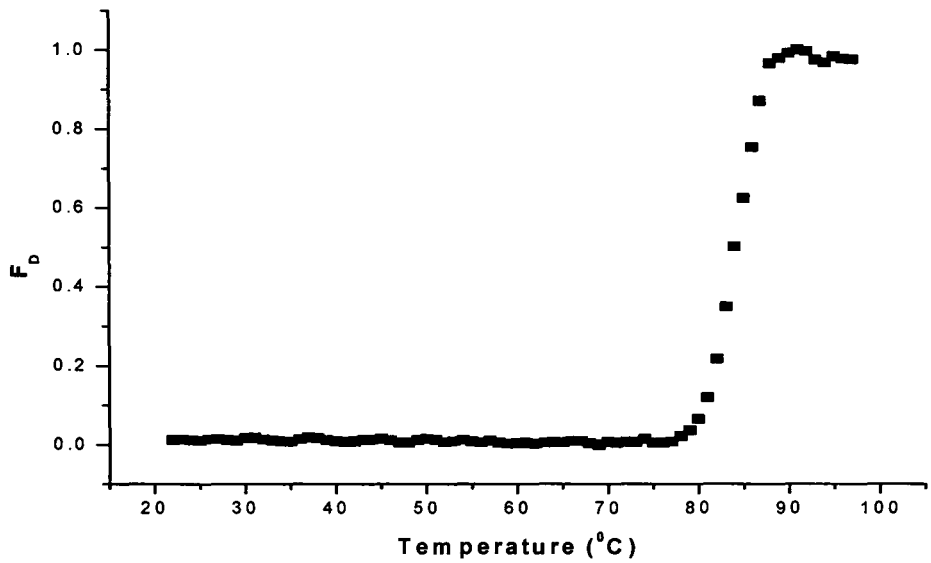

Figure 4.1: Thermal denaturation transition of Human γD Crystallin in 10mM phosphate buffer, 5mM DTE , pH 7.

This also suggested that surface salt bridges are likely to be the major part of free energy barrier for unfolding as most of the surface salt bridges at such high temperatures must be broken to facilitate further hydrophobic solvation facilitating protein denaturation. The thermal denaturation transition was observed to be very cooperative as temperature range for complete transition was found to be only about 10 °C. This further suggests that the major critical centers determining the protein stability must not be dispersed all over the surface, because had this been the case, the denaturation transition would have been less cooperative and would have covered a longer temperature range in transition due to accumulation of intermediates. Instead a cooperative transition at high

mperatures implicated that surface salt bridges might be among the prominent centers ' protein stability. Melting temperature of Human γD crystallin was found to be very gh (~ 85 ^{O}C).

To further explore the stability of Human γD crystallin against high temperature, ıorescence spectra were recorded at every 5 ^{o}C increment of temperature (figure 4.2). uorescence quantum yield was observed to decrease with increase in temperature which rresponded to increase in buffer mediated quenching of buried tryptophans indicating . increased dynamics of the protein. Spectral pattern was changed above 75 ^{o}C and was ry different from the native spectra.

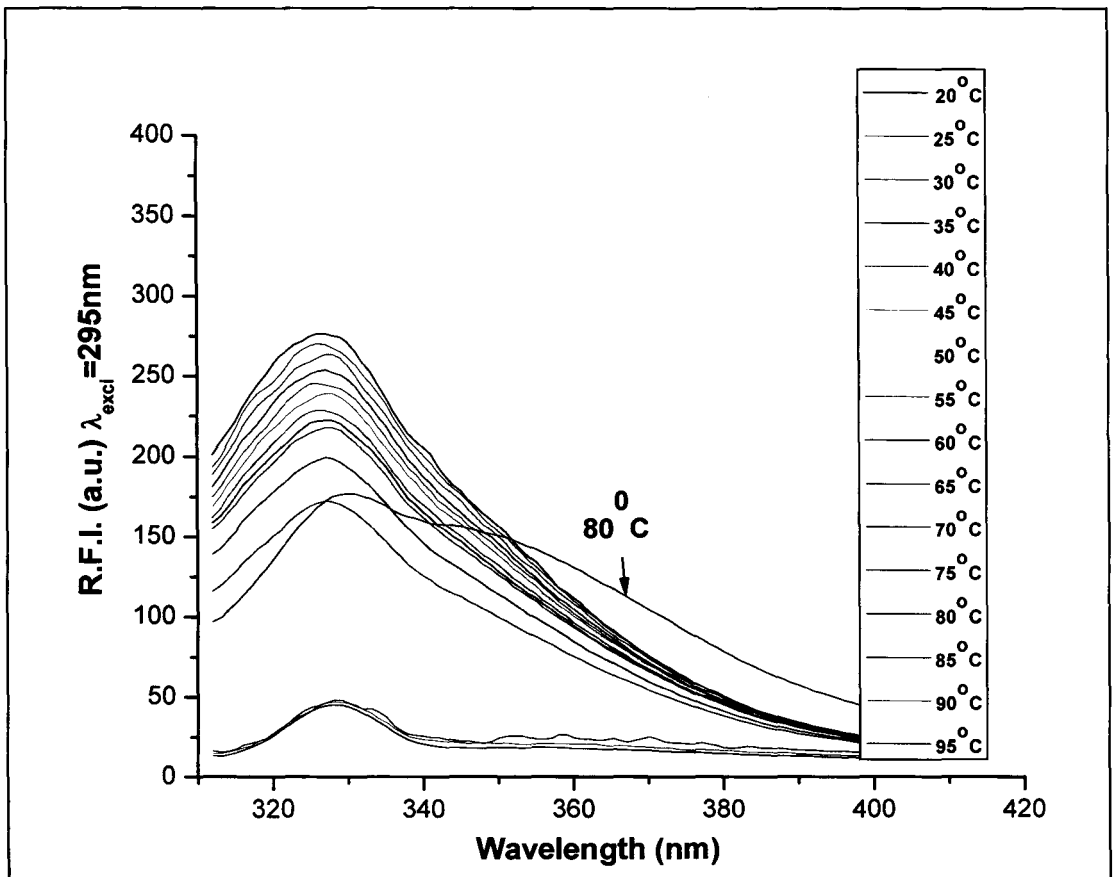

Figure 4.2: Fluorescence spectra of Human γD Crystallin with varying temperature (with every 5 ° increment) in phosphate buffer, pH 7 with λ_{exci}=295 nm.

At 80 ^{0}C, which is the onset of the thermal denaturation transition, fluorescence ectra exhibited an emission maximum at ~325 nm and a prominent shoulder at ~355

nm. This shoulder at 355 nm indicated solvent exposed tryptophans in the protein suggesting the accumulation of partially or completely denatured conformational ensembles. Further increase in temperature above 80 °C led to a significant change in the spectral pattern and not much fluorescence quantum yield was obtained in the spectra recorded. This was unexpected as temperature above 80 °C could only denature the protein further leading to an increased solvation of exposed tryptophans and therefore more prominent peak at ~355nm was expected. Since we were unable to explain this sudden change in the spectral pattern, possibility of aggregation at 80 °C was investigated by recording the scattering of the protein post aggregation by fluorescence spectroscopy.

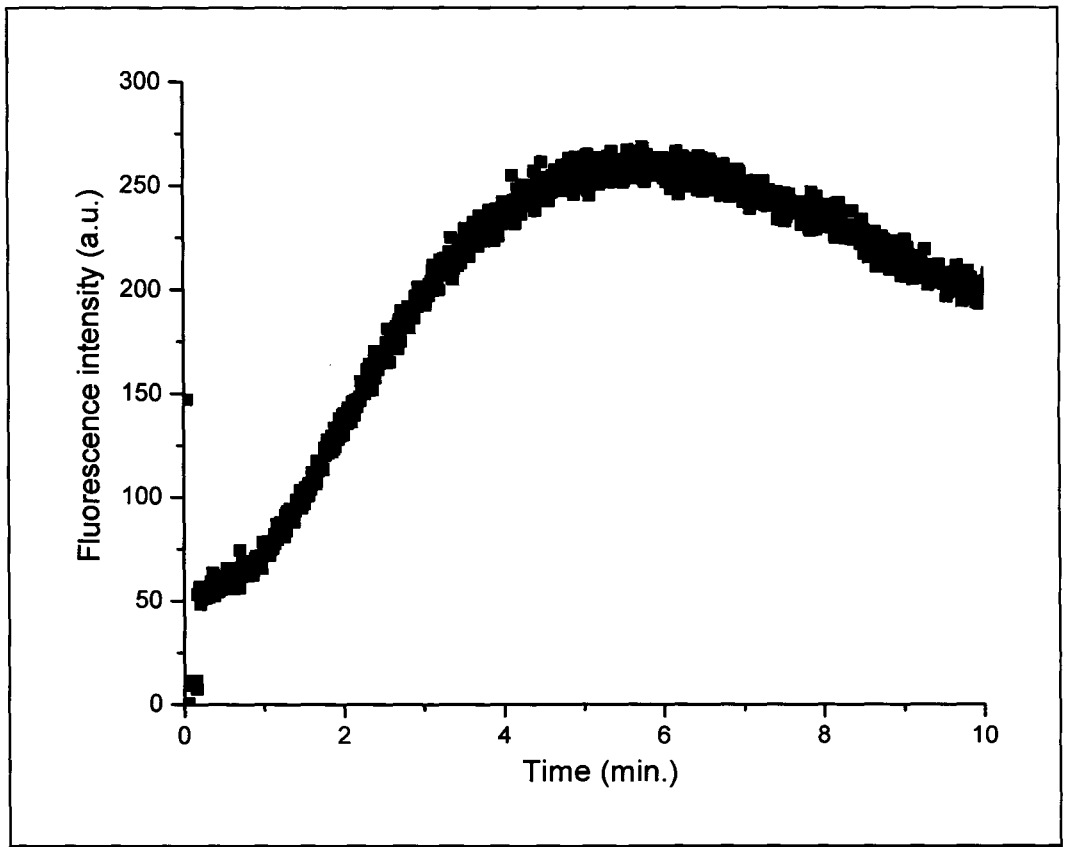

Figure 4.3: Heat induced aggregation kinetics of Human γD Crystallin at pH 7, 80 °C with λ_{exci}=360 nm and $\lambda_{emission}$=360 nm.

Human γD Crystallin when subjected to 80°C was found to aggregate, reaching to its maximum aggregation scattering intensity within 10 min (figure 4.3). The tendency of the protein towards aggregation at 80 °C (which is the temperature of onset of

denaturation) can be explained by the accumulation of partially denatured conformations with their critical surface salt bridges broken but the compact hydrophobic core which is still not loose enough for complete hydrophobic solvation. This tendency of the protein towards aggregation is further enhanced by the slow unfolding kinetics of the protein which facilitates the randomly moving partially unfolded conformational ensembles with open hydrophobic patches to clump together. So, we can conclusively infer from this observation that both kinetic and energetic factors are responsible for thermally induced aggregation. Moreover, as completely denaturing high temperature conditions break open through the surface interactions and lead to forced hydrophobic solvation, intermediate temperature conditions are likely to facilitate aggregation in order to avoid hydrophobic solvation.

Human γD crystallin during its life time is subjected to different kinds of conditions each of which acts on the protein differently and target different forces facilitating or disrupting the native conformation. Present studies were initiated with an objective to explore appropriate conditions/osmolytes which have the potential to counter the aggregation propensity of Human γD crystallin so as to obtain reversible conditions where in the thermodynamics and kinetic characterization of the protein be made feasible. Meanwhile, screening of such conditions/additives also provides an opportunity to ascertain the role of various factors critical for the protein stability and folding.

Effect of pH on stability, folding and aggregation characteristics of Human γD crystallin

Electrostatic network on the surface of Human γD crystallin render it soluble at very high concentration and asymmetric distribution of surface charges leads to dipoles in the proteins which are thought to be responsible for non-specific attractive interaction amongst them (Purkiss et al., 2007). Both these intrinsic properties of the protein make it more susceptible to aggregation Slight changes in these electrostatic forces could therefore lead to the solvent exposure of the hydrophobic core. Thus, both high concentration of the protein and attractive interaction amongst the protein molecules with exposed hydrophobic patches favors aggregation. pH and salts have a marked effect on the stability, solubility, structure and function of many globular proteins due to their

ability to influence the electrostatic interactions (Hawe and Friess, 2007) and changes in pH and ionic strength of the solvent can therefore be used to investigate the relative contribution of the electrostatic interactions in determining protein stability. Human γD crystallin was therefore subjected to conditions that promote aggregation at 80°C and aggregation kinetics was monitored under varying pH conditions (figure 4.4). Heat induced aggregation depicted a pH dependence and is maximum at pI of 5.7. Net charge on the protein under physiological conditions at pH 7 must therefore be positive. This may be due to unsatisfied charges on lysines and arginines present on the protein surface. These unsatisfied charges might seem to compromise the stability of the protein but could prove to be critical in determining proper protein solvation in order to maintain high solubility. Better solvation then may lead to proper hydrophobic packing in the interior leading to an increased protein stability compensating for the reduction in stability due to unsatisfied charges. Though Human γD crystallin have attractive interaction among them, nevertheless, unsatisfied charges on the protein could also be responsible for reducing the aggregation tendency of the protein by surface charge repulsion under destabilizing thermal conditions.

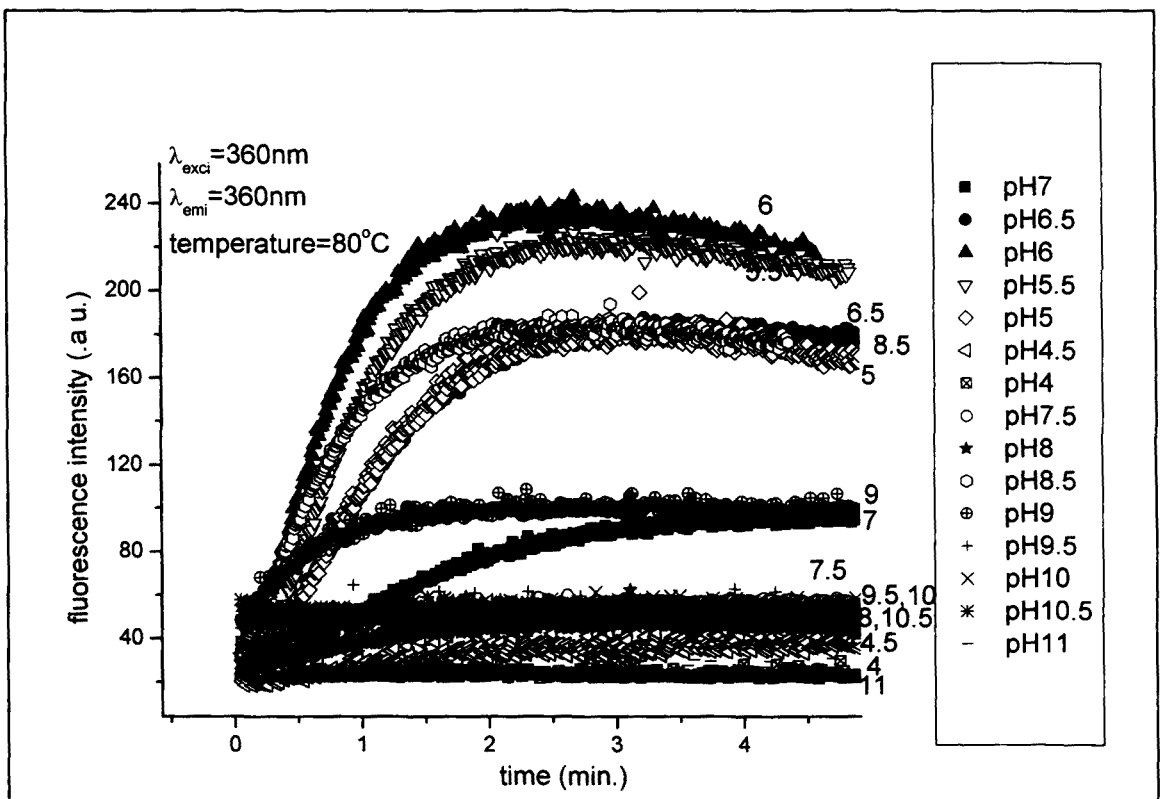

Figure 4.4: Heat induced aggregation kinetics of Human γD Crystallin at 80°C in varying pH conditions (λ_{exci}=360nm and $\lambda_{emission}$=360nm).

Heat induced aggregation decreased on both sides of pI with an enhanced aggregation around pH 8.5. Variation in pH protonates or deprotonates the residues crucial for determining electrostatic interaction thereby perturbing the surface salt bridges and leading to further protein denaturation by exposing the hydrophobic interior to the solvent. This situation with high mobility of the destabilized conformation is expected to promote aggregation but charge-charge repulsion between the protein surfaces counters the aggregation tendency of the molecule. Thus a decrease in aggregation at both sides of pI corresponds to increased repulsions between the charged surface of the protein. Irregularities in aggregation at and around pH 8.5 can be tentatively allocated to free Cysteines having pKa~8.5 as most of the Cysteines are partially or completely buried which can lead to change in their respective pKa's. The change in pKa of cysteine residues however would be very less under destabilizing conditions where Cysteines are solvent exposed. Change in pKa's of the surface residues involved in salt bridges kind of interaction can be another possible reason for abrupt increase in pH dependent aggregation at pH 8.5. This would create a possibility where some critical ionic gatekeepers, which are otherwise involved in surface salt bridges, might get disrupted to abruptly enhance the aggregation tendency of the protein.

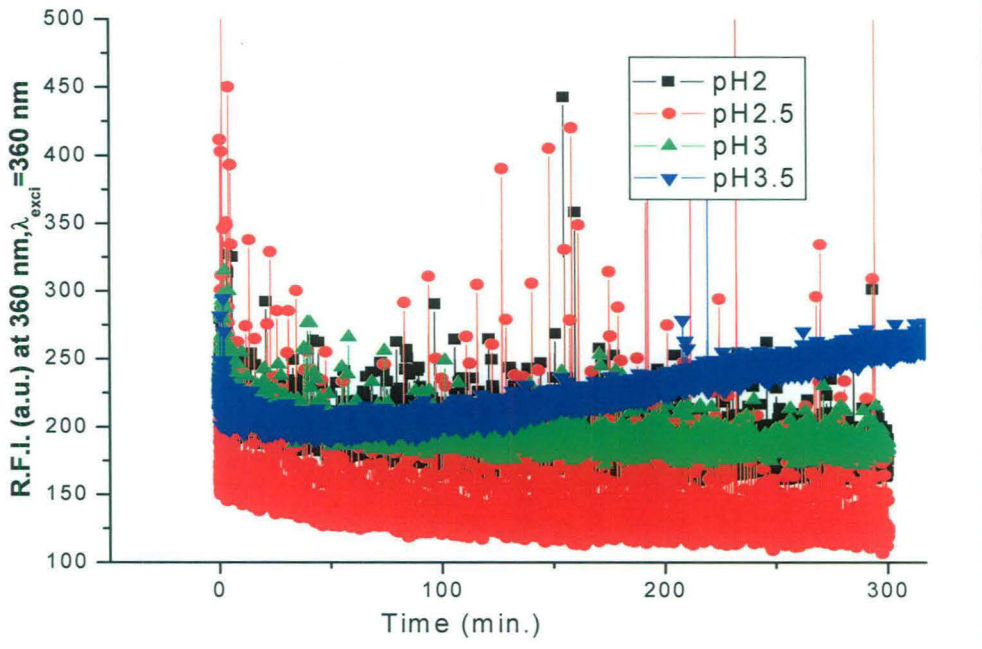

Figure 4.5: Aggregation kinetics of Human γD Crystallin at 25 °C in acidic pH conditions (λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

Thus, the pH dependent thermal aggregation information can be critical in determining the critical surface salt bridges for protein stability as well as determining the role played by the charged residues acting as ionic gatekeepers.

