# Studies on the Refolding and Aggregation of Bovine Carbonic Anhydrase (BCA II) in the presence of Cosolvent Additives

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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January 2010

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# CERTIFICATE

This is to certify that the present work entitled **'Studies on the Refolding and Aggregation of Bovine Carbonic Anhydrase (BCA II) in the presence of Cosolvent Additives'** 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 family*

# Acknowledgements

*With a deep sense of gratitude, I would like to thank my supervisor and my mentor Prof. Rajiv Bhat. All throughout my journey of these many years, I have known him as a sympathetic, kind and enthusiastic person. His enthusiasm and views on research and his mission for providing high-quality work has made a deep impression in my mind. Throughout my stay in the lab, he was always accessible for any discussion. Because of his friendly nature, it is possible for me to be at this stage and I will be able to look further ahead towards the same direction. Without his valuable suggestions it would have been impossible to carry out this work the way it is. His enthusiasm and friendly nature has made the work pleasurable in the lab.*

*I express heartful gratitude to wonderful faculties at the School of Biotechnology. Prof. Rakesh Bhatnagar, Prof. Aprana Dixit, Prof. Santosh Kar, Prof. Uttam Pati, Prof. K. J. Mukharjee, Dr. Devapriya Choudhary, Dr. Maitra, Dr. Ranjana Arya, Dr. Swati Tiwari and Dr. M. S. Rajala for their constant support.*

*I would like to thank my parents, sweet sister Roli, my loving sister-in-laws, Anuradha, Mamta di and late Samta di and my in laws for their endless blessings, love and their untiring support that proved to be a stress buster for me. They were always there besides me to encourage me whenever I needed them the most. Words will not be enough to thank them. My special thank goes to my best friend and my life partner, my husband Kulbhushan. Without his help, this thesis would not have been in the presentable shape as I can see it today. He was always there along with me during entire Ph.D. period and provided me support and strength. His constant love, support and guidance helped me to survive even in the most adverse conditions.*

*I am also thankful to my labmates, Neeraj, Saurabh, Manish, Rajni, Dhiraj, Suman, Dileep, Aruna, Shanti, Akash, Sinjan, Om Prakash, Himanshu, Praveen, Santosh, Saurabh Yadav and Rahul who made my stay compatible and comfortable in the lab.*

*I will remember the company of all the past and present students of SBT and companions including Yogi, Khushoo, Sanjay, Sujeet, Neda, Megha, Sheeba, Tejram Sahu, Anuradha, Anuj, Anoop, Trinath, Amir, Madhu, Kamya, Kiranbala, Shailesh, Puneetprabha, Vibhuti, Preeti, Amit, Sunita, Manpreet, Parul, Kanchan, Amit Rahi,*

*Manish Mishra, Jitendra, Atul, Feroz, Shweta, Arun, Ramendrapati, Pushpendra, Shashi, Hasan, Shivendra and Nitika who made my stay in SBT memorable.*

*Outside SBT, I am thankful to all my seniors and dear friends Brijesh sir, Amit sir, Kushlendra sir, Pooja, Sachin Mittal, Sachin Kumar, Rupali, Manoj, Naveen, Vikas, Bairagi Bapi, Rakhi and Unnati who always cheered me up whenever I felt low.*

*I wish to express my thanks to SBT staff- Manoj ji, Tiwari ji, Nepal Singh ji, Ved Prakah ji, Sindhu madam, Pooja Sachdeva madam, Geeta madam, Yadav ji, Ajay and Sajjajn ji for their full cooperation in official hassles. I would also thank Rajesh ji for his numerous cups of tea and coffee.*

*I am also thankful to Bishu dada, Praveen, Sanjay and Manju for their day to day help. Financial support provided by CSIR is gratefully acknowledged.*

Swapnil Gupta

# *Abbreviations*

| | |
|---|---|
| A | Absorbance |
| A.U. | Arbitary unit |
| BCA | Bovine carbonic anhydrase |
| °C | Degree Celsius |
| CD | Circular dichroism |
| $CH_2$ | methylene group |
| $CH_3$ | methyl group |
| cm | centimeter |
| conc | concentration |
| CS | Citrate synthase |
| CTAB | Cetyltrimethylammonium bromide |
| D | Denatured state of protein |
| Da | Dalton |
| EG | Ethylene glycol |
| Fig | Figure |
| Fl | Fluorescence |
| GdmCl | Guanidine hydrochloride |
| ArgHCl | Arginine hydrochloride |
| hrs | Hours |
| $\Delta G^0$ | Gibbs free energy change |
| k | Rate constant of a reaction |
| kcal $mol^{-1}$ | kilocalories per mole |
| kDa | kilo-dalton |
| °K | Degree Kelvin |
| L | Litre |
| ln | $\log_e$ (natural log) |
| M | Molar |
| m | molality |
| mg | milligram |
| min | Minute |
| mM | Millimolar |
| mol | Mole |
| ml | millilitre |
| mm | millimeter |
| N | Native state of protein |
| nm | nanometer |
| OD | Optical density |
| OH | Hydroxyl ion |
| PDB | Protein data bank |
| PEG | Polyethylene glycol |
| pH | -log $a_{H^+}$ |
| pI | Isoelectric point |
| $pK_a$ | -log $K_a$ |
| RNase | Ribonuclease |
| SDS | Sodium dodecyl sulfate |
| sec | Second |
| STS | Sodium tetradecyl sulfate |
| T | Temperature |

| | |
|---|---|
| $T_m$ | Midpoint of temperature denaturation |
| $T_{agg}$ | Temperature onset of aggregation/denaturation |
| TMAO | Trimethylamine-N-oxide |
| Tris | Tris (hydroxymethyl) amino methane |
| UV | Ultraviolet |
| Zn | Zinc |
| ε | Extinction coefficient |
| μg | Microgram |
| μM | Micromolar |
| μl | Microlitre |
| [Θ] | Molar ellipticity |

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# INTRODUCTION

## Introduction:

Proteins are the most abundant molecules in biology other than water. Human body contains approximately 100,000 types of proteins and they control every chemical process taking place inside the cell (**Branden and Tooze, 1999**). Building blocks of proteins are amino acids and different types of proteins are recognized by the different order of these amino acids in the polypeptide chain. After the biosynthesis, the majority of proteins undergo changes to form tightly folded compact structures in order to function. The interior of the cell is an extraordinary complex environment in which proteins and other molecules are present at a concentration of 300-400 mg/ml (**Ellis and Minton, 2003**). Inside the cell there are present a large number of auxiliary factors that assist in the folding process, including folding catalysts and molecular chaperones (**Gething and Sambrook, 1992**). These factors help polypeptide chains to fold efficiently in the complex and crowded environment. However, they do not determine their native structure which is actually encoded by the amino acid sequences. The question of how the proteins fold into their unique native state from the amino acid sequence they contain is still a challenge in molecular biology. Thus, knowledge of the protein folding process is necessary to understand the role of evolution by which biological systems and ultimately the whole organisms have developed the ability to self assemble (**Vendruscolo *et al.*, 2003**).