Knowing that the free Cysteines could facilitate aggregation at basic pH and the protein is not prone to heat induced aggregation at and before pH 4, aggregation propensity of the molecule at acidic pH was tested at room temperature. Acidic pH range from pH 2 – pH 3 was found to be non- aggregating and stable at room temperature (figure 4.5). At room temperature, protein though is destabilized at acidic pH with several of its electrostatic interactions perturbed, yet protein-protein repulsion due to unsatisfied surface charges dominate over clumping of hydrophobic patches. This aggregation prevention is further facilitated by slow diffusion of the destabilized conformation. On the other hand at high temperature conditions, pH mediated perturbation of the surface electrostatic interactions are also facilitated by high kinetic energy of the solvent to force hydrophobic solvation leading to further protein denaturation. This high energy solvent mediated factors are absent at room temperature and therefore more extreme pH conditions are required to introduce more charges on the surface for protein-protein repulsion. Thus, solvent mediated entropic factors must play a crucial role in determining aggregation propensity of the protein molecule under certain condition.

In order to further investigate the role of surface electrostatic interactions, non-aggregating conditions at pH 2, 2.5 and 3 were found to be suitable to carry out spectroscopic studies. Fluorescence spectral characteristics of the protein changed at acidic pH. A clear red shift in the fluorescence spectra could be detected at acidic pH and red shift increased with decreasing pH indicating the exposure of tryptophans to polar and aqueous environment. Relative contribution of denatured state at 355 nm also increased as a clear shoulder in the spectrum could be observed at pH 2 which indicates pH induced increase in denatured conformations. Also fluorescence spectra at pH 3 was very similar to that observed at pH 7 in contrast to that observed at pH 2 which is more red shifted exhibiting an emission maximum at ~340 nm along with a shoulder at 355 nm. These characteristics indicate towards accumulation of partially denatured intermediate states. Thus, there must be some critical electrostatic interactions being

disrupted between pH 3 and pH 2, which may be responsible for abrupt changes in observed spectral characteristics.

Thermal denaturation of γD crystallin at pH 2, 2.5 and 3 showed that protein at this pH range is stable and has significant interactions enough to show thermal denaturation transition (figure 4.6 B). The profiles at respective pHs' indicate that there are still some electrostatic interactions intact at pH 3, which further break down at relatively lower pH (2.5 and at pH 2). Cooperativity of transition is decreased at pH 3 as compared to that observed at pH 7 but increased at relatively lower pH conditions. Decreased cooperativity of transition at pH 3 can be due to perturbation of those critical surface salt bridges which determines the cooperative melting of the native states and absence of which lead to accumulation of on/off pathway intermediates. For example, perturbation of electrostatic interactions affecting domain-domain interactions directly or indirectly may lead to differential melting transition of the two domains leading to an increased accumulation of intermediates and therefore loss of cooperativity in the thermal denaturation transition. At even lower pH values of 2.5 and at pH 2, there was an earlier onset of denaturation at pH 2 than other higher pH conditions. Cooperativity of thermal denaturation transition at pH2 and pH 2.5 were almost same. Cooperatvity of denaturation transition at these lower pH conditions is however more than that at pH 3 which indicates that energetically there must be at least two sets of salt bridges, one which breaks down at pH 3 leading to an accumulation of more of the intermediates, and the other which breaks down at even lower pH. More cooperative transition at pH 2 compared to that at pH 3 could be due to destabilization of both native and intermediate states. Early onset of denaturation at these pH conditions can be explained by the increased molecular repulsion due to the protein surface charges arising as a result of the perturbed electrostatics which further facilitate protein denaturation with increase in temperature. Normalized fluorescence intensity after transition shows negative temperature dependence with an increasing slope with decrease in pH; which might be due to an increased precipitation of the denatured protein.

The effect of surface electrostatic interactions in determining the kinetic stability of the protein against unfolding can be understood by observing pH induced denaturation kinetics. As could be observed in the figure 4.6 C & D, signals corresponding to native

and denatured states did show altered pH induced unfolding kinetics. At pH 3, there was an increase in native signal reflecting the decreased quenching of tryptophan fluorescence due to partial unfolding of the protein. So, the surrounding water molecules and amino acid residues as quenchers of tryptophan fluorescence are no more available at these conditions. Denatured signal at 360 nm on the other hand was observed to be increased slightly.

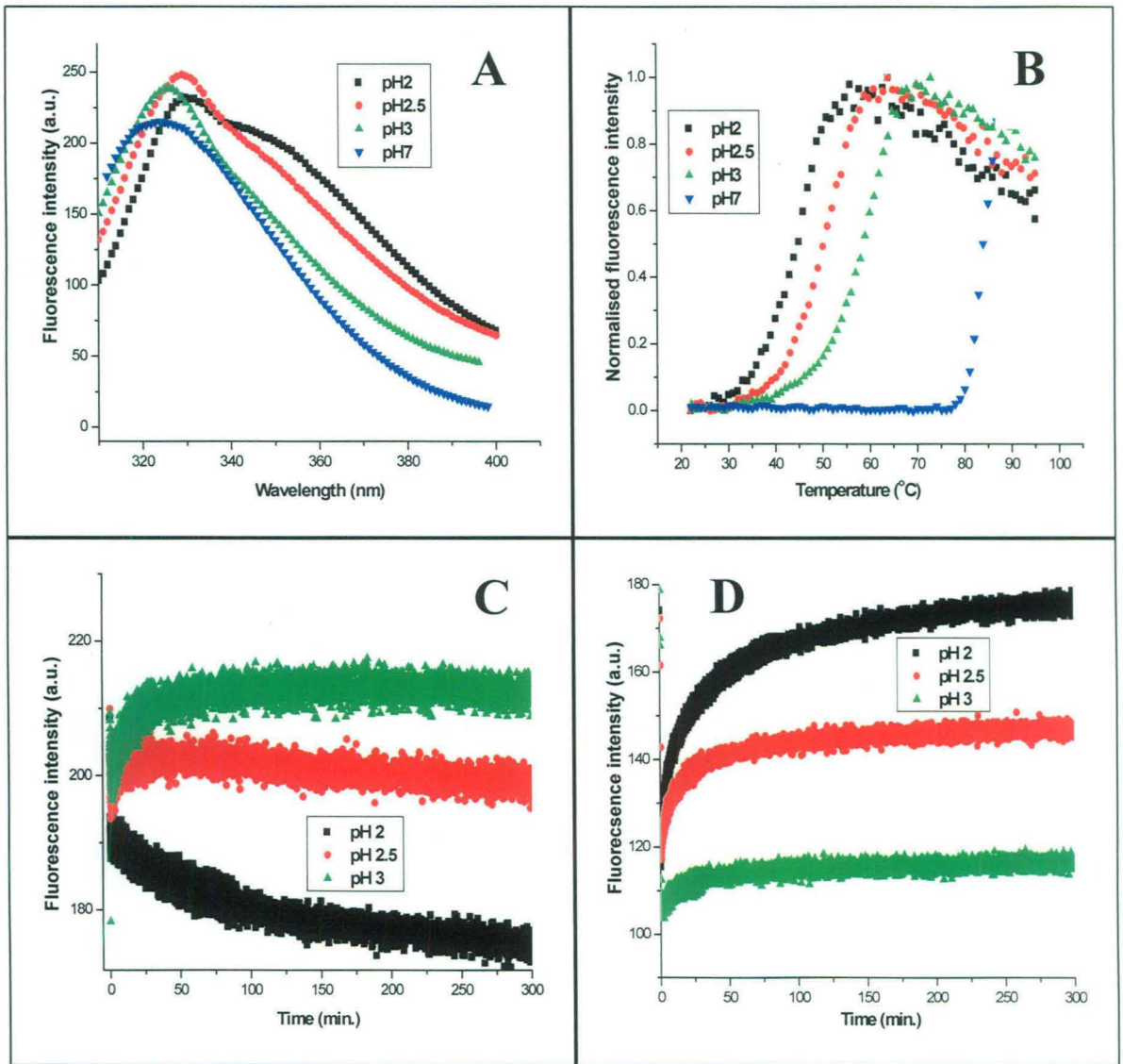

Figure 4.6: A. fluorescence spectra and B. Thermal denaturation transition of Human γD Crystallin in phosphate buffer at pH 7 and Glycine-HCl buffer at pH 2, pH 2.5 and pH 3. pH induced unfolding kinetics: unfolding at pH 2, 2.5 and 3, measured at C. 320nm and D. at 360 nm respectively when excited at 295 nm.

The observation matched with spectral changes at acidic pH suggesting maintenance of most of the native tertiary structure at pH 3 (figure 4.6 A). Native emission at 320 nm underwent a decrease at pH 2, which clearly indicated disruption of electrostatic interactions necessary for maintaining the conformational integrity of the protein. This was supported by a larger increase in 360 nm emission and a shoulder emerging at 355 nm in fluorescence spectrum of the protein at pH 2. pH induced denaturation kinetics at pH 2.5 was observed to be of intermediate characteristics between that of pH 2 and pH 3. Attempts to fit the kinetics data with equations were not successful due to precipitation problem and rates of unfolding therefore could not be derived out of denaturation kinetics data. The data nevertheless suggested that pH induced denaturation was qualitatively different at pH 3 than at pH 2 and different sets of electrostatic interactions were getting disrupted in each case. These sets of electrostatic interactions were however different in their role in determining characteristics of the intermediate conformation. On one hand, interactions getting perturbed at pH 3 seem to be critical in determining native tertiary contacts around tryptophans; those disrupted at pH 2 seemed to be very critical in determining both energetic and kinetic stability of the protein.

Thus, the changes in pH could effectively perturb electrostatic interactions that were critical in determining both thermodynamic and kinetic aspects of the protein stability.

Effect of NaCl on stability, folding and aggregation characteristics of Human γD crystallin

As salts also influence the stability and structure of proteins, effect of NaCl on Human γD crystallin upon thermal aggregation was studied. The effect did not follow a linear concentration dependence (figure 4.7 A). While lower concentrations of NaCl could only delay the heat induced aggregation, higher concentrations of NaCl lead to a faster and more pronounced aggregation of the protein. NaCl effect on heat induced aggregation indicates that electrostatic interactions present on the protein surface are crucial in determining aggregation under thermal stress and respond differently to lower and higher ionic strength of the solvent preferring lower levels (millimolar) of salt concentration.

The delay in heat induced aggregation at lower concentrations of NaCl could be due to screening of the unsatisfied charges on the protein surface leading to further strengthening of the surface electrostatic interactions and therefore resisting heat induced destabilizing effects. This delay of heat induced aggregation at lower NaCl concentration suggest that surface salt bridges are important in determining not only thermodynamic stability but may also provide kinetic stability against thermal unfolding or destabilization of the protein. Higher concentrations of NaCl on the other hand could screen even those charges which are involved in forming surface salt bridges opening the protein interior to solvent and therefore heat induced destabilization would instead facilitate aggregation of solvent exposed hydrophobic patches.

In thermal aggregation conditions protein has destabilized conformation with increased configurational dynamics and solvent has high kinetic energy with high entropy. Thus at higher temperatures, both increased conformational dynamics and solvent entropy factors destabilize the electrostatic interaction. In order to understand conformational entropy of the protein alone as a factor to favor/disfavor aggregation, studies investigating the effect of NaCl on aggregation while refolding of chemically denatured states at room temperature were conducted (figure 4.7 B). Thus the role of solvent mediated entropy factors in disrupting the interactions or forced hydrophobic solvation could be avoided.

Propensity of the heterogeneous conformations to aggregate while refolding the chemically denatured state was more than that of those accumulating at higher temperatures. Aggregation propensity of the folding protein was observed to be so high and fast that slit width for detecting the fluorescence signal had to be reduced from 2.5 nm to 1.5 nm. This suggests that protein conformational heterogeneity when folded native state is in kinetic competition with other conformational states is far more and aggregation causing as compared to those when high solvent entropic factors at higher temperatures determine the conformational heterogeneity and aggregation.

In studies on aggregation while refolding protein from chemically denatured state, presence of NaCl could not effectively delay or counter the protein aggregation under folding stress. In fact, lower concentrations of NaCl enhanced aggregation, that too

within dead time of recording aggregation kinetics. This emphasizes that though electrostatic interactions and tertiary contacts in the form of surface salt bridges are more

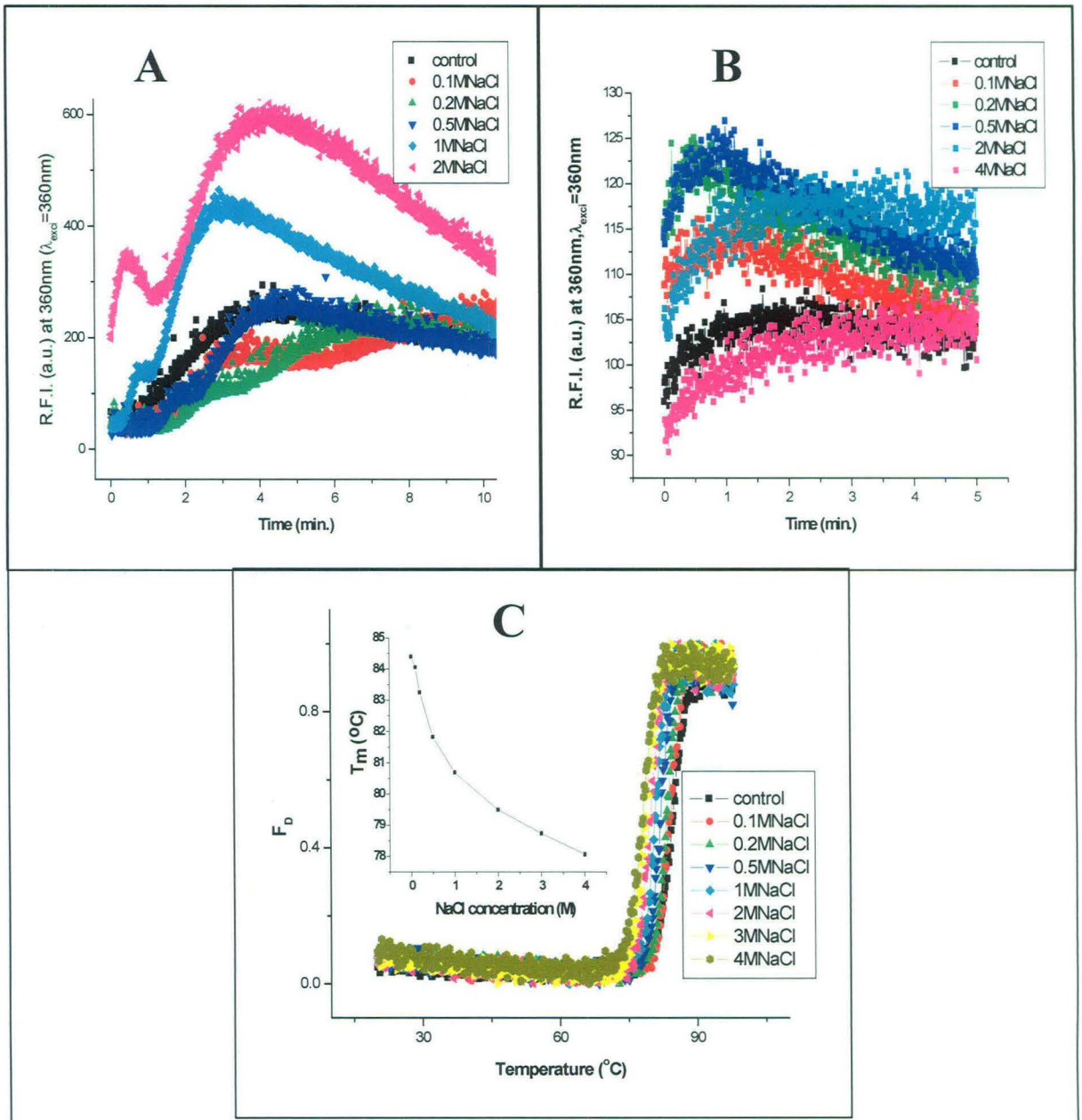

Figure 4.7: A. Heat induced aggregation kinetics of Human γD Crystallin at 80°C in varying concentrations of NaCl. B. Aggregation kinetics of Human γD Crystallin at pH7, 25°C while refolding from chemically denatured state in varying concentrations of NaCl (λ_{exci}=360nm and $\lambda_{emission}$=360nm). C. Thermal denaturation transition of Human γD Crystallin in varying concentrations of NaCl.

likely to be formed at the later stages of folding after establishing proper secondary structural framework, disturbance of some of these electrostatic interactions in presence of NaCl could effectively lead to an enhanced aggregation. Such interactions might therefore be playing critical role in determining the folding trajectory of the protein at relatively earlier stages. Presence of NaCl could only lead to an enhancement in aggregation while refolding chemically denatured state. This enhancement however did not follow concentration dependence. While lower concentrations of NaCl enhanced aggregation, relatively less enhanced aggregation was observed at higher concentration of NaCl. Low concentration of NaCl could interfere with crucial electrostatic interactions to put folding polypeptide towards aggregating conformations. High concentrations of NaCl could lead to screening of all the charges which might disrupt even those electrostatic interactions which would lead the folding pathways towards off-pathway aggregating intermediates. Possible screening of such charges could relatively reduce the aggregation propensity of the refolding protein at high concentrations of NaCl. Thus, different electrostatic interactions of the protein could prove to be critical in determining the alternative routes of folding pathways by determining both on- and off-pathway intermediates selectively.

Aggregation intensity of the protein while folding as well as when induced at high temperature show different patterns at low and high NaCl concentrations addressing different sets of interactions. In order to further investigate the kinetic significance of electrostatic interactions against thermal and chemical induced unfolding stress, protein was subjected to unfold at 95 ^{o}C (figure 4.8) and 5.5 M GdmCl (figure 4.9) separately in varying concentration of NaCl and their respective unfolding kinetics was observed. A clear concentration dependence in both modes of unfolding could be detected which like in aggregation studies did not follow a linear trend. Like in thermal aggregation, lower concentrations of NaCl could slow the rate of unfolding but lead to an enhanced rate of unfolding at higher NaCl concentration. This further confirmed that surface electrostatic interactions might provide kinetic stability to the protein which gets further strengthened at lower concentrations of NaCl leading to a slower rate of unfolding against chemical and thermal stress. The same interactions get disrupted by charge screening at higher concentration of NaCl leading to an enhanced rate of unfolding. The aggregation

enhancement at high NaCl concentration could however be also due to its salting-out effects because at high concentrations salts and the protein compete for solvation water (Kirkwood, 1943)

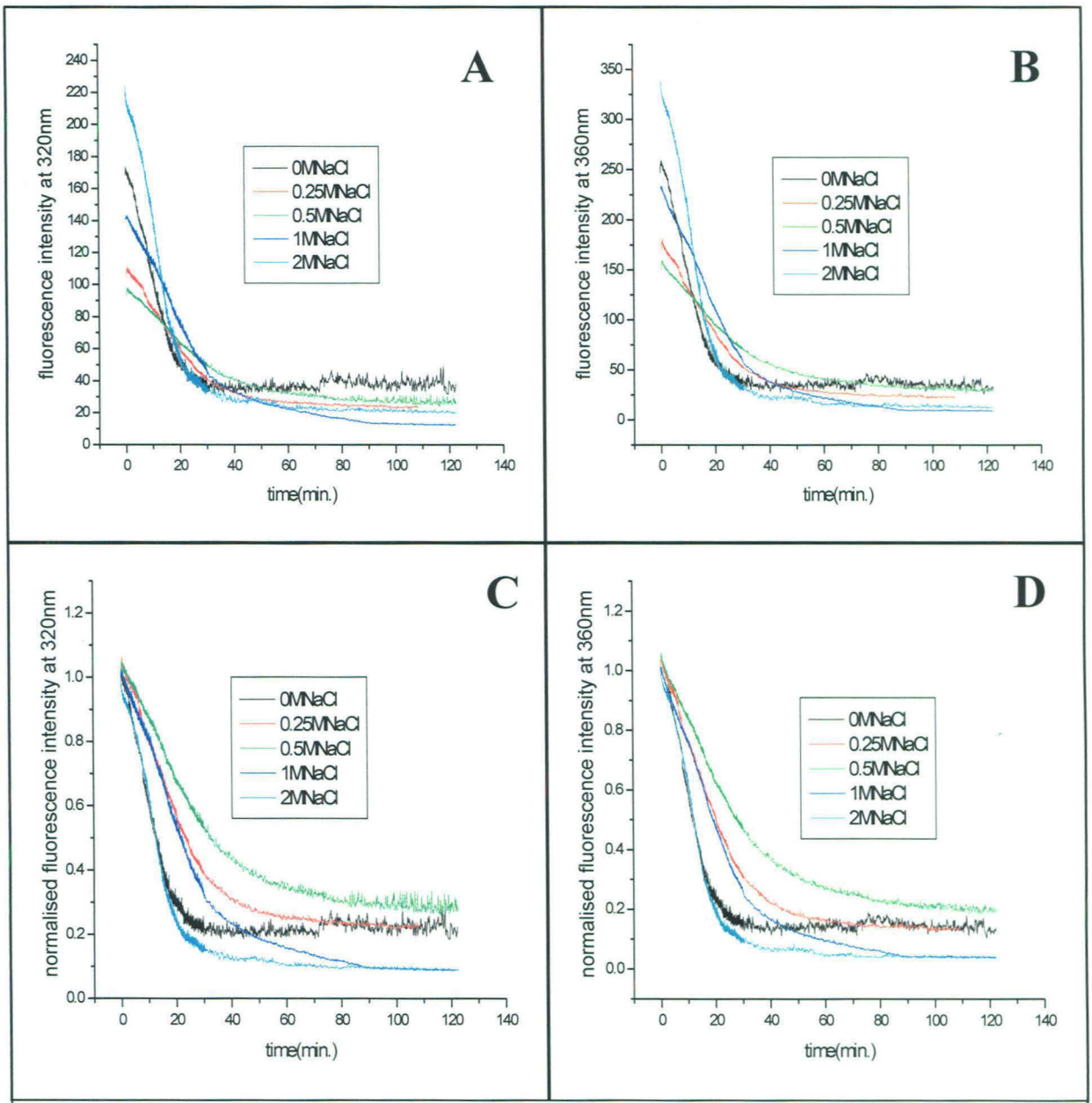

Figure 4.8: Thermal unfolding kinetics of Human γD crystallin at 95°C recorded at A. 320nm and B. at 360nm. C & D. Thermal unfolding kinetics obtained after normalizing fluorescence intensity in each case.

Concentration dependent anomaly on protein aggregation and unfolding kinetics prompted us to investigate the effect of NaCl on protein stability to see whether or not anomaly shows its pattern in melting transition too or not. So, the thermal denaturation transition of the protein was conducted at different concentrations of NaCl. Mid transition temperature of the thermal denaturation transition was observed to decrease with increasing concentration of NaCl. Thus, screening of the charges and therefore disruption of electrostatic interactions lead to reduced protein stability and there was no concentration dependent anomaly, though a linear concentration dependence was not observed. This was in contrast to the effect of NaCl on aggregation tendency and unfolding rate of the protein.

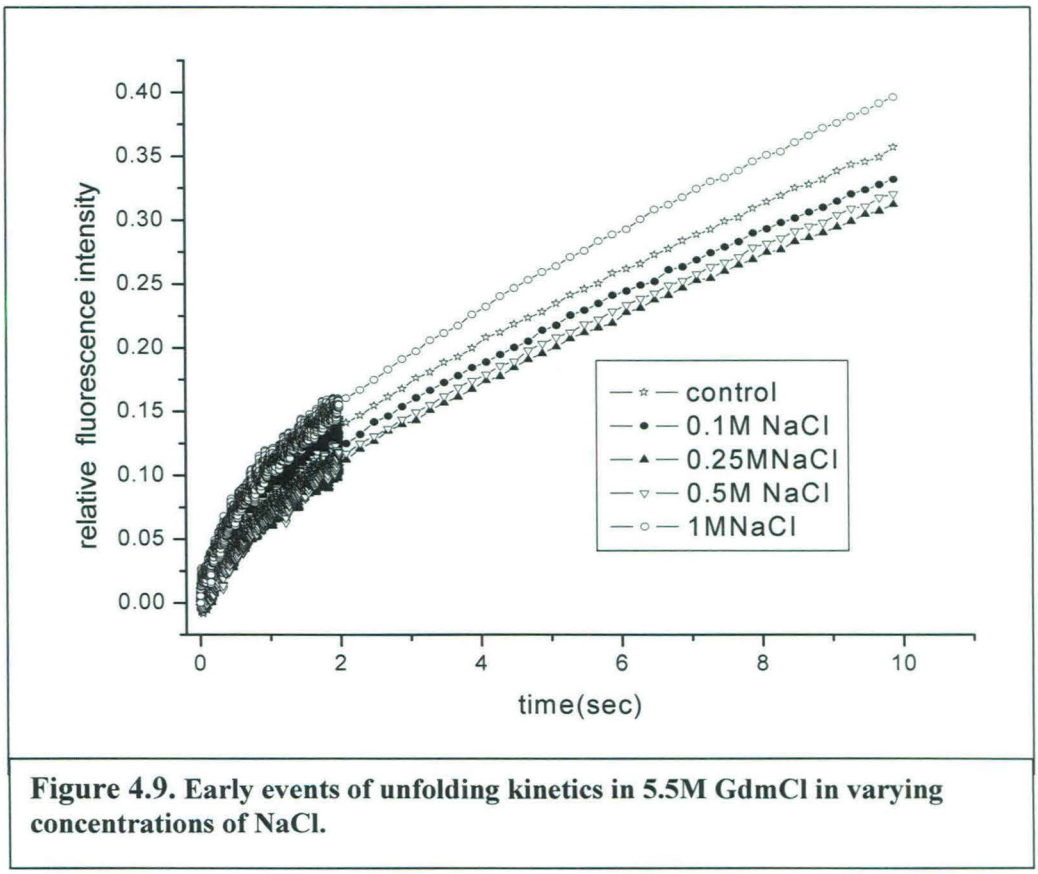

Figure 4.9. Early events of unfolding kinetics in 5.5M GdmCl in varying concentrations of NaCl.

Thus, the electrostatic interactions seem to affect protein stability, folding and aggregation in different ways. On one hand electrostatic interactions seem to be critical in determining protein stability (as is revealed by decreasing T_m with increasing concentrations of NaCl); on the other hand surface electrostatics at lower concentrations

of NaCl seem to get kinetically strengthened to counter heat induced unfolding and aggregation. Similar NaCl concentrations however promote aggregation while refolding chemically denatured state, possibly by interfering with the critical electrostatic interactions of the on-pathway intermediates.

Small Angle X-ray Scattering (SAXS) studies dealing with effect of ionic strength and protein concentration on protein self interactions have revealed that variation in ionic strength can be a good tool to modulate long range and short range protein self interactions leading to alteration in its stability. The competition between long-range attractive and short-range repulsive interactions in varying ionic strength of the aqueous solution have already been reported in case of BSA protein to bring alteration in its stability (Zhang et al., 2007). Human γD cryatallin is known for its long range attractive order among itself and the order is of electrostatic in nature. Presence of NaCl could modulate this electrostatics to alter the long range interactions and therefore could disrupt the interactive order. Any attempt for a charged molecule to be utilized as crystallin stabilizer should be used with caution is it could interfere with the interactive order of crystallin.

Human γD crystallin is enriched with surface electrostatics with residues like arginine, lysine and proline along the hydrophobic sequences at almost regular interval. Naturally occurring aggregating-prone hydrophobic sequences are selectively capped with charged residues called "gatekeeper residues" (Rousseau et al., 2006). The structural gatekeepers are reported to have significant effect on the protein folding as it is found that when gatekeepers are eliminated, the thermodynamic signature of a folding intermediate emerges, and a marked decrease in folding efficiency is observed (Korepanova et al., 2002; Pedersen et al., 2004; Rousseau et al., 2006; Matysiak et al., 2006). The gatekeepers are found to have distinct functions and guide the folding and time-dependent packing of secondary structure elements in the protein. Thus individual gatekeeper residues may thus play a large role in guiding proteins through different folding pathways (Otzen, 2005). They avoid kinetic traps during folding by favoring the formation of "productive topologies" on the way to the native state (Stoycheva et al., 2004). They are also important in determining chaperone selectivity for strongly aggregating regions. Meanwhile, large-scale modulation of thermodynamic protein

folding barriers can be linked to electrostatics as evidenced by the recent reports like in case of alpha-lactalbumin, where stabilization of partially unfolded conformations by electrostatic interactions fact lead to relatively smaller thermodynamic folding barrier (Halskau et al., 2008). This modulation is absent in other proteins like lysozyme indicate that electrostatic surface interactions might be utilized for the modulation of folding barriers in response to special functional requirements within a given structural fold. Human γD crystallin is also provided with such surface electrostatics with gatekeeper residues, alteration in their charges or protonation status could therefore bring changes in stability and aggregation characteristics of the molecule.

Thus the studies dealing with pH and NaCl and their effect on aggregation, folding and stability suggest that electrostatic interactions on the surface of Human γD crystallin play important role in determining its free energy barrier towards unfolding as well as in smoothening kinetic barrier of the folding polypeptide.

Human γD crystallin is known to be a calcium binding protein with 90 μM affinity (Rajini et. al., 2002). The observation that free calcium is found in cataractous lenses, they seem to be necessary for the protein to remain soluble at its high concentration. Thus, the calcium binding property of the protein is expected to influence its response to different solvent conditions. So to find out whether or not the calcium binding property of the protein could rescue it against its aggregating tendency against thermal stress, the protein was subjected to heat induced aggregation at 80 ^{o}C in presence of $CaCl_2$. Presence of $CaCl_2$ was found to delay as well as suppress the thermal aggregation of the protein (figure 4.10). This indicated that the presence of calcium could enhance the thermodynamic and kinetic stability of the protein against thermal stress. Calcium binding sites on the protein are not known to have charged residues on the protein surface and calcium binds through polar but uncharged residues. The fact that the ion-protein interaction can be both charge-dependent and charge-independent (Ciferri, 2008) and that the protein surface electrostatic interactions and protein hydration are coupled aspects, there exists a possibility that together these aspects could determine both protein solubility and stability. Thus, there exists a strong possibility of connection between calcium binding sites and surface electrostatic interactions and the two combining together to alter stability and solubility characteristics of the protein.

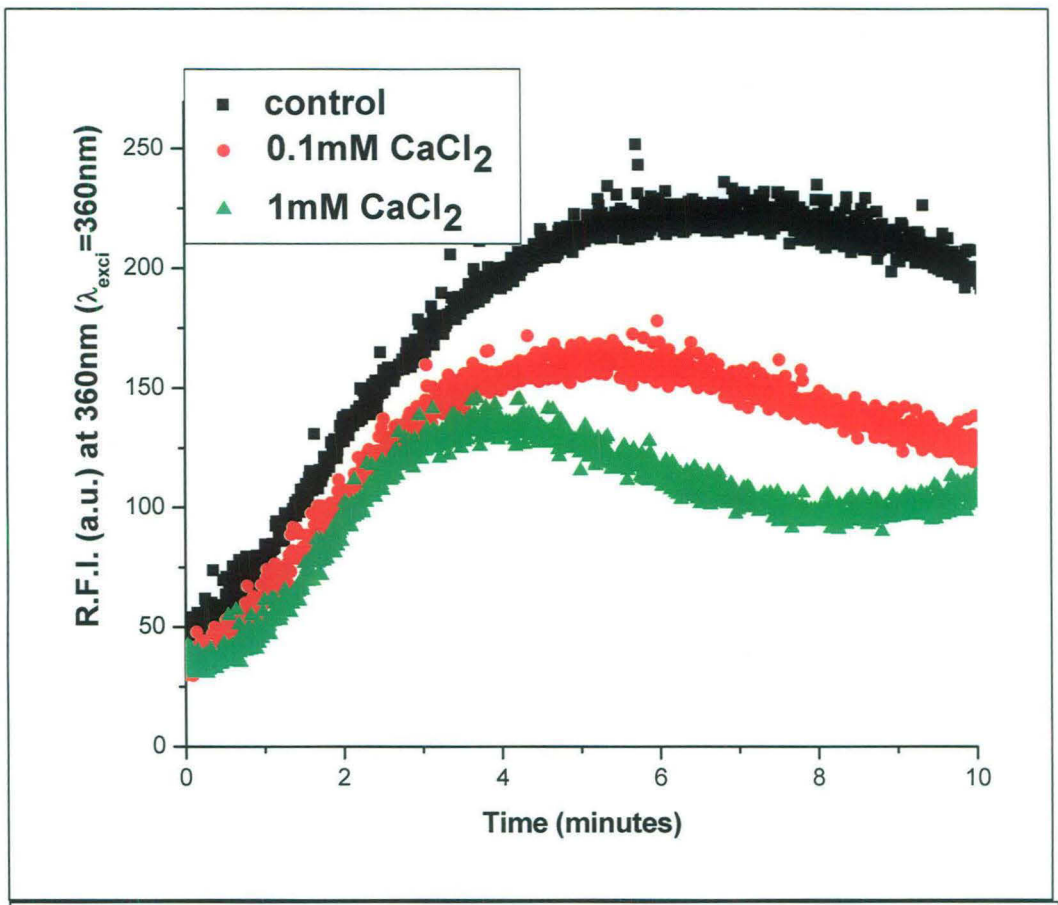

Figure 4.10: Heat induced aggregation kinetics of Human γD Crystallin in phosphate buffer pH 7 at 80 °C, in varying concentrations of $CaCl_2$ (λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

Therefore the delay and suppression of thermal aggregation observed in presence of calcium might be due to the calcium mediated indirect support to protein surface electrostatic interactions and to protein hydration thereby leading to enhanced protein stability and solubility. Further experiments are needed to support or defy this hypothesis.

Chapter 5

Effect of some cosolvents [amino acids & their derivatives, poly(ethylene)glycols and polyols] on Human γD crystallin aggregation

Effect of amino acids on thermal aggregation of Human γD crystallin

Arginine, Proline, TMAO, Glycine and its methyl derivatives etc. are known to effect protein's conformation in different ways. While some of these like TMAO, Proline Sarcosine, Betaine etc. are known for their preferential hydration of the protein's surface leading to its stabilization (Auton et al., 2008; Wang and Bolen, 1997, Taneja and Ahmad, 1994; Arakawa and Timasheff, 1985); some like Arginine are "neutral crowders"

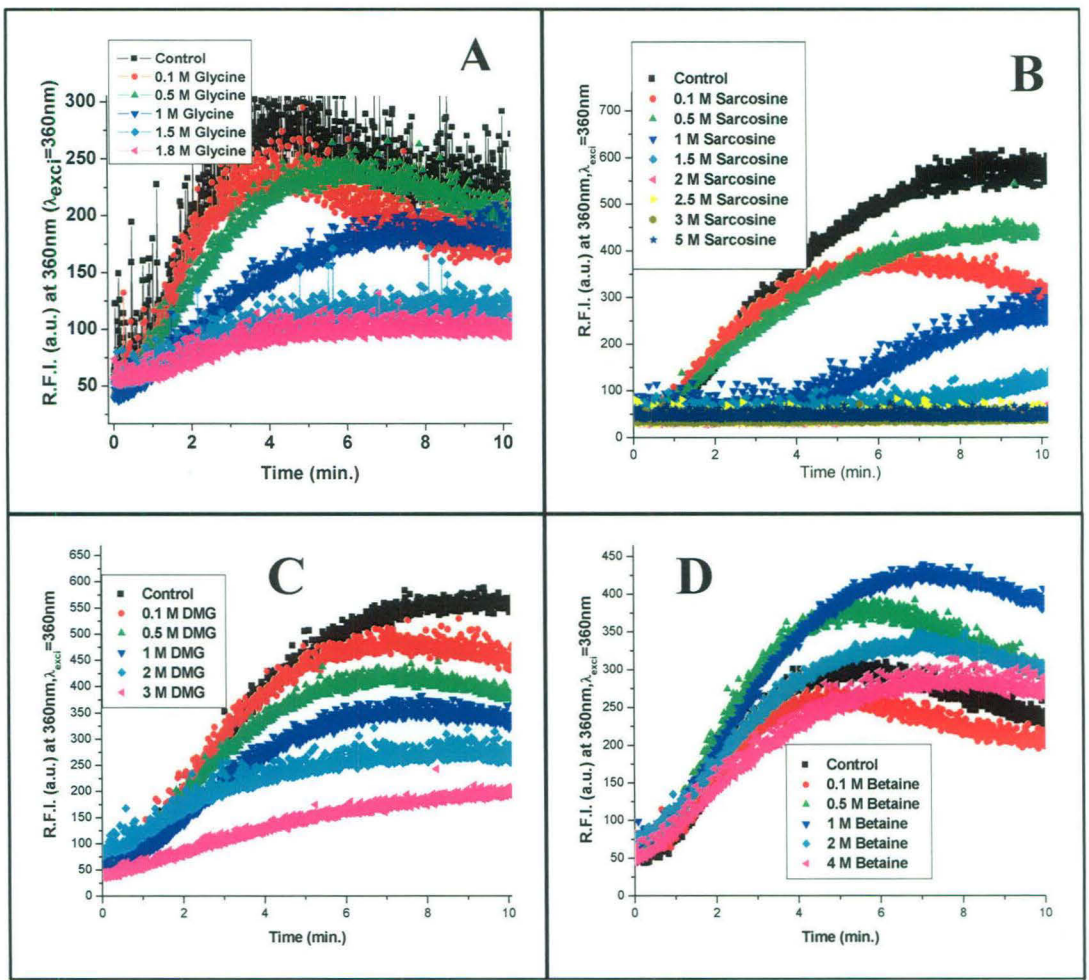

Figure 5.1: Aggregation kinetics of Human γD Crystallin at 80 °C, pH 7 in varying concentration of A. glycine and its methyl derivatives B. Sarcosine C. DMG and D. Betaine(λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

having very small effect on free energy of protein molecules (Baynes et al., 2005; Tsumoto et al., 2005) and suppress heat induced protein aggregation(Arakawa et al., 2006). Effect of these charged cosolvents on an aggregation prone Human γD crystallin having Greek key domain is still not explored.