Expression of recombinant proteins in bacteria often results in the formation of inactive proteins that accumulate inside the cell. The formation of these proteins inside the bacterial cell is independent of the type of protein (**Marston, 1986**). These inactive species associate to form insoluble protein aggregates called inclusion bodies. Recovering the soluble and active protein from these inclusion bodies is a major hurdle in the biotechnology and pharmaceutical industries. To recover an active protein, these inclusion bodies are unfolded by using denaturants like GdmCl or urea and then refolding is done by the removal of denaturant. Unfortunately, during the refolding process, usually there is formation of non native protein aggregates which reduce the refolding yield to a great extent. Thus, understanding of the refolding process and devising strategies for preventing aggregation is of great importance in biotechnology (**Rudolph and Lilie, 1996; Bernardez Clark *et al.*, 1999; De Bernardez Clark, 1998, 2001**).

A growing number of human and animal diseases such as scrapie, Cruetzfeldt-Jakob, familial insomnia, Alzheimer's disease, Parkinson's disease, cystic fibrosis, amylotrophic lateral sclerosis, type II diabetes and many more are known to be caused by either protein misfolding or due to the formation of ordered protein aggregates called amyloids. Prevention of these diseases again requires an in depth knowledge of the folding pathways which would be beneficial in designing new drugs and molecules that could inhibit the side reaction of aggregation thereby opening new frontiers in the development of therapies against several amyloidogenic diseases (**Thomas *et al.*, 1995; Prusiner, 1997; Kelly, 1998; Dobson, 1999; 2001 a, b; Soto, 2001**). In order to understand the folding process it is essential to have understanding of the native, intermediate and the denatured state and the interactions that stabilize or destabilize these states.

**Native state:** Native state of a protein is defined as a biologically active, properly folded three dimensional structure made up of single or multiple domains and could involve monomeric as well as multimeric forms. From the physico-chemical point of view, native state of a protein is highly cooperative in nature and physically represents a macroscopic system (**Privalov, 1992**).

Protein in its native three dimensional structure is a dynamic system fluctuating around a limited number of preferred conformations. The corresponding motion can be of amino acid side chains, stretches of polypeptide chain and domains (**Janin and Wodak, 1983, 1985**). The static structure may not be entropically favorable. However, the dynamic and flexible nature of protein molecules help them to gain entropy without affecting their enthalpic contribution to the stability of the native state (**Makhatadze and Privalov, 1993, 1996**). During formation of native state from its denatured state there is a decrease in the entropy and, hence, the process is thermodynamically unfavorable. To compensate this effect, there is rearrangement of interactions among different atoms (**Privalov, 1992**). The native state of a protein is stabilized by a number of non covalent forces such as hydrophobic interactions, hydrogen bonding, Van der Waals interactions and electrostatic interactions (**Dill, 1990; Jaenicke, 1991**).

Native state of a globular protein may not always represent the compact and tightly packed hydrophobic core. Recent studies have shown that there are many proteins which

do not possess any tertiary structure, have little amount of secondary structure or are unstructured to the extent of being random coil (**Uversky, 2002 a, b**). Proteins of this category are called natively unfolded proteins and they are known to have considerable biological relevance.

**Denatured state:** Protein denaturation was first proposed in 1931 by Hsien Wu (**Wu, 1931**). Denaturation was defined as complete disorganization of native protein molecule which occurs due to the conversion of ordered and rigid state into unordered and more or less open flexible state.

Proteins can be denatured by different methods such as low and high temperature, pressure, acidic and alkaline pH, organic cosolvents and chemical denaturants like urea and guanidinium hydrochloride (GdmCl). Whereas extremes of temperature and pH are unable to denature the protein completely, high concentration of urea and GdmCl are able to convert the fully folded form of native protein into unfolded and random coil state (**Tanford, 1968, 1970; Tanford and Aune, 1970**). The denaturing agents denature proteins either by binding to the proteins directly (**Lee and Timasheff, 1974; Prakash *et al.*, 1981**) or indirectly by altering the solvent water properties (**Schellman, 1987**).

## Fundamentals of protein folding: From the Anfinsen postulate to the new view

### The Anfinsen postulate and the Levinthal paradox: Is protein folding under thermodynamic or kinetic control?

Anfinsen and coworkers made a remarkable achievement by refolding the fully denatured and reduced ribonuclease into a fully active enzyme. This event marked the beginning of modern era of the protein folding problem. Anfinsen concluded from his results that "all the information necessary to achieve the native conformation of a protein in a given environment is contained in its amino acid sequence" (**Anfinsen, 1973**). Anfinsen's postulate was further supported by the thermodynamic control of protein folding according to which the native state is at the minima of Gibbs free energy (**Anfinsen and Scheraga, 1975**). The number of conformation that a newly synthesized polypeptide chain can acquire is astronomically large. A random search through all possible structures

of the native conformation corresponding to the minimum free energy would require a considerably long time which would be incompatible both for the *in vivo* and *in vitro* folding reaction where the folding time is of the order of seconds or minutes. Thus, it is clear that evolution has found an effective solution to this problem. This is referred as Levinthal paradox and it has dominated the protein folding problem for the last nearly 40 years. Different mechanisms were proposed to solve the Levinthal paradox. Among them Levinthal (**Levinthal, 1968**) and Wetlaufer (**Wetlaufer, 1973**) suggested that protein folding is under kinetic control. Levinthal's paradox could be thus overcome by assuming that structured nuclei are formed simultaneously in the different parts of the polypeptide chain, favored by short and medium interactions that would initiate and direct the protein folding process thereby restricting the number of possible conformations.

**Different models of protein folding and pathways of protein folding:**

In order to understand how a newly formed polypeptide chain could overcome the Levinthal's paradox to attain the native conformation, different models of protein folding and pathways have been proposed from time to time by different workers based on the theoretical concepts, experimental data, and computer simulations.

1. **Nucleation-propagation model-** This model is a classical example of helix-coil transition. It involves a nucleation step followed by the rapid propagation, the rate limiting step being the nucleation process. This model explained the folding of ribonuclease A but was forsaken after new kinetic studies of the refolding of ribonuclease were performed (**Kim and Baldwin, 1990**). A nucleation-condensation model, different from the classical one has been proposed by Fersht (**Fersht, 1997**). This model proposes a mechanism in which weak local nucleus is formed which is stabilized by long range interactions.
2. **Stepwise sequential and hierarchical folding process-** In this model, several stretches of structures are formed and assemble at different levels following a unique route (**Jaenicke, 1987; Kim and Baldwin, 1990**). According to this model, the first event is nucleation, which is followed by the formation of secondary structures which in turn associate to generate super secondary structure, then domains and eventually the active monomeric form is generated.

Such a hierarchy of protein folding corresponds to the hierarchy of protein structure.

3. **Framework model-** According to this model the secondary structure is formed in an early step of folding, before tertiary structure is formed, thereby signifying the role of short range interactions in directing the folding process (**Ptitsyn and Rashin, 1973**).
4. **Modular model-** This model is based on the three dimensional structure of protein. It assumes that not only domains but sub domains also participate as folding units which fold independently into a native structure, forming structural modules that assemble to yield the native protein (**Wetlaufer, 1981; Chothia, 1984**).
5. **Diffusion-collision model-** It was developed in 1976 by Karplus and Weaver and was reconsidered in 1994 in the light of more recent experimental data (**Karplus and Weaver, 1994**). According to this model, nucleation starts simultaneously in different parts of the polypeptide chain generating microstructures which then diffuse and associate to form substructures with a native conformation. These microstructures have a lifetime controlled by the segment diffusion. Thus, the folding of a polypeptide chain (100-200 amino acids) can occur within a very short time, less than a second.
6. **Hydrophobic collapse model-** According to this model, the first step in the protein folding is the hydrophobic collapse which occurs before the formation of the secondary structure (**Dill, 1985; Agashe *et al.*, 1995**). In this model hydrophobic effect was considered as the driving force in protein folding and stabilization.
7. **Jigsaw puzzle model-** This model assumes that there are multiple pathways of protein folding to reach the single native conformation. According to this model identification of folding intermediates gives the kinetic description rather than structural one and each intermediate consists of heterogeneous species in rapid equilibrium (**Harrison and Durbin, 1985**).

**Detection and characterization of intermediates in protein folding:** The folding-refolding reaction under equilibrium has been considered as a two state process in which only unfolded and the native species are present. However, there are also present intermediate species which are unstable and poorly populated under equilibrium conditions (**Udgaonkar, 2008**). The two state transitions which are very cooperative in nature are frequently valid for the small proteins. From the two state approximation, the value of $\Delta G^0$, which is variation of free energy of unfolding, was calculated. This value varies between 5-15 kcal/mol, indicating that the stability of proteins is rather weak (**Ghelis and Yon, 1982; Yon 1997**). Even for the two state approximation, the existence of intermediates has been proved from the kinetic studies. In many proteins, there exists monophasic unfolding and multiphasic refolding kinetics which further shows the presence of intermediates in the folding pathway of proteins. In the past decade, several experimental methods have been developed allowing these intermediate species to get characterized e.g. stopped-flow mixing devices coupled with the circular dichroism and NMR using rapid hydrogen-deuterium exchange associated with a mixing system allowing the pulse labeling of transient species. This method is very informative and gives residue specific information (**Roder *et al.*, 1988; Baldwin, 1994**). Protein engineering is another method to probe the particular regions of a protein involved in the folding process (**Matouschek and Fersht, 1991; Ballery *et al.*, 1993; Garcia *et al.*, 1995**). Two major factors in characterizing these intermediate species are a) high cooperativity and b) rapidity of the process, especially in the early part of protein folding. Detection and characterization of the intermediates are prerequisites to solving the protein folding process.

**Early events of protein folding: presence of molten globule state and their characterization.** Initial steps of protein folding are generally characterized by the formation of secondary structures for many proteins (**Ballery *et al.*, 1993; Ptitsyn, 1995**). These early species with high content of secondary structures were named ***"the molten globule"*** by Ohgushi and Wada (**Ohgushi and Wada, 1983**). Ptitsyn (**Ptitsyn, 1995**) suggested that this is a general intermediate in the refolding pathway of proteins. Since the literature for the molten globule definition is rather confusing, Goldberg and colleagues (**Chaffotte *et al.*, 1992**) have introduced the term "***specific molten globule***"

and defined its characteristics. The specific molten globule is a compact intermediate with a high content of secondary structure and a fluctuating tertiary structure. It contains accessible hydrophobic surface area to which a hydrophobic dye such as aniline naphthalene sulphonate can easily bind. As the tertiary structure is not stabilized, the aromatic residues can rotate freely in the symmetrical environment and are thus accessible to the solvent. This was detected by the absence of near-UV circular dichroism of molten globule. Formation of molten globule as an early intermediate has been detected in many proteins such as alpha lactalbumin, carbonic anhydrase, beta lactamase, the $\alpha$ and $\beta_2$ subunits of tryptophan synthase, bovine growth hormone and phosphoglycerate kinase (**Chaffotte *et al.*, 1992; Ballery *et al.*, 1993; Ptitsyn, 1995**).

Several researchers reported the presence of an intermediate species which precedes the molten globule (**Ptitsyn, 1995**). It was termed as ***pre molten globule*** by Englander (**Jeng and Englander, 1991**). This species has a significant content of fluctuating secondary structure; it is less compact than molten globule and displays hydrophobic regions accessible to the solvent. It has been reported to occur during the cold denaturation of $\beta$ lactamase (**Uversky and Ptitsyn, 1994**) and carbonic anhydrase (**Uversky and Ptitsyn, 1996**). Occurrence of such intermediates was also reported during the refolding of several proteins (**Fink, *et al.*, 1994; Fink, 1995**). There are numerous examples which show that protein folding takes place via rapid formation of transient intermediate species, either molten globule or pre molten globule, with a significant content of secondary structure and fluctuating tertiary structure (**Fink, 1995; Ptitsyn, 1995**). It has been proposed that the first event in the protein folding is hydrophobic collapse occurring either before the formation of secondary structure or simultaneously, and being followed by a rearrangement of small number of condensed states (**Dill, 1985**). This view considers both hydrophobic effect and the role of long range interactions in the initiation of the folding process.