When the protein was subjected to heat induced aggregation, the presence of glycine could delay and suppress aggregation by ~75 % at 1.8 M (figure 5.1 A). Higher concentration of glycine could not be tried because of its solubility problems. In order to explore that how high temperature conditions can possibly be modulated by increasing hydrophobic character of the charged cosolvent, effect of methyl derivatives of glycine on thermal aggregation was investigated. It was observed that presence of Sarcosine could also delay and suppress aggregation and could completely inhibit it at 2 M concentration (figure 5.1 B). This way Sarcosine was proved to be a better stabilizer than Glycine both in delaying the onset and suppression of thermal aggregation. Thus, prevention of thermal aggregation by both Glycine and Sarcosine can be explained by their effective stabilization against thermal unfolding both energetically and kinetically. This is because thermodynamic stabilization could be achieved by preferential hydration and the kinetic stability could be improved by preventing rate of protein unfolding. Also, overall aggregation could be restricted by preventing protein-protein interaction between partially denatured conformations through steric exclusion.

Presence of dimethyl glycine (DMG) could suppress the thermal aggregation only moderately but was not affective against onset of aggregation (figure 5.1 C). This might be because DMG could not affect the rate of unfolding to expose hydrophobic patches but could effectively counter protein-protein interaction by binding to already exposed hydrophobic patches. Thus cosolvents seem to address kinetic and energetic factors in a different ways with DMG being moderately affective against energetic factors only. Thus, with Sarcosine being more effective than Glycine, increasing hydrophobicity of the cosolvent is found to be effective against thermal aggregation. But the observation that DMG is less effective than both glycine and sarcosine in preventing thermal aggregation, it can be postulated that there must be a threshold hydrophobic content in the molecule to effectively inhibit different stages of thermal aggregation.

When Betaine (a higher member of the methyl substituted glycine series) with three methyl groups was used against thermal aggregation at 80 ^{o}C, it was not found to be affective against thermal aggregation, instead its presence lead to enhanced aggregation (figure 5.1 D). The enhancement in aggregation though was not in a concentration dependent manner. Betaine at lower concentration lead to enhanced net aggregation with

more or less no effect on onset of aggregation which suggest that rate of protein unfolding at higher temperature remain almost unaffected but betaine could facilitate protein-protein interaction by acting as bridge between already formed hydrophobic patches. Charged status of betaine here could screen free charges on the protein surface (formed by disrupted electrostatic interactions at high temperature), thus facilitating factors associated with hydrophobic aggregation. However, at high concentration, steric exclusion of the free betaine molecules in the solvent (which remain unbound to partially denatured protein) could interfere with protein-protein interaction, leading to a relative decrease in thermal aggregation.

TMAO is known to stabilize proteins against denaturation stress by preferentially hydration of the native protein (Wang and Bolen, 1997). At the same time studies reporting pH dependent stabilization/destabilization by TMAO has also been reported (Singh et al., 2005) which indicate stabilization by TMAO may or may not extend to every condition or conformation. In order to investigate how this stabilizing molecule would respond at high temperature mediated aggregating conditions and whether stabilizing behavior of TMAO would extend to heterogeneous non-native aggregating conformations too, Human γD crystallin was subjected to thermal aggregation conditions in the presence of varying concentration of TMAO.

Presence of lower concentration of TMAO could only delay the onset of aggregation (figure 5.2 C) which indicates that the cosolvent could possibly slow down the rate of thermal unfolding which could be due to known preferential hydration effect of TMAO on the protein. Presence of higher concentration could affectively suppress the thermal aggregation and complete suppression could be possible at 4 M TMAO. Complete suppression of thermal aggregation here can be explained by both stabilization of the native state against thermal unfolding by preferential hydration and inhibition of protein-protein interaction by TMAO remaining free in the solvent and sterically excluding the interacting proteins. The ability of TMAO to suppress thermal aggregation coincides with its natural compatibility towards proteins as it is reported to have no effect on the strength of hydrophobic interactions (Athawale et al., 2005). This could avoid possible interference with hydrating property of TMAO under denaturing conditions.

Studies with Sarcosine, Betaine and TMAO preventing the thermally induced

aggregation of Serpins have been previously reported (Chow et al., 2001). Our results with Sarcosine and TMAO coincide with these reports but Betaine is unable to suppress aggregation. Glycine has no methyl group, Sarcosine has only one, Dimethylglycine has

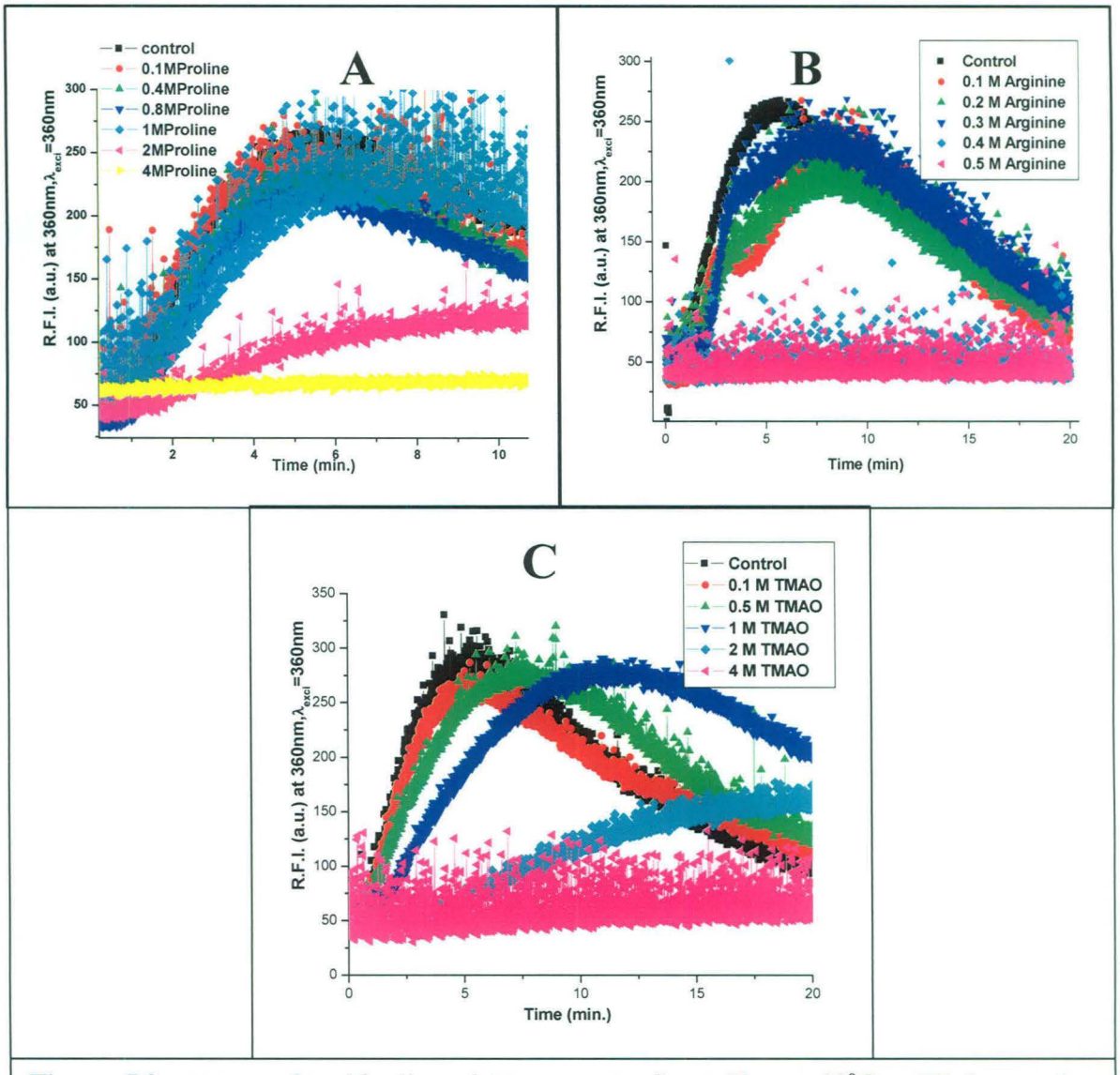

Figure 5.2: Aggregation kinetics of Human γD Crystallin at 80°C, pH7 in varying concentration of A. Proline B. Arginine and C. TMAO (λ_{exci}=360nm and $\lambda_{emission}$=360nm).

two and Betaine having three methyl groups. High hydrophobic content of betaine with three methyl groups could lead to its binding to protein which at high temperature might not facilitate its otherwise hydration or exclusion properties to bring down aggregation. Had the sole criteria behind aggregation suppression be having less of hydrophobic content Glycine would have been the best aggregation suppressor as it has minimum

hydrophobic content. But Sarcosine is better aggregation suppressor than glycine. Therefore, both the charged status and the hydrophobic content together determine whether the molecule would be an aggregation suppressor or an aggregation enhancer. These observations also support the fact that exclusion and preferential hydration of the protein by cosolvents depends on interactions between the protein surface and the cosolvent, and must respond differently to polar and non polar surface areas of the protein (Auton and Bolen, 2005; Singh et al., 2005).

Proline is also known among the amino acid cosolvents to prevent aggregation both *in vitro* and *in vivo* and is believed to act through a combination of solvophobic backbone interactions and favorable side-chain interactions that are not specific to a particular sequence or structure (Ignatova and Gierasch, 2006; Kar and Kishore, 2007). Lower concentration of Proline could not affectively act against thermal aggregation (figure 5.2 A). It was only at 2 M that its presence could suppress aggregation significantly and complete suppression could be observed at 4 M concentration. Thus, on one hand backbone solvophobic property could lead to native state stabilization of the protein, on the other hand favorable side chain interaction of the proline could interact with side chains of partially exposed hydrophobic patches to interfere with protein-protein interaction. Both these properties of proline when combined together enable it to avoid protein-protein interaction meanwhile favoring compact and less aggregation prone states to bring down heat induced aggregation.

Arginine is another amino acid which is commonly used to prevent aggregation while protein refolding to increase refolding yield. Presence of arginine could suppress thermal aggregation very efficiently and a complete suppression was observed at 0.4 M concentration (figure 5.2 B). Thus, on subjecting protein to high temperatures in the presence of these amino acids, Arginine was found to be the most effective amino acid against the heat induced aggregation. Sarcosine could completely suppress aggregation at ~ 2 M concentration. While betaine could not lead to any suppression in aggregation, DMG even at 3 M could not completely suppress, Glycine was partially effective at ~ 2 M and Proline and TMAO did succeed in preventing aggregation completely but only at higher concentration of 4 M. Thus, the order of effectiveness of these cosolvents to suppress preventing heat induced aggregation completely was determined to be Arginine

> Sarcosine > Proline ~ TMAO.

Arginine is the most effective amino acid against aggregation against thermal stress indicates that their property of forming molecular clusters (Das et al., 2007) might be playing an important role in enhancing their efficacy in countering thermal aggregation as rest of the amino acids like betaine or TMAO are not reported to show any hydrophobic self-association behavior in aqueous solution or form molecular clusters (Paolantoni et al., 2006). These molecular clusters of Arginine are neutral and relatively bigger in size and therefore result in better steric exclusion of the protein surface which acts against protein-protein interaction. Thus, Arginine inspite of not being a protein stabilizer (like TMAO, Sarcosine etc.), could possibly suppress heat induced aggregation by avoiding protein-protein interactions with its molecular cluster forming property.

Thus Arginine is distinguished from other amino acid aggregation suppressors in the aspect where self associating cluster forming tendency makes it a good cosolvent to be used *in vitro*. But TMAO and glycine derivatives remain the choice *in vivo* as the absence of hydrophobic behavior in the aqueous solution make them more compatible and non-interfering with biochemical processes in the cell even if they exist at high concentrations (Paolantoni et al., 2006).

Effect of series of polyethylene glycols and polyols on aggregation of Human γD crystallin

Polyethylene glycols are known for their crowding effects and have varying hydrophobic chain lengths. They tend to preferentially exclude the protein surface Arakawa and Timasheff, 1985; Lee and Lee, 1987). Preferential exclusion of PEGs from proteins is due principally to the steric exclusion of PEG from the protein domain, although favorable interactions with protein surface residues, in particular nonpolar ones, may compete with the exclusion (Bhat and Timasheff, 1992; Arakawa and Timasheff, 1985). These thermodynamically unfavorable preferential exclusion interactions lead to the action of PEGs as precipitants, although they may destabilize protein structure at higher temperatures. In our experiments with polyethylene glycols (PEGs) on thermally induced aggregation of Human γ D crystallin (figure 5.3), PEG 400 was found to be most effective against heat induced aggregation. PEG 200 was also effective and could

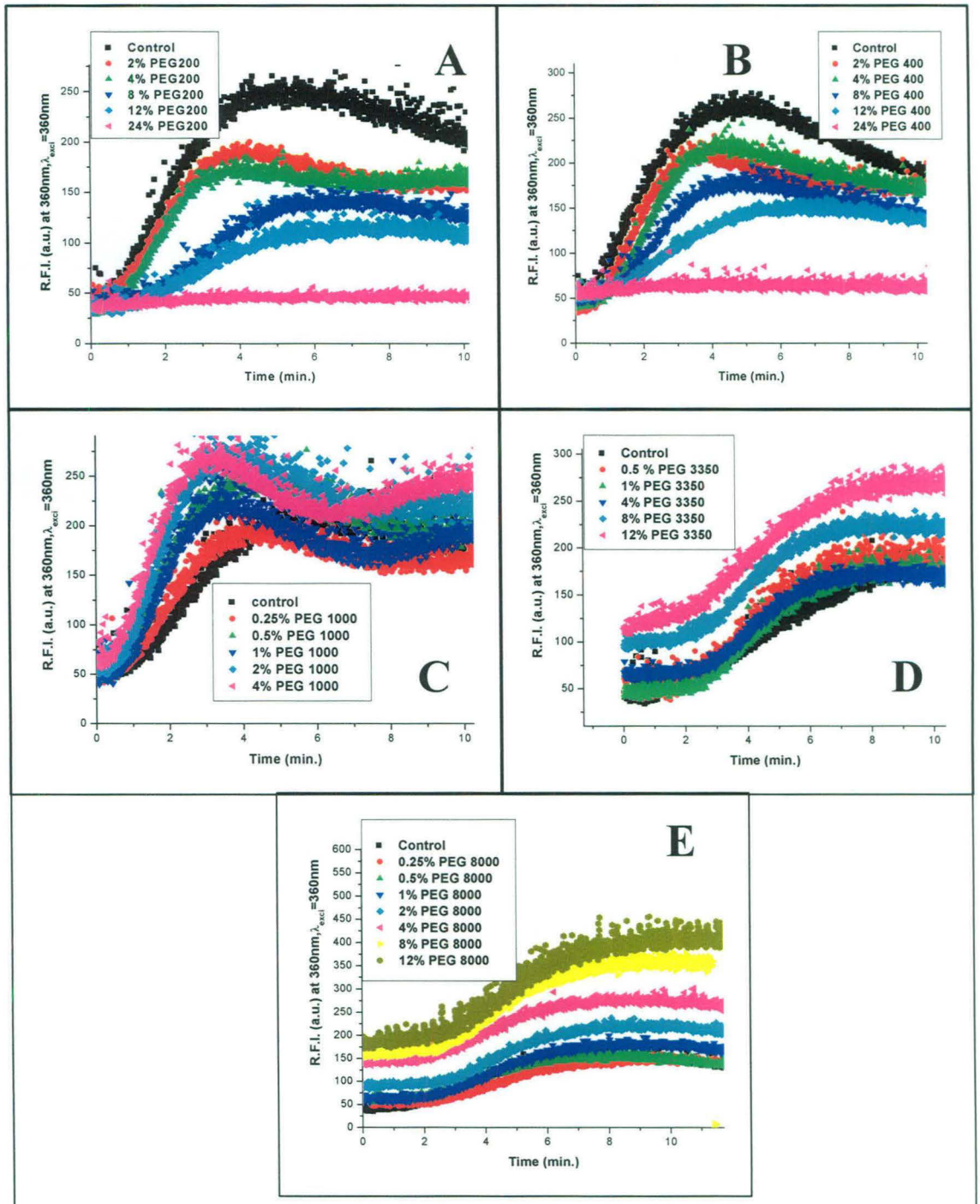

Figure 5.3: Aggregation kinetics of Human γD Crystallin at 80 °C, pH 7 in presence of varying concentration of increasing size of PEGs. A. PEG 200. B. PEG 400. C. PEG 1000. D. PEG 3350 and E. PEG 8000 (λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