Classical rapid mixing techniques such as stopped-flow, continuous flow and quenched flow are limited to the millisecond range. Therefore, ultra fast or very fast events of the protein folding occurring within the burst phase of protein folding cannot be detected by these techniques. Most of the secondary structure formation is completed within the dead time of a stopped-flow device. The upper limit for the rate of protein

folding has been evaluated to be around one microsecond (**Hagen *et al.*, 1996**), which is in agreement with the theoretical estimate (**McCammon, 1996**). This is very inconvenient as the crucial part in the protein folding studies is the characterization of the very early intermediate species. Severeal advances in the technology have improved the resolution time in the kinetic studies of protein folding (**Plaxco and Dobson, 1996; Englander, 2000; Bartlett and Radford, 2009**). Nonmixing techniques such as classical T-jump, nanosecond infrared laser induced T-jump and picosecond T- jump have been used to study the refolding of cold denatured proteins. Besides, other rapid techniques such as nanosecond laser photolysis, optical electron transfer and dynamic NMR methods have brought down the detection limit of very fast protein folding events in the time scale of less than one microsecond. Thus, it can be concluded that the very fast events in the protein folding start with the hydrophobic collapse which may or may not be followed by the formation of secondary structures, depending upon the protein.

**Late events in the protein folding:** On the folding time scale, intermediate events take place after the formation of the molten globule and before the rate limiting step of the folding process which finally forms the native and functional state. In the last limiting step of the folding process, the proteins attain their native conformations by regaining the enzymatic activity or restoring its full functionality. These last events correspond to the precise ordering of the secondary structure (**Matouschek *et al.*, 1990**), correct packing of the hydrophobic core (**Lecomte and Matthews, 1993**), subunit assembly in oligomeric proteins (**Ptitsyn, 1991**), the correct pairing of the domains in multidomain proteins (**Jaenicke, 1987**), and the reshuffling of the disulphide bridges (**Levitt and Chothia, 1976**). Folding is governed by the noncovalent interactions and disulphide bridges help in the stabilization of the structure, however, proline isomerization has been reported to be the rate limiting step in the folding of several proteins (**Brandts *et al.*, 1975**).

**Domains and sub domains in protein folding:** Domains are compact substructures within a protein molecule and have been considered as folding units forming structural modules called sub domains that fold independently and assemble to generate the native structure (**Wetlaufer, 1973, 1981**). Many experimental data have shown that domains behave as independent folding units (**Wetlaufer, 1981; Ghelis and Yon, 1982; Jaenicke, 1987, 1991**), including the N-terminal domain of gamma D crystallin (**Sharma**

*et al.*, **1990**), etc. However, even though isolated domains can refold independently, it has been proved through a variety of experiments, that it is not possible to reproduce functionally active protein from the two separate domains and it has been found possible only in case of certain proteins like thioredoxin (**Slaby and Holmgren, 1979**), elastase (**Ghelis *et al.*, 1978**) and methionyl t-RNA synthetase (**Shiba and Schimmel, 1992**). Thus, it seems that, for many proteins, an interaction between isolated domains along with their correct folding is required to allow the structural reorganization that could yield a functional protein.

It has been proposed that subdomains, which have a compact region within a protein, and are smaller than domains could also fold autonomously, forming folded module that assemble to generate the native conformation (**Dill *et al.*, 1995**).These sub domains are considered to be a condensed state in which the close packing of the atoms in the hydrophobic core of the molecule has not yet taken place.

**The new view of protein folding: energy landscape and the folding funnel**

The new view of protein folding has emerged in the past few years from the combination of both the experiment and theory by using simple mechanical models and is illustrated by the concept of folding funnel introduced by Wolynes and coworkers (**Wolynes *et al.*, 1995**). The model is represented in the form of energy landscape and describes the thermodynamic and kinetic behavior of the transformation of an ensemble of unfolded molecules to a predominantly native state. Depending upon the energetic parameters and conditions, a wide variety of folding patterns can evolve from the energy landscape. The folding rate is slowed down due to ripples in the energy landscape corresponding to the energy minima populated by transiently stable intermediates. According to this model, there are parallel micro pathways and each individual polypeptide chain can follow its own route. Towards the bottom of the folding funnel, the number of protein conformations decreases with decrease in the chain entropy. The steeper is the slope, faster the folding is. Contrary to this, a rugged energy landscape consists of several kinetic traps formed by the energy barriers. In this case folding can be multistate and can be slower (**Dill and Chan, 1997**). When local energy barriers are high enough, protein molecules can be trapped and possibly can also aggregate. This new view has

progressively replaced the older view of protein folding pathways. The energy landscape model provides the conceptual understanding of both two state and multistate kinetics and also protein misfolding and subsequent aggregation behavior.

**The main rules of protein folding:**

Understanding the transfer of information from amino acid sequences to the fully functional three dimensional structure of proteins (the folding mechanism), requires the complete characterization of the dynamics of partially folded proteins. A general guideline for the protein folding can be deduced from the enormous information accumulated through *in vitro* studies;

1. The information needed by a newly formed polypeptide chain resides in its amino acid sequences (**Anfinsen, 1973**).
2. For most of the proteins, the native structure is under the thermodynamic control.
3. Folding pathway involves partially folded intermediates under kinetic control. According to the new view of protein folding, the newly synthesized polypeptide chain navigates the folding routes towards the native state through intermediates which consist of heterogeneous population of partially folded species whose number decreases as the protein reaches towards the energy minimum landscape. It is possible that some folding species may get trapped in the local minima, slowing down the folding process. In the very first step of protein folding, there occurs a hydrophobic collapse which might generate heterogeneous population with native and non native structure in equilibrium, with internal rearrangements as in jigsaw puzzle or the diffusion collision method. This is followed by the association of correctly folded nuclei to generate the sub domains which further diffuse and associate. Eventually a molten globule state may be formed which in turn may consist of heterogeneous population in rapid equilibrium. All these early events occur very rapidly. From the molten globule species, protein folding can take certain restricted pathways including the formation of tertiary structure, domain pairing and last conformational rearrangement to produce the native protein in slow folding step.

**Folding in the biological environment: presence of molecular chaperones**

It has long been accepted that the *in vitro* protein folding rules are also applicable to the *in vivo* folding and proteins fold in the similar way inside the cellular environment. However, after the discovery of molecular chaperones (**Ellis, 1991**) this view was changed.

More than 20 protein families are now known to work as molecular chaperones. It is well established that in chaperone assisted system of protein folding, no further information or external energy is required for the correct folding of protein. The ATP hydrolyses by GroEL is used for the conformational changes in the chaperone which in turn releases the folded protein. By transiently binding with stress destabilized or translocated polypeptide chain, molecular chaperones help in preventing the improper folding as well as the aggregation of proteins. Chaperones do not interact with the folded protein nor do they carry any information that can direct a protein to assume a structure different from that coded by amino acid sequence. These chaperones transiently associate with an early folding intermediate, probably a molten globule or a pre molten globule, through hydrophobic interaction. Thus, chaperones increase the refolding yield but not the rate of refolding reactions.

## Protein folding and protein misfolding

Synthesis of each protein molecule takes place on the ribosome inside the living cell after which it has to fold into specific conformational state which is encoded in its amino acid sequences in order to carry out its biological function. Protein folding inside the cell is still the most intriguing question in the biological sciences (**Dobson, 2004**). Protein folding takes place in the highly crowded and complex environment of the cell in the presence of several auxiliary proteins (**Gething and Sambrook, 1992**; **Ellis and Hartl, 1999**). Inside the cell, protein synthesis takes place in the organelles known as ribosomes from the information contained in the cellular DNA. *In vivo*, the folding process of some nascent polypeptide chain begins when it is still attached to the ribosome (**Hardesty and Kramer, 2001**). There are some proteins which undergo major part of the protein folding in the cytoplasm after the release from the ribosome, while yet others fold in the specific

compartments known as endoplasmic reticulum (ER) following translocation through membranes **(Hartl and Hartl, 2002)**. The surrounding environment of the cell affects the folding process of proteins and however, the fundamental principle behind the folding process remains almost same in all cases. During folding, the incompletely folded polypeptide chain has some exposed hydrophobic regions that otherwise remain buried inside in the native state; such species are prone to make contact with other molecules and can give rise to some non native interactions. To cope with this problem more generally, living systems have evolved a range of elaborate strategies to prevent interaction with other molecules prior to the completion of the folding process **(Gething and Sambrook, 1992; Hartl and Hartl, 2002; Dobson, 2003)**.

Large numbers of molecular chaperones are present in all types of cells and cellular compartments. These chaperones have general role in enabling the efficient folding and assembly, however, their specific function can differ substantially and it is evident that many types of chaperones work in tandem with one another **(Hartl and Hartl, 2002).** Some types of molecular chaperones have been found to interact with nascent polypeptide chain as they emerge from the ribosome, and bind non-specifically to protect aggregation-prone regions rich in hydrophobic residues. Other chaperones help in guiding the later stages of protein folding, particularly for complex proteins including oligomeric and multimolecular assemblies. In addition to the molecular chaperones, there are present several classes of folding catalysts that simply accelerate the otherwise extremely slow steps of protein folding. The most important ones are peptidylprolyl isomerases, that increase the rate of cis/trans isomerisation of peptide bonds involving proline residues, and protein disulphide isomerases which help in the formation of disulphide bond and their reorganization within proteins **(Gething and Sambrook 1992; Hartl and Hartl, 2002)**.

*In vitro,* folding process starts from the newly formed polypeptide chain that has been fully unfolded by using chemical denaturant such as urea or GdmCl. In both the cases, the polypeptide chain is highly disordered before folding is initiated. The folding of small proteins (less than 100 amino acid residues), appears to be limited by the time required to search for the most appropriate interactions that are needed to permit the rapid progression towards the native state. Folding process for the larger proteins is rather a

much more complex process and it can involve the population of one or more partially folded intermediate states (**Dobson *et al.*, 1999**). The protein folding process becomes gradually more complex as the size and complexity of the protein is increased. There can be accumulation of intermediates which are only partially folded and have enhanced lifetimes also. In addition to this, the considerably long time taken by the polypeptide chain to search for the stable native like conformation could eventually lead to the events termed as 'misfolding' (**Dobson, 2000**).

Therefore protein misfolding, or its folding into intermediates that may form undesirable aggregates, provides an insight into potential problems that could arise during protein folding even in the best designed environment. Folding and unfolding events are supposed to be coupled in many cases such as translocation of proteins across the membranes, protein trafficking, secretion of extracellular proteins, and the control and regulation of cell cycle (**Radford and Dobson, 1999**). Thus, the failure of proteins to fold or to remain correctly folded under the physiological conditions may give rise to malfunctions and several diseases (**Thomas *et al.*, 1995; Dobson 1999, 2003 a, b**). A selection of such diseases is given in Table 1.

**Table 1.** *Representative protein folding diseases*

| Disease | Protein | Site of folding |
|---|---|---|
| Huntington's disease | Huntingtin | Cytosol |
| Cystic fibrosis | Cystic fibrosis trans-membrane regulator | ER |
| Phenylketonuria | Phenylalanine hydroxylase | Cytosol |
| Hypercholesterolemia | Low-density lipoprotein receptor | ER |
| α1-Antitrypsin deficiency | α1-Antitrypsin | ER |
| Osteogenesis imperfecta | Procollagen | ER |
| Sickle cell anemia | Hemoglobin | Cytosol |
| Marfan syndrome | Fibrillin | ER |
| Tay–Sachs disease | β-Hexosaminidase | ER |
| Familial amyloidoses | Transthyretin/ lysozyme | ER |
| Alzheimer's disease | Amyloid β-peptide/tau | ER |
| Parkinson's disease | α-Synuclein | Cytosol |
| Scrapie/Creutzfeldt–Jacob disease | Prion protein | ER |
| Scurvy | Collagen | ER |
| Cancer | p53 | |
| Cataracts | Crystallins | Cytosol |
| Retinitis pigmentosa | Rhodopsin | ER |

## Protein aggregation

Protein aggregation applies to the formation of insoluble precipitates that can be 'pathological' in nature. Some process like salting out, which is insolubility of the native state due to protein concentration exceeding the solubility limit or the intermolecular association involved in the formation of native oligomers can also be categorized under aggregation phenomena. In case of pathological aggregation the initial material may be

soluble aggregates, but eventually become insoluble in nature on exceeding a certain size. Aggregation of proteins can be classified into the following categories: *in vivo* and *in vitro* and ordered and disordered. Amyloid fibrils (both *in vivo* and *in vitro*) are example of ordered aggregates, whereas inclusion bodies are example of *in vivo* disordered aggregates. Corresponding disordered aggregate *in vitro* are those formed during the refolding of the denaturant unfolded protein at high protein concentration, or under weakly native conditions at high protein concentrations; these are often referred as folding aggregates.

Native, folded proteins can also undergo aggregation process under certain conditions, mostly due to salting out and isoelectric precipitation (when the net charge on the protein is zero). The precipitates formed by the native proteins can be distinguished from the pathological aggregates by their solubility in buffer under native like conditions. Pathological aggregates however dissociate and dissolve only in the high concentration of denaturant or detergent. Protein aggregation is assumed to involve either unfolded or the native states. For example, during protein folding, formation of inclusion body can arise from the hydrophobic aggregation of the unfolded or denatured states, whereas amyloid fibrils and other extracellular aggregate formation can take place from the native like conformation in a process analogous to the polymerization of hemoglobin S (**Wetzel, 1994**). Recent studies suggest that aggregation is much more likely to arise from the specific, partially folded intermediates. Thus, it can be concluded that aggregation will be favored by factors and conditions that favor the population of these intermediates. Also the properties of these intermediates are important in determining whether aggregation will occur or not.