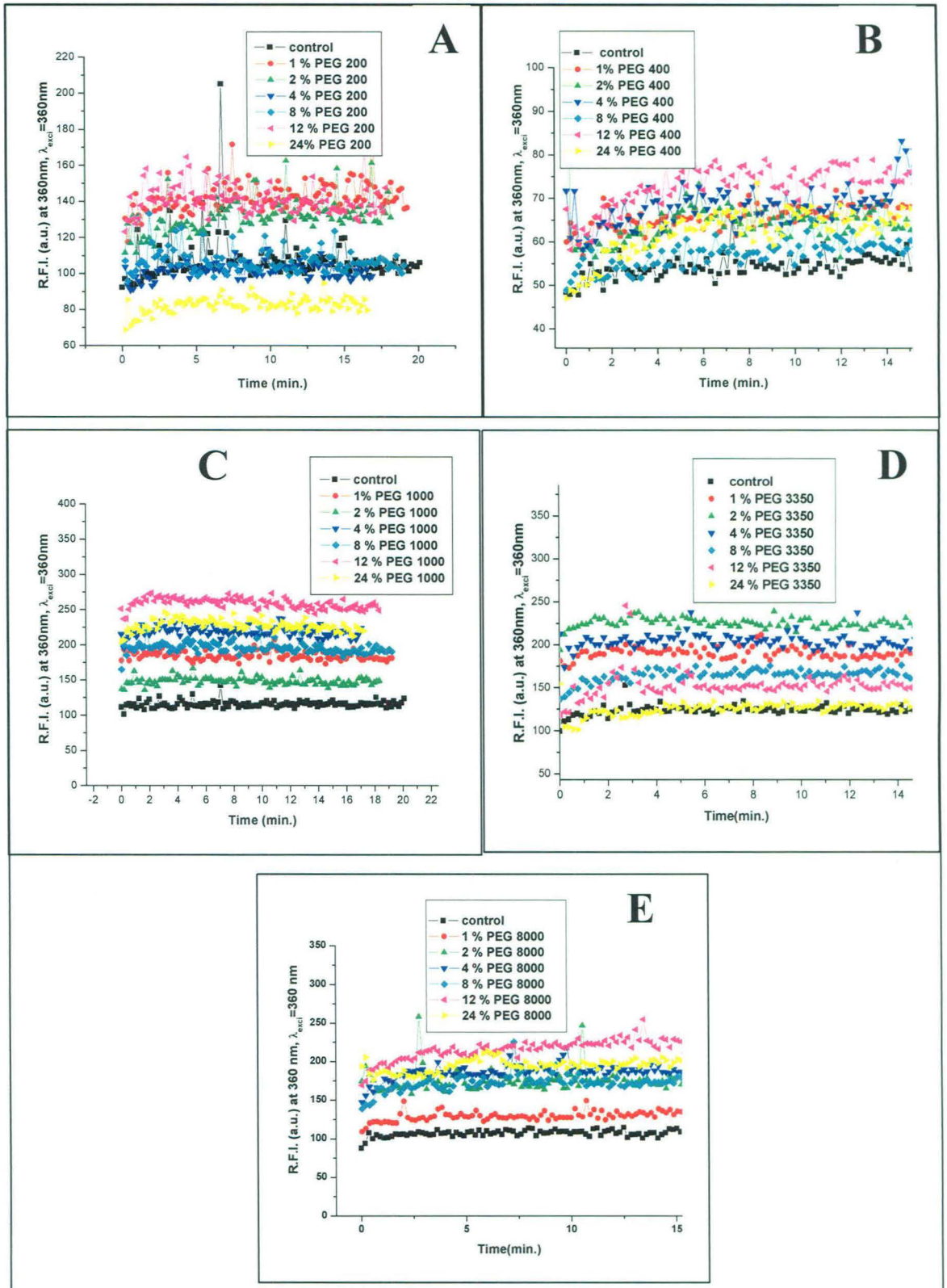

Figure 5.4: Aggregation kinetics of Human γD Crystallin while refolding chemical denatured protein in presence of varying concentration of increasing size of PEGs at 25 °C, pH 7. A. PEG 200. B. PEG 400. C. PEG 1000. D. PEG 3350 and E. PEG 8000. (λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

completely suppress the thermal aggregation but was found to be lesser effective than PEG 200. Higher members of the PEG series- PEG 1000, PEG 3350 and PEG 8000 were not effective against thermal aggregation at all. Also, the presence of some higher molecular weight PEGs (PEG 8000 and PEG 3350) lead to an enhanced thermal aggregation (figure 5.3 D & E).

Polyethylene glycols are the polymers of ethylene glycol. On comparing the effect of ethylene glycol with polyethylene glycols on thermal aggregation, while Ethylene Glycol was able to suppress thermal aggregation completely at 2.5 M (figure 5.5 A), PEG 200 could do the same at ~1 M and PEG 400 even at lower concentration of ~0.6 M, PEG 1000 being almost neutral towards thermal aggregation, and PEG 3350 and PEG 8000 enhanced aggregation. This indicates that the molecular lengths of PEGs are critical in determining aggregation behavior of the protein at higher temperature where the hydrophobic patches of the protein are all exposed. Providing these open hydrophobic patches with high hydrophobicity of larger chains of PEG 3350 and PEG 8000 could only further facilitate bridging of the partially unfolded protein. On the other hand, small chain size members with relatively lesser hydrophobicity could avoid this intermolecular bridging or aggregating effect under high entropic conditions of high temperatures leading to reduced thermal aggregation of the protein. This is also supported by observation from other members of the polyol series which are hydrophilic, small in size and chemically neutral in nature and could effectively counter the high temperature aggregating conditions (figure 5.5). Thus the heat induced aggregation is well countered by molecules of intermediate hydrophobic characteristics, intermediate size and high hydrophilic content.

PEG size dependence on thermal aggregation effect can be explained by its increasing hydrophobicity with increasing size. This coincides with water's increasing hydrogen bonding propensity with increasing hydrophobic characteristics of the molecule which also follow the temperature dependence of the hydrophobic effect (Xu and Dill, 2005). The fact that dissolving a nonpolar solute in hot water has positive impact on its entropy also supports the observation that aggregation increased with PEG's increasing size and hydrophobicity. This was expected because increased entropy would only

facilitate the aggregation propensity of the hydrophobic patches on the protein due to increased diffusion rate of the protein at high temperature conditions.

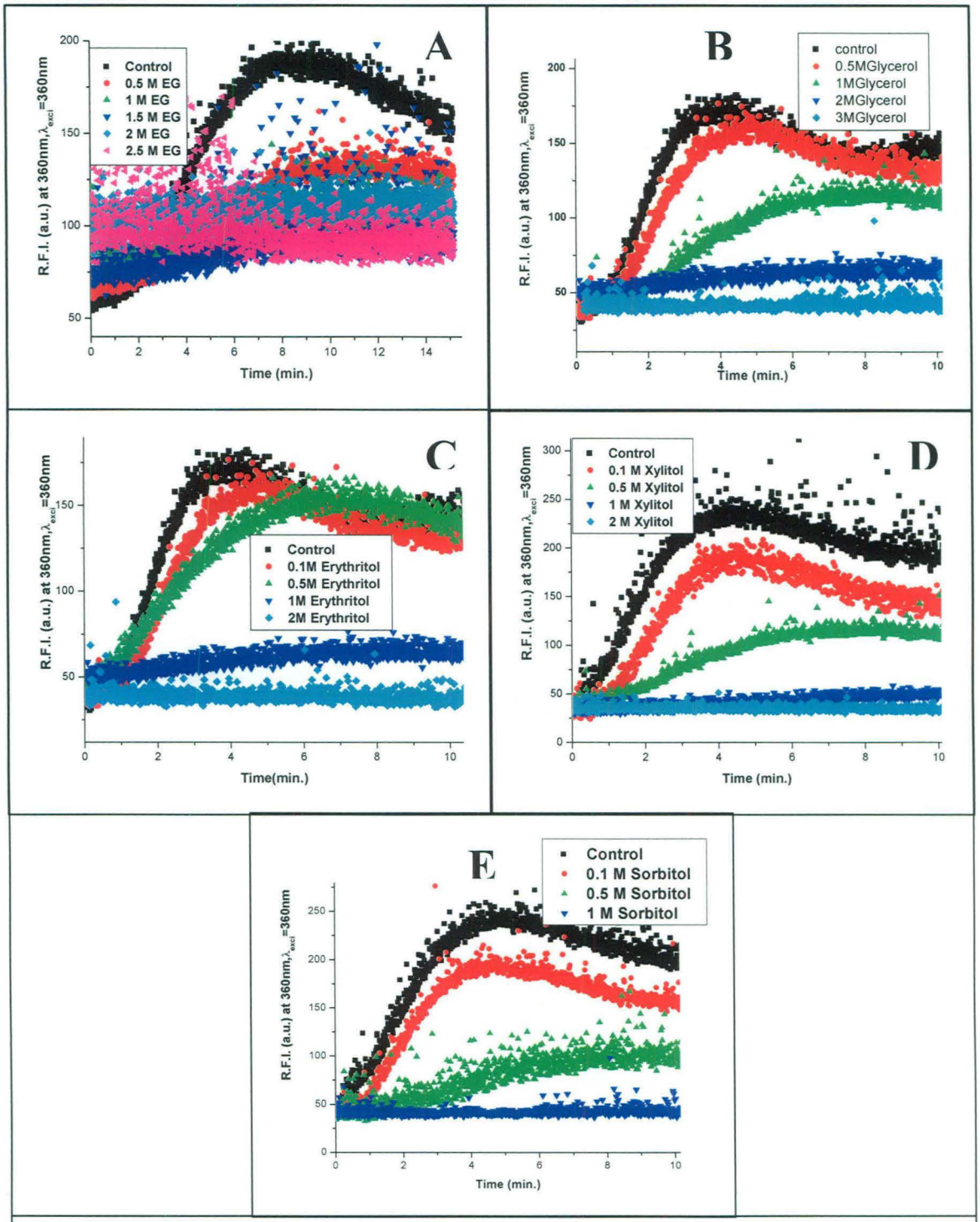

Figure 5.5: Aggregation kinetics of Human γD Crystallin at 80 °C, pH 7 in varying concentration of polyols. A. Ethylene Glycol B. Glycerol C. Eryhthritol D. Xylitol and E. Sorbitol (λ_{exci}=360 nm and $\lambda_{emission}$=360 nm).

In order to discriminate between the aggregation propensity of the protein under thermal stress and while refolding, and to find out how increasing hydrophobicity with increasing size of PEG molecule is able to address the two forms of aggregation, protein was subjected to aggregation while refolding chemically denatured protein under native conditions in presence of PEG. Results with Polyethylene Glycols on aggregation while refolding chemically denatured protein revealed that none of the polyethylene glycols (except by PEG 200 at high concentration with very less aggregation suppression) could effectively counter the aggregation while refolding, while some of the higher molecular weight members even enhanced the aggregation (figure 5.4). Comparison of thermally induced aggregation with aggregation while refolding and the effect of polyethylene glycols therein suggests that temperature induced high entropy conditions could be effectively countered by lower members of the PEGs which were small (but larger than Ethylene Glycol) and not too hydrophobic to bind to the partially unfolded protein and in the process got effectively excluded; on the other hand while refolding from chemically denatured state PEGs did not get effectively excluded from the protein surface in absence of such high entropy. Presence of far more heterogeneous and kinetically competing conformations (with open hydrophobic patches) in case of refolding (than under thermal stress) could only add into inefficiency of hydrophobic PEGs to suppress aggregation.

Polyol co-solvents such as glycerol increase the thermal stability of proteins (Tiwari and Bhat, 2006; Haque et al., 2005; Kaushik and Bhat, 2003; Gekko and Koga, 1981, 1982), are able to counter thermal induced aggregation (Singh et al., 2004, Mishra et al., 2007) and act as chemical chaperone to prevent aggregation while a protein is refolding (Mishra et al., 2005; Yu and Li, 2003). Our experiments in the presence of members of the Polyol series and their effect against thermal aggregation, sorbitol was found to be most effective member suppressing thermal aggregation at 1 M concentration (figure 5.5). Ethylene glycol could completely suppress aggregation at 2.5 M. The effect could be due to ethylene glycol mediated destabiliastion of the protein bringing protein to non-aggregating region in thermal transition thereby leading to less aggregation. For other polyols, complete thermal aggregation by 3M Glycerol, 2M erythritol, 2M xylitol and 1M Sorbitol could be acheived. Thus, with exception of ethylene glycol, increase in the numbers of hydroxyl groups in the polyols lead to increased suppression of thermally

induced aggregation.

These observations suggest that more hydrophilic characteristics of the chemically neutral polyols facilitate their protein's hydration and exclusion property to effectively facilitate thermal stability of the protein. Previous reports that the presence of polyols could induce structure into the unstructured segments of the protein (Kumar et al., 2007) and favor structure formation in loosely structured folding intermediates to facilitate protein folding (Mishra et al., 2007) further strengthens and supports our conclusion about the effectiveness of polyols to counter aggregation propensity of the protein. Polyols and its protein excluding and hydrating characteristics can be safely utilized to counter aggregation of Human γD crystallin.

Summary and conclusions

Critical role of the surface salt bridges and electrostatics of Human γD crystallin is already established in determining its attractive interaction order within itself. The pH and NaCl experiments conducted by us could further establish their importance and involvement in determining various aspects of protein stability. The observation of high mid point of transition temperature (T_m) during thermal denaturation, high mid point of denaturant concentration (C_m) during equilibrium denaturation by GdmCl and delay in onset of thermally induced aggregation at low concentration of NaCl further indicate their possible role and importance in altering protein stability. Effect of NaCl on aggregation while refolding indicated the possibility of an interplay of molecular charges in determining the on- and off- pathway intermediates which could effectively alter the folding trajectory of the protein. pH and NaCl effects thus could help us figure out the possible role of electrostatic interactions in determining folding and stability associated factors of Human γD crystallin.

Presence of proline and other ionic gatekeepers, tight packing density of the hydrophobic greek-key domain with high kinetic barrier for folding and buried cysteines are all important components governing the aggregation propensity and the kinetic aspects of the Human γD crystallin stability. This is further evident by the observation that aggregation while refolding is faster and more pronounced than thermally induced aggregation which again explains the fact that a protein in presence of significant kinetic barrier (to bring non-local secondary and tertiary contacts of a geek key structure in place), in absence of proper folding or assistance of chaperones, is highly prone to aggregation. High hydrophobic content of the core of Human γD crystallin in presence of molecular heterogeneity during folding could only increase the aggregation propensity of the molecule.

Use of cosolvents and investigation of their effect on protein aggregation is an important tool to explore the characteristics of the aggregation prone protein and the factors governing it. Arginine which can form supra-molecular assemblies was the most effective among the amino acids studied against thermal aggregation. The effect is a strong indication towards steric exclusion as an important factor in determining the aggregation suppressing ability of a cosolvent molecule. Meanwhile, aggregation experiments in the presence of glycine and its methyl derivatives clearly suggest the

criticality of both charge and the hydrophobic content of the cosolvent molecule in determining its effectiveness against protein aggregation.

Aggregation in the presence of polyethylene glycols revealed that thermally induced aggregation was well countered by polyethylene glycols with intermediate hydrophobic characteristics and intermediate molecular size. This effect of polyethylene glycols however could not be observed on aggregation while refolding from a chemically denatured protein. This implies that steric exclusion may be an important factor in determining aggregation suppressing efficiency of the cosolvent but there must exist a threshold and a limit to its hydrophobicity. Under aggregating conditions, molecules with hydrophobicity above a threshold are likely to disrupt the hydrophobic core of the protein. On the other hand cosolvents with high hydrophobicity could also act as a bridge between already formed hydrophobic patches. The limit in hydrophobicity therefore ensures the neutrality of the cosolvent towards hydrophobic aggregating conformations.

Polyols are known for their preferential hydration of the protein. Effectiveness of polyols in preventing thermally induced aggregation further strengthened their preferential hydrating and stabilizing ability to counter high entropy conditions of thermal stress. More the number of hydroxyl groups in the polyols and larger their sizes, more was their effectiveness against thermal aggregation. Thus increasing size of the polyols and their number of hydroxyl groups were found to be directly proportional to steric exclusion and hydration of the protein respectively.

The studies therefore indicate towards a combination of properties like steric exclusion, hydration and the cosolvents' ability to weakly interact with the protein (on the basis of charges and hydrophobicity of cosolvent and the protein) as some of the properties which a cosolvent molecule should possess in order to prevent Human γD crystallin from destabilization or aggregation.

Bibliography

Abel, R., Young, T., Farid, R., Berne, B. J. & Friesner, R. A. (2008). Role of the active-site solvent in the thermodynamics of factor Xa ligand binding. *J Am Chem Soc* 130, 2817-31.

Agarwal, P. K. (2005). Role of protein dynamics in reaction rate enhancement by enzymes. *J Am hem Soc* 127, 15248-56.

Ahmad, F. B. & Williams, P. A. (1999). Effect of sugars on the thermal and rheological properties of sago starch. *Biopolymers* 50, 401-12.

Ahmed, S., Kapoor, D., Singh, B. & Guptasarma, P. (2008). Conformational behavior of polypeptides derived through simultaneous global conservative site-directed mutagenesis of chymotrypsin inhibitor 2. *Biochim Biophys Acta* 1784, 796-805.

Alber, T. & Matthews, B. W. (1987). Structure and thermal stability of phage T4 lysozyme. *Methods Enzymol* 154, 511-33.

Almagor, A., Priev, A., Barshtein, G., Gavish, B. & Yedgar, S. (1998). Reduction of protein volume and compressibility by macromolecular cosolvents: dependence on the cosolvent molecular weight. *Biochim Biophys Acta* 1382, 151-6.

Alonso, D. O. & Dill, K. A. (1991). Solvent denaturation and stabilization of globular proteins. *Biochemistry* 30, 5974-85.

Anderson, D. E., Becktel, W. J. & Dahlquist, F. W. (1990). pH-induced denaturation of proteins: a single salt bridge contributes 3-5 kcal/mol to the free energy of folding of T4 lysozyme. *Biochemistry* 29, 2403-8.

Arakawa, T., Kita, Y., Ejima, D., Tsumoto, K. & Fukada, H. (2006). Aggregation suppression of proteins by arginine during thermal unfolding. *Protein Pept Lett* 13, 921-7.

Arakawa, T. & Timasheff, S. N. (1982). Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry* 21, 6545-52.

Arakawa, T. & Timasheff, S. N. (1982). Stabilization of protein structure by sugars. *Biochemistry* 21, 6536-44.

Arakawa, T. & Timasheff, S. N. (1983). Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Arch Biochem Biophys* 224, 169-77.

Arakawa, T. & Timasheff, S. N. (1984). Protein stabilization and destabilization by guanidinium salts. *Biochemistry* 23, 5924-9.

Arakawa, T. & Timasheff, S. N. (1985). Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry* 24, 6756-62.