**Mechanism of protein aggregation:** Earliest study on protein aggregation was done on the enzyme tryptophanase, which showed the presence of an intermediate at moderate denaturant concentration that forms aggregate (**London *et al.*, 1974**). The specificity of the aggregation was proved by the addition of other folded proteins that did not affect the amount of aggregated tryptophanase. Finally, the idea that the partially folded intermediates are responsible for the aggregation emerged as the key point during protein aggregation process (**Mitraki and King 1989; Wetzel, 1994, 1996; Speed *et al.*, 1995, King *et al.*, 1996**). Studies by several research groups have supported the above idea

which suggests the generality of the phenomena (**Hurle *et al.*, 1994; Oberg *et al.*, 1994; Kim and Yu, 1996**).

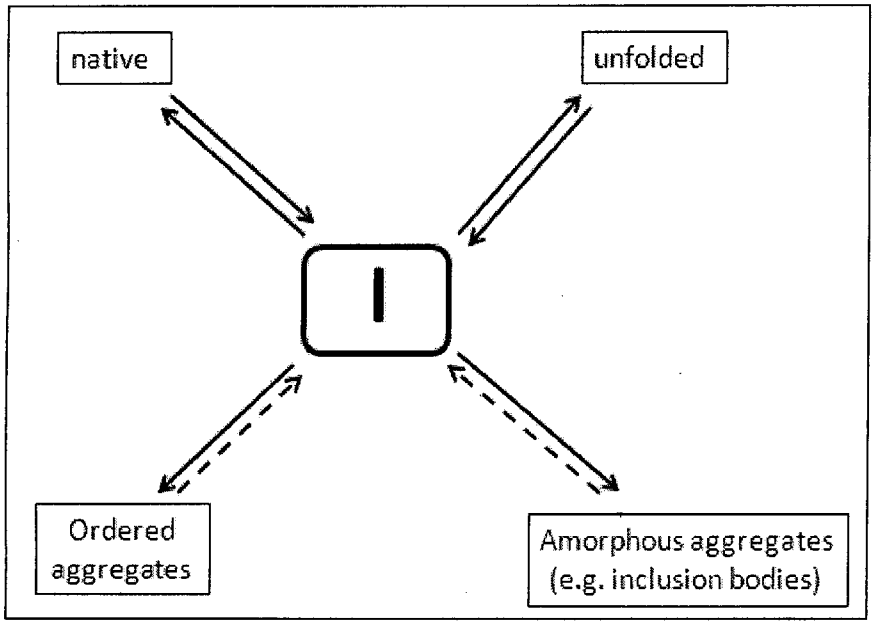



***Fig. 1:*** *Basic model for protein aggregation (Adapted from **Fink, 1998**). The box I represents a partially folded intermediate, which can be populated either in the folding direction (from unfolded) or from the native state. The intermediate I has a strong propensity to aggregate, leading to either ordered or disordered (amorphous) aggregates.*

The most accepted model for the formation and structure of protein aggregates is the specific intermolecular interactions between hydrophobic surfaces of structural subunits in partially folded intermediates (**Fink, 1998**). According to this model protein folding involves intermediates, each consisting of an ensemble of closely related substate (the various substates of a given intermediate can be characterized by having a common secondary structure and most likely a common core of relatively native like structure, with the remaining polypeptide chain disordered or in unstable structural units). The substructural units, which can be considered as the building blocks (typically subdomains) and can be stable or metastable on their own, undergo sequential interactions with each other to give rise to the native state (**Fink, 1995; 1998**). Formation of native state involves the intramolecular interaction of the hydrophobic faces of

structural subunits. Aggregation occurs when these hydrophobic surfaces start interacting in an intermolecular manner. Thus the initial stages of aggregation are quite specific as they involve interaction of specific surface element of the structural subunits of one molecule with the matching hydrophobic surface areas of structural subunit of a neighboring molecule. Three dimensional propagation of this process leads to large aggregates. Initially the aggregates (e.g. dimers and tetramers) will be soluble, but eventually their size will exceed the solubility limit. The intermediates are more prone to aggregation than the unfolded state because in the unfolded state the hydrophobic side chains are randomly scattered into many small hydrophobic regions, whereas in the partially folded intermediates there will be large patches of contiguous surface hydrophobicity, which will have much stronger propensity for the aggregation.

Aggregation is often considered to be irreversible phenomena but actually it reflects a very slow rate of disaggregation and the fact that equilibrium lies far away in the favor of aggregates rather than its soluble monomeric form. Under certain conditions, aggregates including *in vivo* amyloids can be reversed (**Deyoung *et al.*, 1993 and Tennent *et al.*, 1995**). In general, however, once the insoluble aggregates are formed, the process is effectively irreversible under native like conditions.

**Factors favoring aggregation:** Protein aggregation is majorly favored by those factors which result in the accumulation of partially folded intermediates. One important factor is mutation in the polypeptide chain that leads to differential destabilization of the native state relative to the partially folded intermediates. Environmental conditions also affect the rate of aggregation. Thus, the important factors that determine whether a protein will aggregate, and the extent and rate of the aggregation are: the protein amino acid sequence, the pH, the temperature, the ionic strength, the concentration of the protein, the presence of cosolutes (e.g. denaturants such as urea, other chaotropes or kosmotropes – including the osmolytes and ligands that interact selectively with native or non native conformations of the protein or the aggregated form and the presence (or absence) of various molecular chaperones.

**Towards an energy landscape for aggregation:** There exists a much more complex energy landscape for aggregation based on the various theoretical studies of the equilibrium and kinetic behavior of proteins and peptides at high concentration as

compared to that for the folding (**Wolynes *et al.*, 1995; Dinner *et al.*, 2000; Brooks *et al.*, 2001**). Small globular proteins are characterized by a funnel like landscape that illustrates their ability to reach the functional state rapidly and reliably. The funnel shape of the protein folding energy landscape arises from the evolutionary process that results in the selection of polypeptide chains for which the native contacts are on average more favorable than the non native ones (**Bryngelson and Wolynes, 1987; Dill and Chan, 1997**). However, at higher protein concentration there is a competition between the native contacts with a large number of alternative intermolecular interactions that increase the ruggedness of the protein aggregation landscape.

The high energy and high entropy region of the energy landscape for the aggregation corresponds to the situation where soluble monomers and small oligomers of different sizes are present in the solution. These species are highly dynamic in nature and interconvertible to the heterogeneous ensemble of conformation with different amounts of native and non native, as well as inter and intramolecular contacts (**Bitan *et al.*, 2003; Calamai *et al.*, 2005**). The region characterized by low energy and low entropy is partitioned into three distinct types of assemblies: a) crystal structures, which are stabilized mainly by the formation of intramolecular interactions; b) ordered aggregates (amyloid fibrils), in which intermolecular interactions are more dominant; c) amorphous aggregates, which are characterized by the irregular packing of polypeptide chains.

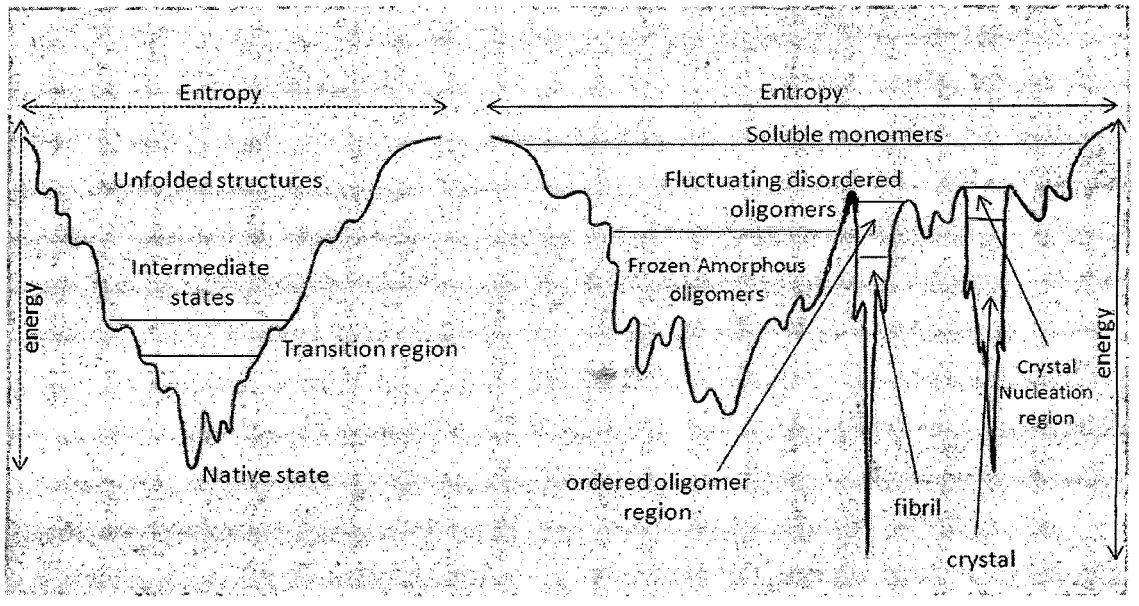



***Fig. 2:*** *Illustrations of the energy landscape for the protein folding (left) and protein aggregation (right). Adapted from* ***(Gsponer and Vendruscolo, 2006).***

**Kinetics and thermodynamics of protein misfolding and aggregation:** Misfolding of polypeptide chain leading to aggregation as well as the stability of the aggregates formed during the folding pathways is of considerable interest. The knowledge of the stability of aggregates and the kinetics of aggregation are crucial in order to design the therapeutics aimed at for preventing aggregation associated with a numerous diseases as well as in designing, purifying and formulating manufactured protein products (**Murphy and Kendrick, 2007**).

Protein aggregation pathway can be divided into two alternatives: rapid hydrophobic collapse into quasi stable aggregate or rare conformational conversion from a natively folded protein to a partially misfolded "nucleus" followed by a rapid growth. Four general and somewhat overlapping pathway for the protein aggregation has been suggested: a) monomer-directed conversion, in which collision of two monomers induces conversion to an aggregation prone conformation, b) nucleated polymerization, in which a rare thermodynamically unfavorable conformer appears and nucleates further growth by monomer addition, c) templated assembly, in which monomers assemble on the pre existing aggregate, and d) nucleated conformation conversion, in which collapse of monomers into oligomers precedes a conformational conversion to a structured aggregate

(**Kelly, 1998**). Interestingly, it is noted that quaternary structure greatly stabilizes proteins against aggregation, and that dissociation of stable oligomers to monomers initiates aggregation.

**Kinetic competition between folding and aggregation:** In most of the cases of aggregation, especially *in vivo*, there is a kinetic competition between aggregation and other processes such as folding (Fig. 3)

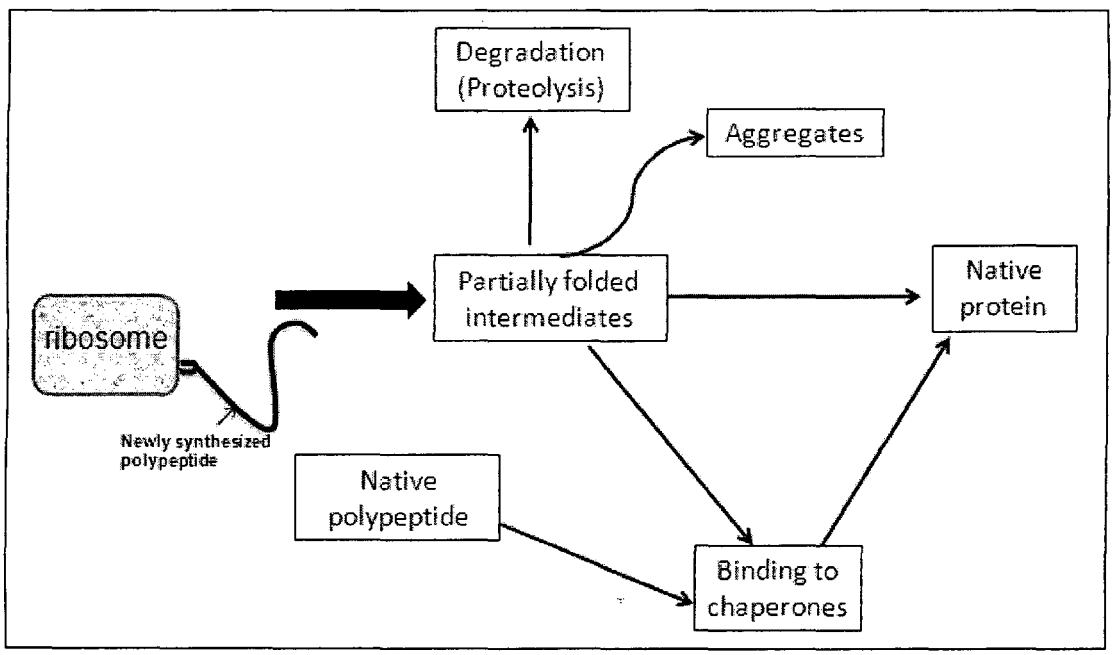



***Fig. 3:*** *Schematic diagram showing that aggregation usually involves kinetic competition which shows different pathways of partially folded intermediates leading to spontaneous folding and the native state, aggregation, binding to chaperones and proteolytic degradation.*

The protein concentration and the environmental conditions significantly affect the rate of intermolecular association. In case of physiological aggregation, the potential role of post-translational modification may be critical. In many cases when aggregation occurs from the solution of native protein, it is partially folded intermediates in equilibrium with the native state that are immediate precursors of the aggregates.