Athawale, M. V., Dordick, J. S. & Garde, S. (2005). Osmolyte trimethylamine-N-oxide does not affect the strength of hydrophobic interactions: origin of osmolyte compatibility. *Biophys J* 89, 858-66.

Auffray, C. & Rougeon, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* 107, 303-14.

Auton, M. & Bolen, D. W. (2005). Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc Natl Acad Sci U S A* 102, 15065-8.

Auton, M., Bolen, D. W. & Rosgen, J. (2008). Structural thermodynamics of protein preferential solvation: Osmolyte solvation of proteins, aminoacids, and peptides. *Proteins*.

Auton, M., Ferreon, A. C. & Bolen, D. W. (2006). Metrics that differentiate the origins of osmolyte effects on protein stability: a test of the surface tension proposal. *J Mol Biol* 361, 983-92.

Basharov, M. A. (2003). Protein folding. *J Cell Mol Med* 7, 223-37.

Baynes, B. M. & Trout, B. L. (2004). Rational design of solution additives for the prevention of protein aggregation. *Biophys J* 87, 1631-9.

Baynes, B. M., Wang, D. I. & Trout, B. L. (2005). Role of arginine in the stabilization of proteins against aggregation. *Biochemistry* 44, 4919-25.

Bhat, R. & Timasheff, S. N. (1992). Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Sci* 1, 1133-43.

Bloemendal, H. & de Jong, W. W. (1991). Lens proteins and their genes. *Prog Nucleic Acid Res Mol Biol* 41, 259-81.

Bolanos-Garcia, V. M., Renault, A. & Beaufils, S. (2008). Surface rheology and adsorption kinetics reveal the relative amphiphilicity, interfacial activity, and stability of human exchangeable apolipoproteins. *Biophys J* 94, 1735-45.

Boonyaratanakornkit, B. B., Park, C. B. & Clark, D. S. (2002). Pressure effects on intra- and intermolecular interactions within proteins. *Biochim Biophys Acta* 1595, 235-49.

Cacace, M. G., Landau, E. M. & Ramsden, J. J. (1997). The Hofmeister series: salt and solvent effects on interfacial phenomena. *Q Rev Biophys* 30, 241-77.

Cai, Y. Q., Mao, H. K., Chow, P. C., Tse, J. S., Ma, Y., Patchkovskii, S., Shu, J. F., Struzhkin, V., Hemley, R. J., Ishii, H., Chen, C. C., Jarrige, I., Chen, C. T., Shieh, S. R., Huang, E. P. & Kao, C. C. (2005). Ordering of hydrogen bonds in high-pressure low-temperature H2O. *Phys Rev Lett* 94, 025502.

Campioni, S., Mossuto, M. F., Torrassa, S., Calloni, G., de Laureto, P. P., Relini, A., Fontana, A. & Chiti, F. (2008). Conformational properties of the aggregation precursor state of HypF-N. *J Mol Biol* 379, 554-67.

Carrea, G., Bovara, R., Pasta, P. & Cremonesi, P. (1982). The effect of Hofmeister anions and protein concentration on the activity and stability of some immobilized NAD-dependent dehydrogenases. *Biotechnol Bioeng* 24, 1-7.

Cecconi, F., Guardiani, C. & Livi, R. (2008). Analyzing pathogenic mutations of C5 domain from cardiac myosin binding protein C through MD simulations. *Eur Biophys J* 37, 683-91.

Cellmer, T., Bratko, D., Prausnitz, J. M. & Blanch, H. (2005). Thermodynamics of folding and association of lattice-model proteins. *J Chem Phys* 122, 174908.

Chakraborty, S. & Peng, Z. (2000). Hierarchical unfolding of the alpha-lactalbumin molten globule: presence of a compact intermediate without a unique tertiary fold. *J Mol Biol* 298, 1-6.

Chang, L., Bertelsen, E. B., Wisen, S., Larsen, E. M., Zuiderweg, E. R. & Gestwicki, J. E. (2008). High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK. *Anal Biochem* 372, 167-76.

Chaudhuri, T. K. & Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. *Febs J* 273, 1331-49.

Chen, B. L. & Arakawa, T. (1996). Stabilization of recombinant human keratinocyte growth factor by osmolytes and salts. *J Pharm Sci* 85, 419-26.

Chen, J., Lu, Z., Sakon, J. & Stites, W. E. (2004). Proteins with simplified hydrophobic cores compared to other packing mutants. *Biophys Chem* 110, 239-48.

Chen, L., Ferreira, J. A., Costa, S. M., Cabrita, G. J., Otzen, D. E. & Melo, E. P. (2006). Compaction of ribosomal protein S6 by sucrose occurs only under native conditions. *Biochemistry* 45, 2189-99.

Chi, E. Y., Krishnan, S., Kendrick, B. S., Chang, B. S., Carpenter, J. F. & Randolph, T. W. (2003). Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor. *Protein Sci* 12, 903-13.

Cho, J. H., Sato, S., Horng, J. C., Anil, B. & Raleigh, D. P. (2008). Electrostatic interactions in the denatured state ensemble: their effect upon protein folding and protein stability. *Arch Biochem Biophys* 469, 20-8.

Cho, S. S., Weinkam, P. & Wolynes, P. G. (2008). Origins of barriers and barrierless folding in BBL. *Proc Natl Acad Sci U S A* 105, 118-23.

Chow, M. K., Devlin, G. L. & Bottomley, S. P. (2001). Osmolytes as modulators of conformational changes in serpins. *Biol Chem* 382, 1593-9.

Ciferri, A. (2008). Charge-dependent and charge-independent contributions to ion-protein interaction. *Biopolymers* 89, 700-9.

Cioni, P., Bramanti, E. & Strambini, G. B. (2005). Effects of sucrose on the internal dynamics of azurin. *Biophys J* 88, 4213-22.

Clark, L. A. (2005). Protein aggregation determinants from a simplified model: cooperative folders resist aggregation. *Protein Sci* 14, 653-62.

Colombo, G., Meli, M. & De Simone, A. (2008). Computational studies of the structure, dynamics and native content of amyloid-like fibrils of ribonuclease A. *Proteins* 70, 863-72.

Compiani, M., Capriotti, E. & Casadio, R. (2004). Dynamics of the minimally frustrated helices determine the hierarchical folding of small helical proteins. *Phys Rev E Stat Nonlin Soft Matter Phys* 69, 051905.

Costenaro, L., Zaccai, G. & Ebel, C. (2002). Link between protein-solvent and weak protein-protein interactions gives insight into halophilic adaptation. *Biochemistry* 41, 13245-52.

Covalt, J. C., Jr., Cao, T. B., Magdaroag, J. R., Gross, L. A. & Jennings, P. A. (2005). Temperature, media, and point of induction affect the N-terminal processing of interleukin-1beta. *Protein Expr Purif* 41, 45-52.

Daggett, V., Li, A., Itzhaki, L. S., Otzen, D. E. & Fersht, A. R. (1996). Structure of the transition state for folding of a protein derived from experiment and simulation. *J Mol Biol* 257, 430-40.

Daidone, I., Ulmschneider, M. B., Di Nola, A., Amadei, A. & Smith, J. C. (2007). Dehydration-driven solvent exposure of hydrophobic surfaces as a driving force in peptide folding. *Proc Natl Acad Sci U S A* 104, 15230-5.

Das, U., Hariprasad, G., Ethayathulla, A. S., Manral, P., Das, T. K., Pasha, S., Mann, A., Ganguli, M., Verma, A. K., Bhat, R., Chandrayan, S. K., Ahmed, S., Sharma, S., Kaur, P., Singh, T. P. & Srinivasan, A. (2007). Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. *PLoS ONE* 2, e1176.

Delaye, M. & Tardieu, A. (1983). Short-range order of crystallin proteins accounts for eye lens transparency. *Nature* 302, 415-7.

Deniz, A. A., Laurence, T. A., Beligere, G. S., Dahan, M., Martin, A. B., Chemla, D. S., Dawson, P. E., Schultz, P. G. & Weiss, S. (2000). Single-molecule protein folding: diffusion fluorescence resonance energy transfer studies of the denaturation of chymotrypsin inhibitor 2. *Proc Natl Acad Sci U S A* 97, 5179-84.

Derrick, T., Grillo, A. O., Vitharana, S. N., Jones, L., Rexroad, J., Shah, A., Perkins, M., Spitznagel, T. M. & Middaugh, C. R. (2007). Effect of polyanions on the structure and stability of repifermin (keratinocyte growth factor-2). *J Pharm Sci* 96, 761-76.

Di Michele, A., Freda, M., Onori, G., Paolantoni, M., Santucci, A. & Sassi, P. (2006). Modulation of hydrophobic effect by cosolutes. *J Phys Chem B* 110, 21077-85.

Diamant, S., Eliahu, N., Rosenthal, D. & Goloubinoff, P. (2001). Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. *J Biol Chem* 276, 39586-91.

Dill, K. A. (1990). Dominant forces in protein folding. *Biochemistry* 29, 7133-55.

Dill, K. A. (1990). The meaning of hydrophobicity. *Science* 250, 297-8.

Djikaev, Y. S. & Ruckenstein, E. (2007). A ternary nucleation model for the nucleation pathway of protein folding. *J Chem Phys* 126, 175103.

Djikaev, Y. S. & Ruckenstein, E. (2008). Effect of ionized protein residues on the nucleation pathway of protein folding. *J Chem Phys* 128, 025103.

Doan-Nguyen, V. & Loria, J. P. (2007). The effects of cosolutes on protein dynamics: the reversal of denaturant-induced protein fluctuations by trimethylamine N-oxide. *Protein Sci* 16, 20-9.

Dong, X. Y., Huang, Y. & Sun, Y. (2004). Refolding kinetics of denatured-reduced lysozyme in the presence of folding aids. *J Biotechnol* 114, 135-42.

Efimova, Y. M., Haemers, S., Wierczinski, B., Norde, W. & van Well, A. A. (2007). Stability of globular proteins in H2O and D2O. *Biopolymers* 85, 264-73.

Elcock, A. H. (1999). Realistic modeling of the denatured states of proteins allows accurate calculations of the pH dependence of protein stability. *J Mol Biol* 294, 1051-62.

Fadrna, E., Hladeckova, K. & Koca, J. (2005). Long-range electrostatic interactions in molecular dynamics: an endothelin-1 case study. *J Biomol Struct Dyn* 23, 151-62.

Fan, H., Vitharana, S. N., Chen, T., O'Keefe, D. & Middaugh, C. R. (2007). Effects of pH and polyanions on the thermal stability of fibroblast growth factor 20. *Mol Pharm* 4, 232-40.

Fink, A. L. (1995). Compact intermediate states in protein folding. *Annu Rev Biophys Biomol Struct* 24, 495-522.

Fitter, J. & Heberle, J. (2000). Structural equilibrium fluctuations in mesophilic and thermophilic alpha-amylase. *Biophys J* 79, 1629-36.

Fitter, J., Herrmann, R., Dencher, N. A., Blume, A. & Hauss, T. (2001). Activity and stability of a thermostable alpha-amylase compared to its mesophilic homologue: mechanisms of thermal adaptation. *Biochemistry* 40, 10723-31.

Flaugh, S. L., Kosinski-Collins, M. S. & King, J. (2005). Interdomain side-chain interactions in human gammaD crystallin influencing folding and stability. *Protein Sci* 14, 2030-43.

Font, J., Torrent, J., Ribo, M., Laurents, D. V., Balny, C., Vilanova, M. & Lange, R. (2006). Pressure-jump-induced kinetics reveals a hydration dependent folding/unfolding mechanism of ribonuclease A. *Biophys J* 91, 2264-74.

Frauenfelder, H., Fenimore, P. W., Chen, G. & McMahon, B. H. (2006). Protein folding is slaved to solvent motions. *Proc Natl Acad Sci U S A* 103, 15469-72.

Funahashi, J., Sugita, Y., Kitao, A. & Yutani, K. (2003). How can free energy component analysis explain the difference in protein stability caused by amino acid substitutions? Effect of three hydrophobic mutations at the 56th residue on the stability of human lysozyme. *Protein Eng* 16, 665-71.

Galka, J. J., Baturin, S. J., Manley, D. M., Kehler, A. J. & O'Neil, J. D. (2008). Stability of the glycerol facilitator in detergent solutions. *Biochemistry* 47, 3513-24.

Gallagher, K. R. & Sharp, K. A. (2003). A new angle on heat capacity changes in hydrophobic solvation. *J Am Chem Soc* 125, 9853-60.

Gekko, K. (1982). Calorimetric study on thermal denaturation of lysozyme in polyol-water mixtures. *J Biochem* 91, 1197-204.

Gekko, K. & Morikawa, T. (1981). Thermodynamics of polyol-induced thermal stabilization of chymotrypsinogen. *J Biochem* 90, 51-60.

Gloss, L. M., Topping, T. B., Binder, A. K. & Lohman, J. R. (2008). Kinetic folding of Haloferax volcanii and Escherichia coli dihydrofolate reductases: haloadaptation by unfolded state destabilization at high ionic strength. *J Mol Biol* 376, 1451-62.

Golovanov, A. P., Vergoten, G. & Arseniev, A. S. (2000). Stabilization of proteins by enhancement of inter-residue hydrophobic contacts: lessons of T4 lysozyme and barnase. *J Biomol Struct Dyn* 18, 477-91.

Golub, N. V., Markossian, K. A., Kasilovich, N. V., Sholukh, M. V., Orlov, V. N. & Kurganov, B. I. (2008). Thermal inactivation, denaturation and aggregation of mitochondrial aspartate aminotransferase. *Biophys Chem* 135, 125-31.

Gorski, S. A., Le Duff, C. S., Capaldi, A. P., Kalverda, A. P., Beddard, G. S., Moore, G. R. & Radford, S. E. (2004). Equilibrium hydrogen exchange reveals extensive hydrogen bonded secondary structure in the on-pathway intermediate of Im7. *J Mol Biol* 337, 183-93.

Green, S. M., Meeker, A. K. & Shortle, D. (1992). Contributions of the polar, uncharged amino acids to the stability of staphylococcal nuclease: evidence for mutational effects on the free energy of the denatured state. *Biochemistry* 31, 5717-28.

Griko, Y. V. & Privalov, P. L. (1992). Calorimetric study of the heat and cold denaturation of beta-lactoglobulin. *Biochemistry* 31, 8810-5.

Grimsley, G. R., Shaw, K. L., Fee, L. R., Alston, R. W., Huyghues-Despointes, B. M., Thurlkill, R. L., Scholtz, J. M. & Pace, C. N. (1999). Increasing protein stability by altering long-range coulombic interactions. *Protein Sci* 8, 1843-9.

Gross, M. & Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. *Eur J Biochem* 221, 617-30.

Grottesi, A., Ceruso, M. A., Colosimo, A. & Di Nola, A. (2002). Molecular dynamics study of a hyperthermophilic and a mesophilic rubredoxin. *Proteins* 46, 287-94.

Guo, Z. & Thirumalai, D. (1997). The nucleation-collapse mechanism in protein folding: evidence for the non-uniqueness of the folding nucleus. *Fold Des* 2, 377-91.

Haas, E. (2005). The study of protein folding and dynamics by determination of intramolecular distance distributions and their fluctuations using ensemble and single-molecule FRET measurements. *Chemphyschem* 6, 858-70.

Hall, D. & Minton, A. P. (2003). Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochim Biophys Acta* 1649, 127-39.

Halskau, O., Jr., Perez-Jimenez, R., Ibarra-Molero, B., Underhaug, J., Munoz, V., Martinez, A. & Sanchez-Ruiz, J. M. (2008). Large-scale modulation of thermodynamic protein folding barriers linked to electrostatics. *Proc Natl Acad Sci U S A* 105, 8625-30.

Haque, I., Singh, R., Ahmad, F. & Moosavi-Movahedi, A. A. (2005). Testing polyols' compatibility with Gibbs energy of stabilization of proteins under conditions in which they behave as compatible osmolytes. *FEBS Lett* 579, 3891-8.

Hardy, J. & Gwinn-Hardy, K. (1998). Genetic classification of primary neurodegenerative disease. *Science* 282, 1075-9.

Harper, J. D. & Lansbury, P. T., Jr. (1997). Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu Rev Biochem* 66, 385-407.

Hau, V. S., Huber, J. D., Campos, C. R., Lipkowski, A. W., Misicka, A. & Davis, T. P. (2002). Effect of guanidino modification and proline substitution on the in vitro stability and blood-brain barrier permeability of endomorphin II. *J Pharm Sci* 91, 2140-9.

Hawe, A. & Friess, W. (2007). Stabilization of a hydrophobic recombinant cytokine by human serum albumin. *J Pharm Sci* 96, 2987-99.

Hendsch, Z. S. & Tidor, B. (1994). Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Sci* 3, 211-26.

Hernandez, G., Jenney, F. E., Jr., Adams, M. W. & LeMaster, D. M. (2000). Millisecond time scale conformational flexibility in a hyperthermophile protein at ambient temperature. *Proc Natl Acad Sci U S A* 97, 3166-70.

Herrmann, L., Bowler, B. E., Dong, A. & Caughey, W. S. (1995). The effects of hydrophilic to hydrophobic surface mutations on the denatured state of iso-1-cytochrome c: investigation of aliphatic residues. *Biochemistry* 34, 3040-7.

Hill, J. J., Shalaev, E. Y. & Zografi, G. (2005). Thermodynamic and dynamic factors involved in the stability of native protein structure in amorphous solids in relation to levels of hydration. *J Pharm Sci* 94, 1636-67.

Hirano, A., Hamada, H., Okubo, T., Noguchi, T., Higashibata, H. & Shiraki, K. (2007). Correlation between thermal aggregation and stability of lysozyme with salts described by molar surface tension increment: an exceptional propensity of ammonium salts as aggregation suppressor. *Protein J* 26, 423-33.

Hoiberg-Nielsen, R., Fuglsang, C. C., Arleth, L. & Westh, P. (2006). Interrelationships of glycosylation and aggregation kinetics for Peniophora lycii phytase. *Biochemistry* 45, 5057-66.

Hong, J., Capp, M. W., Anderson, C. F., Saecker, R. M., Felitsky, D. J., Anderson, M. W. & Record, M. T., Jr. (2004). Preferential interactions of glycine betaine and of urea with DNA: implications for DNA hydration and for effects of these solutes on DNA stability. *Biochemistry* 43, 14744-58.

Honig, B. (1999). Protein folding: from the levinthal paradox to structure prediction. *J Mol Biol* 293, 283-93.

Honig, B. & Yang, A. S. (1995). Free energy balance in protein folding. *Adv Protein Chem* 46, 27-58.

Hoyer, W., Cherny, D., Subramaniam, V. & Jovin, T. M. (2004). Rapid self-assembly of alpha-synuclein observed by in situ atomic force microscopy. *J Mol Biol* 340, 127-39.

Hu, C. Q., Sturtevant, J. M., Thomson, J. A., Erickson, R. E. & Pace, C. N. (1992). Thermodynamics of ribonuclease T1 denaturation. *Biochemistry* 31, 4876-82.

Ibarra-Molero, B., Zitzewitz, J. A. & Matthews, C. R. (2004). Salt-bridges can stabilize but do not accelerate the folding of the homodimeric coiled-coil peptide GCN4-p1. *J Mol Biol* 336, 989-96.

Idiyatullin, D., Nesmelova, I., Daragan, V. A. & Mayo, K. H. (2003). Heat capacities and a snapshot of the energy landscape in protein GB1 from the pre-denaturation temperature dependence of backbone NH nanosecond fluctuations. *J Mol Biol* 325, 149-62.

Ignatova, Z. & Gierasch, L. M. (2006). Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant. *Proc Natl Acad Sci U S A* 103, 13357-61.

Jackson, R. M. & Sternberg, M. J. (1994). Application of scaled particle theory to model the hydrophobic effect: implications for molecular association and protein stability. *Protein Eng* 7, 371-83.

Jacob, M., Geeves, M., Holtermann, G. & Schmid, F. X. (1999). Diffusional barrier crossing in a two-state protein folding reaction. *Nat Struct Biol* 6, 923-6.

Jaenicke, R. (2000). Do ultrastable proteins from hyperthermophiles have high or low conformational rigidity? *Proc Natl Acad Sci U S A* 97, 2962-4.

Jayaraman, S., Gantz, D. & Gursky, O. (2005). Structural basis for thermal stability of human low-density lipoprotein. *Biochemistry* 44, 3965-71.

Kar, K. & Kishore, N. (2007). Enhancement of thermal stability and inhibition of protein aggregation by osmolytic effect of hydroxyproline. *Biopolymers* 87, 339-51.

Kaushik, J. K. & Bhat, R. (1999). A mechanistic analysis of the increase in the thermal stability of proteins in aqueous carboxylic acid salt solutions. *Protein Sci* 8, 222-33.

Kaushik, J. K. & Bhat, R. (2003). Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. *J Biol Chem* 278, 26458-65.

Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. *Adv Protein Chem* 14, 1-63.

Kellis, J. T., Jr., Nyberg, K. & Fersht, A. R. (1989). Energetics of complementary side-chain packing in a protein hydrophobic core. *Biochemistry* 28, 4914-22.

Kellis, J. T., Jr., Nyberg, K., Sali, D. & Fersht, A. R. (1988). Contribution of hydrophobic interactions to protein stability. *Nature* 333, 784-6.

Kendrick, B. S., Carpenter, J. F., Cleland, J. L. & Randolph, T. W. (1998). A transient expansion of the native state precedes aggregation of recombinant human interferon-gamma. *Proc Natl Acad Sci U S A* 95, 14142-6.

Kerwin, B. A., Heller, M. C., Levin, S. H. & Randolph, T. W. (1998). Effects of Tween 80 and sucrose on acute short-term stability and long-term storage at -20 degrees C of a recombinant hemoglobin. *J Pharm Sci* 87, 1062-8.

Kim, B., Young, T., Harder, E., Friesner, R. A. & Berne, B. J. (2005). Structure and dynamics of the solvation of bovine pancreatic trypsin inhibitor in explicit water: a comparative study of the effects of solvent and protein polarizability. *J Phys Chem B* 109, 16529-38.

Kim, S. H., Yan, Y. B. & Zhou, H. M. (2006). Role of osmolytes as chemical chaperones during the refolding of aminoacylase. *Biochem Cell Biol* 84, 30-8.

Kirkwood, J. G., In: Cohn, E. J., Edsall J. T., editors. Proteins, amino acids and peptides as ions and dipolar ions. New York: Reinhold Publishing Corporation,. (1943) p 586–622.

Koo, E. H., Lansbury, P. T., Jr. & Kelly, J. W. (1999). Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc Natl Acad Sci U S A* 96, 9989-90.

Korepanova, A., Douglas, C. & Logan, T. M. (2002). Glutamine 53 is a gatekeeper residue in the FK506 binding protein. *J Mol Biol* 323, 285-96.

Kosinski-Collins, M. S., Flaugh, S. L. & King, J. (2004). Probing folding and fluorescence quenching in human gammaD crystallin Greek key domains using triple tryptophan mutant proteins. *Protein Sci* 13, 2223-35.

Kosinski-Collins, M. S. & King, J. (2003). In vitro unfolding, refolding, and polymerization of human gammaD crystallin, a protein involved in cataract formation. *Protein Sci* 12, 480-90.

Kruse, S., Kleineidam, R. G., Roggentin, P. & Schauer, R. (1996). Expression and purification of a recombinant "small" sialidase from Clostridium perfringens A99. *Protein Expr Purif* 7, 415-22.

Kumar, R., Serrette, J. M., Khan, S. H., Miller, A. L. & Thompson, E. B. (2007). Effects of different osmolytes on the induced folding of the N-terminal activation domain (AF1) of the glucocorticoid receptor. *Arch Biochem Biophys* 465, 452-60.

Kumar, S. & Nussinov, R. (2002). Close-range electrostatic interactions in proteins. *Chembiochem* 3, 604-17.

Kuo, N. N., Huang, J. J., Miksovska, J., Chen, R. P., Larsen, R. W. & Chan, S. I. (2005). Effects of turn stability on the kinetics of refolding of a hairpin in a beta-sheet. *J Am Chem Soc* 127, 16945-54.

Lange, C., Patil, G. & Rudolph, R. (2005). Ionic liquids as refolding additives: N'-alkyl and N'-(omega-hydroxyalkyl) N-methylimidazolium chlorides. *Protein Sci* 14, 2693-701.

Lansbury, P. T., Jr. (1999). Evolution of amyloid: what normal protein folding may tell us about fibrillogenesis and disease. *Proc Natl Acad Sci U S A* 96, 3342-4.

Lazaridis, T., Lee, I. & Karplus, M. (1997). Dynamics and unfolding pathways of a hyperthermophilic and a mesophilic rubredoxin. *Protein Sci* 6, 2589-605.

Lee, C. F., Allen, M. D., Bycroft, M. & Wong, K. B. (2005). Electrostatic interactions contribute to reduced heat capacity change of unfolding in a thermophilic ribosomal protein l30e. *J Mol Biol* 348, 419-31.

Lee, J. C. & Timasheff, S. N. (1981). The stabilization of proteins by sucrose. *J Biol Chem* 256, 7193-201.

Lee, L. L. & Lee, J. C. (1987). Thermal stability of proteins in the presence of poly(ethylene glycols). *Biochemistry* 26, 7813-9.

Levy, Y., Jortner, J. & Becker, O. M. (2001). Solvent effects on the energy landscapes and folding kinetics of polyalanine. *Proc Natl Acad Sci U S A* 98, 2188-93.

Lim, W. A., Fox, R. O. & Richards, F. M. (1994). Stability and peptide binding affinity of an SH3 domain from the Caenorhabditis elegans signaling protein Sem-5. *Protein Sci* 3, 1261-6.

Lim, W. A. & Sauer, R. T. (1991). The role of internal packing interactions in determining the structure and stability of a protein. *J Mol Biol* 219, 359-76.

Lin, C. C. & Chang, J. Y. (2007). Pathway of oxidative folding of bovine alpha-interferon: predominance of native disulfide-bonded folding intermediates. *Biochemistry* 46, 3925-32.

Lindman, S., Linse, S., Mulder, F. A. & Andre, I. (2007). pK(a) values for side-chain carboxyl groups of a PGB1 variant explain salt and pH-dependent stability. *Biophys J* 92, 257-66.

Lomize, A. L., Reibarkh, M. Y. & Pogozheva, I. D. (2002). Interatomic potentials and solvation parameters from protein engineering data for buried residues. *Protein Sci* 11, 1984-2000.

Lu, D. & Liu, Z. (2008). Oscillatory molecular driving force for protein folding at high concentration: a molecular simulation. *J Phys Chem B* 112, 2686-93.

Lucent, D., Vishal, V. & Pande, V. S. (2007). Protein folding under confinement: a role for solvent. *Proc Natl Acad Sci U S A* 104, 10430-4.

Maity, H., Maity, M. & Englander, S. W. (2004). How cytochrome c folds, and why: submolecular foldon units and their stepwise sequential stabilization. *J Mol Biol* 343, 223-33.

Maldonado, S., Irun, M. P., Campos, L. A., Rubio, J. A., Luquita, A., Lostao, A., Wang, R., Garcia-Moreno, E. B. & Sancho, J. (2002). Salt-induced stabilization of apoflavodoxin at neutral pH is mediated through cation-specific effects. *Protein Sci* 11, 1260-73.

Manno, M., San Biagio, P. L. & Palma, M. U. (2004). The role of pH on instability and aggregation of sickle hemoglobin solutions. *Proteins* 55, 169-76.

Marques, M. I., Borreguero, J. M., Stanley, H. E. & Dokholyan, N. V. (2003). Possible mechanism for cold denaturation of proteins at high pressure. *Phys Rev Lett* 91, 138103.

Matousek, W. M., Ciani, B., Fitch, C. A., Garcia-Moreno, B., Kammerer, R. A. & Alexandrescu, A. T. (2007). Electrostatic contributions to the stability of the GCN4 leucine zipper structure. *J Mol Biol* 374, 206-19.

Matsumura, M., Becktel, W. J. & Matthews, B. W. (1988). Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature* 334, 406-10.

Matysiak, S. & Clementi, C. (2006). Minimalist protein model as a diagnostic tool for misfolding and aggregation. *J Mol Biol* 363, 297-308.

Meehan, S., Berry, Y., Luisi, B., Dobson, C. M., Carver, J. A. & MacPhee, C. E. (2004). Amyloid fibril formation by lens crystallin proteins and its implications for cataract formation. *J Biol Chem* 279, 3413-9.

Melo, E. P., Chen, L., Cabral, J. M., Fojan, P., Petersen, S. B. & Otzen, D. E. (2003). Trehalose favors a cutinase compact intermediate off-folding pathway. *Biochemistry* 42, 7611-7.

Mertz, E. L. & Leikin, S. (2004). Interactions of inorganic phosphate and sulfate anions with collagen. *Biochemistry* 43, 14901-12.

Minton, A. P. (2000). Effect of a concentrated "inert" macromolecular cosolute on the stability of a globular protein with respect to denaturation by heat and by chaotropes: a statistical-thermodynamic model. *Biophys J* 78, 101-9.

Minton, A. P. (2001). The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* 276, 10577-80.

Minton, A. P. (2005). Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited. *Biophys J* 88, 971-85.

Mishra, R., Bhat, R. & Seckler, R. (2007). Chemical chaperone-mediated protein folding: stabilization of P22 tailspike folding intermediates by glycerol. *Biol Chem* 388, 797-804.

Mishra, R., Seckler, R. & Bhat, R. (2005). Efficient refolding of aggregation-prone citrate synthase by polyol osmolytes: how well are protein folding and stability aspects coupled? *J Biol Chem* 280, 15553-60.

Missimer, J. H., Steinmetz, M. O., Baron, R., Winkler, F. K., Kammerer, R. A., Daura, X. & van Gunsteren, W. F. (2007). Configurational entropy elucidates the role of salt-bridge networks in protein thermostability. *Protein Sci* 16, 1349-59.

Moult, J. & Unger, R. (1991). An analysis of protein folding pathways. *Biochemistry* 30, 3816-24.

Mozhaev, V. V., Heremans, K., Frank, J., Masson, P. & Balny, C. (1996). High pressure effects on protein structure and function. *Proteins* 24, 81-91.

Mukaiyama, A., Koga, Y., Takano, K. & Kanaya, S. (2008). Osmolyte effect on the stability and folding of a hyperthermophilic protein. *Proteins* 71, 110-8.

Myers, J. K. & Oas, T. G. (2001). Preorganized secondary structure as an important determinant of fast protein folding. *Nat Struct Biol* 8, 552-8.

Nakagawa, H., Joti, Y., Kitao, A. & Kataoka, M. (2008). Hydration affects both harmonic and anharmonic nature of protein dynamics. *Biophys J.*

Nakai, K., Kidera, A. & Kanehisa, M. (1988). Cluster analysis of amino acid indices for prediction of protein structure and function. *Protein Eng* 2, 93-100.

Nakamura, H. (1996). Roles of electrostatic interaction in proteins. *Q Rev Biophys* 29, 1-90.

Nguyen, H., Jager, M., Moretto, A., Gruebele, M. & Kelly, J. W. (2003). Tuning the free-energy landscape of a WW domain by temperature, mutation, and truncation. *Proc Natl Acad Sci U S A* 100, 3948-53.

Niccolai, A., Fontani, S., Kapat, A. & Olivieri, R. (2003). Maximization of recombinant Helicobacter pylori neutrophil activating protein production in Escherichia coli: improvement of a chemically defined medium using response surface methodology. *FEMS Microbiol Lett* 221, 257-62.

Nielsen, A. D., Borch, K. & Westh, P. (2007). Thermal stability of Humicola insolens cutinase in aqueous SDS. *J Phys Chem B* 111, 2941-7.

Nishimura, C., Uversky, V. N. & Fink, A. L. (2001). Effect of salts on the stability and folding of staphylococcal nuclease. *Biochemistry* 40, 2113-28.

Okanojo, M., Shiraki, K., Kudou, M., Nishikori, S. & Takagi, M. (2005). Diamines prevent thermal aggregation and inactivation of lysozyme. *J Biosci Bioeng* 100, 556-61.

Otzen, D. E. (2005). Conformational detours during folding of a collapsed state. *Biochim Biophys Acta* 1750, 146-53.

Oxender DL, Fox CF. 1987. Protein Engineering. New York: Wiley

Pace, C. N. (1990). Conformational stability of globular proteins. *Trends Biochem Sci* 15, 14-7.

Pace, C. N., Alston, R. W. & Shaw, K. L. (2000). Charge-charge interactions influence the denatured state ensemble and contribute to protein stability. *Protein Sci* 9, 1395-8.

Pace, C. N., Trevino, S., Prabhakaran, E. & Scholtz, J. M. (2004). Protein structure, stability and solubility in water and other solvents. *Philos Trans R Soc Lond B Biol Sci* 359, 1225-34; discussion 1234-5.

Pande, A., Pande, J., Asherie, N., Lomakin, A., Ogun, O., King, J. & Benedek, G. B. (2001). Crystal cataracts: human genetic cataract caused by protein crystallization. *Proc Natl Acad Sci U S A* 98, 6116-20.

Papp, E. & Csermely, P. (2006). Chemical chaperones: mechanisms of action and potential use. *Handb Exp Pharmacol*, 405-16.

Parker, M. J. & Clarke, A. R. (1997). Amide backbone and water-related H/D isotope effects on the dynamics of a protein folding reaction. *Biochemistry* 36, 5786-94.

Pauling, L. & Corey, R. B. (1951). The polypeptide-chain configuration in hemoglobin and other globular proteins. *Proc Natl Acad Sci U S A* 37, 282-5.

Pedersen, J. S., Christensen, G. & Otzen, D. E. (2004). Modulation of S6 fibrillation by unfolding rates and gatekeeper residues. *J Mol Biol* 341, 575-88.

Perez-Jimenez, R., Godoy-Ruiz, R., Ibarra-Molero, B. & Sanchez-Ruiz, J. M. (2004). The efficiency of different salts to screen charge interactions in proteins: a Hofmeister effect? *Biophys J* 86, 2414-29.

Permyakov, S. E., Makhatadze, G. I., Owenius, R., Uversky, V. N., Brooks, C. L., Permyakov, E. A. & Berliner, L. J. (2005). How to improve nature: study of the electrostatic properties of the surface of alpha-lactalbumin. *Protein Eng Des Sel* 18, 425-33.

Pradeep, L. & Udgaonkar, J. B. (2004). Effect of salt on the urea-unfolded form of barstar probed by m value measurements. *Biochemistry* 43, 11393-402.

Pradeep, L. & Udgaonkar, J. B. (2007). Diffusional barrier in the unfolding of a small protein. *J Mol Biol* 366, 1016-28.

Priev, A., Almagor, A., Yedgar, S. & Gavish, B. (1996). Glycerol decreases the volume and compressibility of protein interior. *Biochemistry* 35, 2061-6.

Purkiss, A. G., Bateman, O. A., Wyatt, K., Wilmarth, P. A., David, L. L., Wistow, G. J. & Slingsby, C. (2007). Biophysical properties of gammaC-crystallin in human and mouse eye lens: the role of molecular dipoles. *J Mol Biol* 372, 205-22.

Radha, C., Muralidhara, B. K., Kumar, P. R., Tasneem, R. & Prakash, V. (1998). Thermal stabilization of multimeric proteins: a case study with alpha-globulin. *Indian J Biochem Biophys* 35, 76-85.

Ragone, R. (2001). Hydrogen-bonding classes in proteins and their contribution to the unfolding reaction. *Protein Sci* 10, 2075-82.

Rajini, B., Shridas, P., Sundari, C. S., Muralidhar, D., Chandani, S., Thomas, F. & Sharma, Y. (2001). Calcium binding properties of gamma-crystallin: calcium ion binds at the Greek key beta gamma-crystallin fold. *J Biol Chem* 276, 38464-71.

Raman, B., Ramakrishna, T. & Rao, C. M. (1997). Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. *FEBS Lett* 416, 369-72.

Ramirez-Carrozzi, V. R. & Kerppola, T. K. (2001). Long-range electrostatic interactions influence the orientation of Fos-Jun binding at AP-1 sites. *J Mol Biol* 305, 411-27.

Ramos, C. H. & Baldwin, R. L. (2002). Sulfate anion stabilization of native ribonuclease A both by anion binding and by the Hofmeister effect. *Protein Sci* 11, 1771-8.

Ratnaparkhi, G. S. & Varadarajan, R. (2000). Thermodynamic and structural studies of cavity formation in proteins suggest that loss of packing interactions rather than the hydrophobic effect dominates the observed energetics. *Biochemistry* 39, 12365-74.

Remmele, R. L., Jr., Zhang-van Enk, J., Dharmavaram, V., Balaban, D., Durst, M., Shoshitaishvili, A. & Rand, H. (2005). Scan-rate-dependent melting transitions of interleukin-1 receptor (type II): elucidation of meaningful thermodynamic and kinetic parameters of aggregation acquired from DSC simulations. *J Am Chem Soc* 127, 8328-39.f

Reubsaet, J. L., Beijnen, J. H., Bult, A., van Maanen, R. J., Marchal, J. A. & Underberg, W. J. (1998). Analytical techniques used to study the degradation of proteins and peptides: chemical instability. *J Pharm Biomed Anal* 17, 955-78.

Rezaei-Ghaleh, N., Ramshini, H., Ebrahim-Habibi, A., Moosavi-Movahedi, A. A. & Nemat-Gorgani, M. (2008). Thermal aggregation of alpha-chymotrypsin: role of hydrophobic and electrostatic interactions. *Biophys Chem* 132, 23-32.

Rishi, V., Anjum, F., Ahmad, F. & Pfeil, W. (1998). Role of non-compatible osmolytes in the stabilization of proteins during heat stress. *Biochem J* 329 (Pt 1), 137-43.

Rivas, G. & Minton, A. P. (2004). Non-ideal tracer sedimentation equilibrium: a powerful tool for the characterization of macromolecular interactions in crowded solutions. *J Mol Recognit* 17, 362-7.

Rochet, J. C. (2007). Novel therapeutic strategies for the treatment of protein-misfolding diseases. *Expert Rev Mol Med* 9, 1-34.

Rodriguez-Larrea, D., Minning, S., Borchert, T. V. & Sanchez-Ruiz, J. M. (2006). Role of solvation barriers in protein kinetic stability. *J Mol Biol* 360, 715-24.

Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H. & Zehfus, M. H. (1985). Hydrophobicity of amino acid residues in globular proteins. *Science* 229, 834-8.

Roseman, M. A. (1988). Hydrophobicity of the peptide C=O.H-N hydrogen-bonded group. *J Mol Biol* 201, 621-3.

Rosgen, J. (2007). Molecular basis of osmolyte effects on protein and metabolites. *Methods Enzymol* 428, 459-86.

Rosgen, J., Pettitt, B. M. & Bolen, D. W. (2004). Uncovering the basis for nonideal behavior of biological molecules. *Biochemistry* 43, 14472-84.

Rosgen, J., Pettitt, B. M. & Bolen, D. W. (2005). Protein folding, stability, and solvation structure in osmolyte solutions. *Biophys J* 89, 2988-97.

Rousseau, F., Serrano, L. & Schymkowitz, J. W. (2006). How evolutionary pressure against protein aggregation shaped chaperone specificity. *J Mol Biol* 355, 1037-47.

Royer, C. A. (2002). Revisiting volume changes in pressure-induced protein unfolding. *Biochim Biophys Acta* 1595, 201-9.

Russo, A. T., Rosgen, J. & Bolen, D. W. (2003). Osmolyte effects on kinetics of FKBP12 C22A folding coupled with prolyl isomerization. *J Mol Biol* 330, 851-66.

Saluja, A. & Kalonia, D. S. (2008). Nature and consequences of protein-protein interactions in high protein concentration solutions. *Int J Pharm* 358, 1-15.

Sanchez-Ruiz, J. M., Lopez-Lacomba, J. L., Cortijo, M. & Mateo, P. L. (1988). Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. *Biochemistry* 27, 1648-52.

Sandberg, W. S. & Terwilliger, T. C. (1991). Energetics of repacking a protein interior. *Proc Natl Acad Sci U S A* 88, 1706-10.

Santra, M. K., Banerjee, A., Krishnakumar, S. S., Rahaman, O. & Panda, D. (2004). Multiple-probe analysis of folding and unfolding pathways of human serum albumin. Evidence for a framework mechanism of folding. *Eur J Biochem* 271, 1789-97.

Sasahara, K., Yagi, H., Naiki, H. & Goto, Y. (2007). Heat-induced conversion of beta(2)-microglobulin and hen egg-white lysozyme into amyloid fibrils. *J Mol Biol* 372, 981-91.

Scharnagl, C., Reif, M. & Friedrich, J. (2005). Stability of proteins: temperature, pressure and the role of the solvent. *Biochim Biophys Acta* 1749, 187-213.

Schell, D., Tsai, J., Scholtz, J. M. & Pace, C. N. (2006). Hydrogen bonding increases packing density in the protein interior. *Proteins* 63, 278-82.

Schellman, J. A. (2005). Destabilization and stabilization of proteins. *Q Rev Biophys* 38, 351-61.

Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S. & Kolodziejczyk, E. (2007). Whey protein soluble aggregates from heating with NaCl: physicochemical, interfacial, and foaming properties. *Langmuir* 23, 4155-66.

Schoeffler, A. J., Joubert, A. M., Peng, F., Khan, F., Liu, C. C. & LiCata, V. J. (2004). Extreme free energy of stabilization of Taq DNA polymerase. *Proteins* 54, 616-21.

Schurr, J. M., Rangel, D. P. & Aragon, S. R. (2005). A contribution to the theory of preferential interaction coefficients. *Biophys J* 89, 2258-76.

Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A. & Fersht, A. R. (1992). The folding of an enzyme. II. Substructure of barnase and the contribution of different interactions to protein stability. *J Mol Biol* 224, 783-804.

Shea, J. E., Onuchic, J. N. & Brooks, C. L., 3rd. (2002). Probing the folding free energy landscape of the Src-SH3 protein domain. *Proc Natl Acad Sci U S A* 99, 16064-8.

Shi, Z., Krantz, B. A., Kallenbach, N. & Sosnick, T. R. (2002). Contribution of hydrogen bonding to protein stability estimated from isotope effects. *Biochemistry* 41, 2120-9.

Shimizu, O., Watanabe, J., Naito, S. & Shibata, Y. (2006). Quenching mechanism of Rose Bengal triplet state involved in photosensitization of oxygen in ethylene glycol. *J Phys Chem A* 110, 1735-9.

Shimizu, S. (2004). Estimating hydration changes upon biomolecular reactions from osmotic stress, high pressure, and preferential hydration experiments. *Proc Natl Acad Sci U S A* 101, 1195-9.

Shimizu, S. & Boon, C. L. (2004). The Kirkwood-Buff theory and the effect of cosolvents on biochemical reactions. *J Chem Phys* 121, 9147-55.

Shire, S. J., Shahrokh, Z. & Liu, J. (2004). Challenges in the development of high protein concentration formulations. *J Pharm Sci* 93, 1390-402.

Shirley, B. A., Stanssens, P., Hahn, U. & Pace, C. N. (1992). Contribution of hydrogen bonding to the conformational stability of ribonuclease T1. *Biochemistry* 31, 725-32.

Shortle, D. & Meeker, A. K. (1986). Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation. *Proteins* 1, 81-9.

Shortle, D., Stites, W. E. & Meeker, A. K. (1990). Contributions of the large hydrophobic amino acids to the stability of staphylococcal nuclease. *Biochemistry* 29, 8033-41.

Shulgin, I. L. & Ruckenstein, E. (2005). Relationship between preferential interaction of a protein in an aqueous mixed solvent and its solubility. *Biophys Chem* 118, 128-34.

Silow, M. & Oliveberg, M. (2003). High concentrations of viscogens decrease the protein folding rate constant by prematurely collapsing the coil. *J Mol Biol* 326, 263-71.

Silva, J. L., Silveira, C. F., Correia Junior, A. & Pontes, L. (1992). Dissociation of a native dimer to a molten globule monomer. Effects of pressure and dilution on the association equilibrium of arc repressor. *J Mol Biol* 223, 545-55.

Singh, R., Haque, I. & Ahmad, F. (2005). Counteracting osmolyte trimethylamine N-oxide destabilizes proteins at pH below its pKa. Measurements of thermodynamic parameters of proteins in the presence and absence of trimethylamine N-oxide. *J Biol Chem* 280, 11035-42.

Singh, S., Singh, A., Aziz, M. A., Waheed, S. M., Bhat, R. & Bhatnagar, R. (2004). Thermal inactivation of protective antigen of Bacillus anthracis and its prevention by polyol osmolytes. *Biochem Biophys Res Commun* 322, 1029-37.

Sinibaldi, R., Ortore, M. G., Spinozzi, F., de Souza Funari, S., Teixeira, J. & Mariani, P. (2008). SANS/SAXS study of the BSA solvation properties in aqueous urea solutions via a global fit approach. *Eur Biophys J* 37, 673-81.

Skolnick, J. & Kolinski, A. (1991). Dynamic Monte Carlo simulations of a new lattice model of globular protein folding, structure and dynamics. *J Mol Biol* 221, 499-531.

Soares, C. M., Teixeira, V. H. & Baptista, A. M. (2003). Protein structure and dynamics in nonaqueous solvents: insights from molecular dynamics simulation studies. *Biophys J* 84, 1628-41.

Spencer, D. S., Xu, K., Logan, T. M. & Zhou, H. X. (2005). Effects of pH, salt, and macromolecular crowding on the stability of FK506-binding protein: an integrated experimental and theoretical study. *J Mol Biol* 351, 219-32.

Stickle, D. F., Presta, L. G., Dill, K. A. & Rose, G. D. (1992). Hydrogen bonding in globular proteins. *J Mol Biol* 226, 1143-59.

Stoycheva, A. D., Brooks, C. L., 3rd & Onuchic, J. N. (2004). Gatekeepers in the ribosomal protein s6: thermodynamics, kinetics, and folding pathways revealed by a minimalist protein model. *J Mol Biol* 340, 571-85.

Stradner, A., Sedgwick, H., Cardinaux, F., Poon, W. C., Egelhaaf, S. U. & Schurtenberger, P. (2004). Equilibrium cluster formation in concentrated protein solutions and colloids. *Nature* 432, 492-5.

Street, T. O., Bolen, D. W. & Rose, G. D. (2006). A molecular mechanism for osmolyte-induced protein stability. *Proc Natl Acad Sci U S A* 103, 13997-4002.

Sun, D. P., Soderlind, E., Baase, W. A., Wozniak, J. A., Sauer, U. & Matthews, B. W. (1991). Cumulative site-directed charge-change replacements in bacteriophage T4 lysozyme suggest that long-range electrostatic interactions contribute little to protein stability. *J Mol Biol* 221, 873-87.

Svingor, A., Kardos, J., Hajdu, I., Nemeth, A. & Zavodszky, P. (2001). A better enzyme to cope with cold. Comparative flexibility studies on psychrotrophic, mesophilic, and thermophilic IPMDHs. *J Biol Chem* 276, 28121-5.

Takano, K., Scholtz, J. M., Sacchettini, J. C. & Pace, C. N. (2003). The contribution of polar group burial to protein stability is strongly context-dependent. *J Biol Chem* 278, 31790-5.

Tan, Y. J., Oliveberg, M., Davis, B. & Fersht, A. R. (1995). Perturbed pKA-values in the denatured states of proteins. *J Mol Biol* 254, 980-92.

Tanaka, M., Machida, Y., Niu, S., Ikeda, T., Jana, N. R., Doi, H., Kurosawa, M., Nekooki, M. & Nukina, N. (2004). Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat Med* 10, 148-54.

Tanaka, M., Machida, Y. & Nukina, N. (2005). A novel therapeutic strategy for polyglutamine diseases by stabilizing aggregation-prone proteins with small molecules. *J Mol Med* 83, 343-52.

Taneja, S. & Ahmad, F. (1994). Increased thermal stability of proteins in the presence of amino acids. *Biochem J* 303 (Pt 1), 147-53.

Tardieu, A., Veretout, F., Krop, B. & Slingsby, C. (1992). Protein interactions in the calf eye lens: interactions between beta-crystallins are repulsive whereas in gamma-crystallins they are attractive. *Eur Biophys J* 21, 1-12.

Thirumangalathu, R., Krishnan, S., Brems, D. N., Randolph, T. W. & Carpenter, J. F. (2006). Effects of pH, temperature, and sucrose on benzyl alcohol-induced aggregation of recombinant human granulocyte colony stimulating factor. *J Pharm Sci* 95, 1480-97.

Tiana, G., Provasi, D. & Broglia, R. A. (2003). Role of bulk and of interface contacts in the behavior of lattice model dimeric proteins. *Phys Rev E Stat Nonlin Soft Matter Phys* 67, 051909.

Tiwari, A. & Bhat, R. (2006). Stabilization of yeast hexokinase A by polyol osmolytes: correlation with the physicochemical properties of aqueous solutions. *Biophys Chem* 124, 90-9.

Tollinger, M., Crowhurst, K. A., Kay, L. E. & Forman-Kay, J. D. (2003). Site-specific contributions to the pH dependence of protein stability. *Proc Natl Acad Sci U S A* 100, 4545-50.

Tong, W. Y., Yao, S. J., Zhu, Z. Q. & Yu, J. (2001). An improved procedure for production of human epidermal growth factor from recombinant E. coli. *Appl Microbiol Biotechnol* 57, 674-9.

Toth, K., Sedlak, E., Sprinzl, M. & Zoldak, G. (2008). Flexibility and enzyme activity of NADH oxidase from Thermus thermophilus in the presence of monovalent cations of Hofmeister series. *Biochim Biophys Acta* 1784, 789-95.

Trefethen, J. M., Pace, C. N., Scholtz, J. M. & Brems, D. N. (2005). Charge-charge interactions in the denatured state influence the folding kinetics of ribonuclease Sa. *Protein Sci* 14, 1934-8.

Tsai, A. M., Udovic, T. J. & Neumann, D. A. (2001). The inverse relationship between protein dynamics and thermal stability. *Biophys J* 81, 2339-43.

Tsumoto, K., Ejima, D., Kita, Y. & Arakawa, T. (2005). Review: Why is arginine effective in suppressing aggregation? *Protein Pept Lett* 12, 613-9.

van der Spoel, D., van Maaren, P. J., Larsson, P. & Timneanu, N. (2006). Thermodynamics of hydrogen bonding in hydrophilic and hydrophobic media. *J Phys Chem B* 110, 4393-8.

Vihinen, M. (1987). Relationship of protein flexibility to thermostability. *Protein Eng* 1, 477-80.

Wagner, G., Kalb, A. J. & Wuthrich, K. (1979). Conformational studies by 1H nuclear magnetic resonance of the basic pancreatic trypsin inhibitor after reduction of the disulfide bond between Cys-14 and Cys-38. Influence of charged protecting groups on the stability of the protein. *Eur J Biochem* 95, 249-53.

Wang, A. & Bolen, D. W. (1997). A naturally occurring protective system in urea-rich cells: mechanism of osmolyte protection of proteins against urea denaturation. *Biochemistry* 36, 9101-8.

Wang, A., Robertson, A. D. & Bolen, D. W. (1995). Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A. *Biochemistry* 34, 15096-104.

Wang, P. & Klimov, D. K. (2008). Lattice simulations of cotranslational folding of single domain proteins. *Proteins* 70, 925-37.

Webb, J. N., Webb, S. D., Cleland, J. L., Carpenter, J. F. & Randolph, T. W. (2001). Partial molar volume, surface area, and hydration changes for equilibrium unfolding and formation of aggregation transition state: high-pressure and cosolute studies on recombinant human IFN-gamma. *Proc Natl Acad Sci U S A* 98, 7259-64.

Weijers, M., Barneveld, P. A., Cohen Stuart, M. A. & Visschers, R. W. (2003). Heat-induced denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics. *Protein Sci* 12, 2693-703.

White, G. W., Gianni, S., Grossmann, J. G., Jemth, P., Fersht, A. R. & Daggett, V. (2005). Simulation and experiment conspire to reveal cryptic intermediates and a slide from the nucleation-condensation to framework mechanism of folding. *J Mol Biol* 350, 757-75.

Winzor, D. J., Deszczynski, M., Harding, S. E. & Wills, P. R. (2007). Nonequivalence of second virial coefficients from sedimentation equilibrium and static light scattering studies of protein solutions. *Biophys Chem* 128, 46-55.

Wright, D. B., Banks, D. D., Lohman, J. R., Hilsenbeck, J. L. & Gloss, L. M. (2002). The effect of salts on the activity and stability of Escherichia coli and Haloferax volcanii dihydrofolate reductases. *J Mol Biol* 323, 327-44.

Xia, Y., Park, Y. D., Mu, H., Zhou, H. M., Wang, X. Y. & Meng, F. G. (2007). The protective effects of osmolytes on arginine kinase unfolding and aggregation. *Int J Biol Macromol* 40, 437-43.

Xu, H. & Dill, K. A. (2005). Water's hydrogen bonds in the hydrophobic effect: a simple model. *J Phys Chem B* 109, 23611-7.

Yamada, H., Kanaya, E., Ueno, Y., Ikehara, M., Nakamura, H. & Kikuchi, M. (1994). Contribution of a hydrogen bond to the thermal stability of the mutant human lysozyme C77/95S. *Biol Pharm Bull* 17, 612-6.

Yang, A. S. & Honig, B. (1993). On the pH dependence of protein stability. *J Mol Biol* 231, 459-74.

Yang, A. S., Sharp, K. A. & Honig, B. (1992). Analysis of the heat capacity dependence of protein folding. *J Mol Biol* 227, 889-900.

Yang, D. S., Yip, C. M., Huang, T. H., Chakrabartty, A. & Fraser, P. E. (1999). Manipulating the amyloid-beta aggregation pathway with chemical chaperones. *J Biol Chem* 274, 32970-4.

Yoshimoto, N., Hashimoto, T., Felix, M. M., Umakoshi, H. & Kuboi, R. (2003). Artificial chaperone-assisted refolding of bovine carbonic anhydrase using molecular assemblies of stimuli-responsive polymers. *Biomacromolecules* 4, 1530-8.

Yu, Z. & Li, B. (2003). The effect of polyols on the reactivation of guanidium chloride-denatured arginine kinase from shrimp feneropenaeus chinensis muscle. *Protein Pept Lett* 10, 199-211.

Yutani, K., Ogasahara, K., Tsujita, T. & Sugino, Y. (1987). Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit. *Proc Natl Acad Sci U S A* 84, 4441-4.

Zavodszky, P., Kardos, J., Svingor & Petsko, G. A. (1998). Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. *Proc Natl Acad Sci U S A* 95, 7406-11.

Zhang, F., Skoda, M. W., Jacobs, R. M., Martin, R. A., Martin, C. M. & Schreiber, F. (2007). Protein interactions studied by SAXS: effect of ionic strength and protein concentration for BSA in aqueous solutions. *J Phys Chem B* 111, 251-9.

Zhang, H., Skinner, M. M., Sandberg, W. S., Wang, A. H. & Terwilliger, T. C. (1996). Context dependence of mutational effects in a protein: the crystal structures of the V35I, I47V and V35I/I47V gene V protein core mutants. *J Mol Biol* 259, 148-59.

Zhang, J., Qin, M. & Wang, W. (2005). Multiple folding mechanisms of protein ubiquitin. *Proteins* 59, 565-79.

Zhou, H. X. (2005). Interactions of macromolecules with salt ions: an electrostatic theory for the Hofmeister effect. *Proteins* 61, 69-78.

Zhou, Y. & Karplus, M. (1999). Interpreting the folding kinetics of helical proteins. *Nature* 401, 400-3.

Zhou, Z., Feng, H. & Bai, Y. (2006). Detection of a hidden folding intermediate in the focal adhesion target domain: Implications for its function and folding. *Proteins* 65, 259-65.