The propensity for a given protein to aggregate, either *in vivo* or *in vitro*, may well be determined in part by the lifetime of partially folded intermediates (**Fink, 1998**). Those intermediates which have longer life time are more prone to aggregate for two

reasons: first, there is a greater chance of interaction with another such partially folded intermediate, and second, in the *in vivo* situation, the molecular chaperones involved in preventing aggregation by interacting with the partially folded species may become saturated, and thus there will be depletion of chaperone to bind to the additional newly synthesized protein. Several studies indicate that *in vivo* and *in vitro* aggregate formation during refolding give rise to similar aggregates, suggesting the presence of common partially folded intermediate for both types of aggregates (**Teschke and King, 1993; Oberg *et al.*, 1994; King *et al.*, 1996**).

**Some common examples of *in vivo* aggregates**

**1) *In vivo* aggregates: <u>inclusion bodies</u>**

Inclusion body formation is not only common in prokaryotic and eukaryotic cells, but also for both heterologous and homologous over expression, showing that over expression itself is responsible for the aggregation. For example, *in vivo* aggregation of beta-lactamase is only observed when the rate of expression exceeds 2.5% of the total protein synthesis rate (**Georgiou *et al.*, 1986**). These results are in accordance with the *in vitro* refolding system, which show that aggregation increases as the protein concentration increases (**Bowden and Georgiou, 1990; Georgiou *et al.*, 1994**). Inclusion bodies are frequently refractory to renaturation. To obtain the functional protein requires denaturation and solubilization, often with disulphide reducing agents, and subsequent renaturation.

Among the several factors responsible for the inclusion bodies formation, few are: high local concentration of proteins; a reducing environment in the cytoplasm; lack of post-translational modifications; improper interactions with chaperones and other enzymes involved in *in vivo* folding; and intermolecular cross linking via disulphide (although inclusion bodies can also be formed in the proteins lacking cysteine). Three possible mechanisms have been proposed for the inclusion bodies formation: aggregation of native protein of limited solubility; aggregation of the unfolded state; aggregation of partially folded intermediate states (**Wetzel, 1996**).

**2) *In vivo* aggregates: <u>prions</u>**

The prion diseases are caused by a protein that undergoes conformational changes from its normal conformation (PrPc or PrP-sens) to its pathological form (PrPsc or PrP-res). The pathological form is protease resistant and insoluble, usually forming amyloid fibrils (**Lansbury and Caughey, 1996; Harrison *et al.*, 1997**). Prions were first detected in sheep disease called scrapie and have now been known to identified in, or transmitted to, a number of mammals, including humans, and a analogous phenomena has been found in yeast involving Sup35 protein (**Patino *et al.*, 1996**).

A very interesting model for prion-protein aggregation, involving the conversion of regions of the polypeptide that are helical in the soluble form into beta sheet in the insoluble, protease resistant prion form, has been proposed (**Cohen *et al.*, 1994**). In case of prion proteins and peptides, insoluble form acts as a template to catalyze the conversion of the soluble form into the aggregate form. An investigation of the conformation of a partially folded intermediate of the scrapie prion proteins at low denaturant concentration led to the suggestion that the insoluble prion protein is an aggregated form of a partially folded intermediate on the folding pathway (**Safar *et al.*, 1994**).

**3) *In vivo* aggregates: <u>amyloids</u>**

Many diseases listed in the Table 2 are associated with the deposition of proteinaceous aggregates in a variety of organs such as the liver, heart and brain **(Prusiner, 1997; Kelly, 1998).** Many of these diseases are described as 'amyloidoses' because the aggregated material stains with dye such as Congo red in a manner similar to starch (amylase), the aggregates are referred to as '*amyloid*' and the typical fibrous structures as '*amyloid* fibrils'. A list of known amyloid disease is given in Table 2, along with the protein component that is associated with the extracellular aggregates in each case (**Sunde *et al.*, 1997**).

**Table 2.** *Fibril protein components and precursors in amyloid diseases*

| Clinical syndrome | Fibril component |
|---|---|
| Alzheimer's disease | A β peptide, 1-42,1-43 |
| Lysozyme variants | full length Lysozyme amyloidosis |
| Primary systemic amyloidosis | Intact light chain or fragments |
| Secondary systemic amyloidosis | 76- residue fragment of amyloid A protein |
| Familial amyloidoticpoly Neuropathy I | transthyretin variants and fragments |
| senile systemic amyloidosis | wild-type transthyretin and fragments |
| Hereditary cerebral amyloid angiopathy | fragment of cystatin-C |
| Haemodialysis -related amyloidosis | β 2- microglobulin |
| Familial amyloidotic polyneuropathyII | fragments of apolipoprotein A-I |
| Finnish hereditary amyloidosis | 71-residuefragment of gelsolin |
| type II diabetes | fragment of islet-associated polypeptide |
| insulin-related amyloid | full length insulin |
| Fibrinogen α- chain amyloidosis | fibrinogen α-chain variants |
| Spongiform encephalopathies | full length prion or fragments |
| Medullary carcinoma of the thyroid | fragments of calcitonin |
| Atrial amyloidosis | atrial natriuretic factor |

Some of the amyloid diseases are familial (probability of misfolding is often greater in mutational variants), some associated with medical treatment (e.g. haemodialysis) or infection (the prion diseases), and some are sporadic (e.g. most forms of Alzheimer's). Many of them can be found in both familial and sporadic form (e.g. transthyretin). In addition to these there are some diseases like Parkinson's and Huntington's diseases

(**Perutz, 1999**) that appear to involve very similar aggregates but which are intracellular not extracellular and are therefore not included in the strict definition of amyloids.

**Characteristics of amyloids:** Amyloid deposits show specific optical behaviors (such as birefringence) on binding with certain dye molecules such as Congo red. The fibrillar structures typical of many of the aggregates have very similar morphologies (long, unbranched and often twisted structures a few nanometers in diameters) and a characteristic cross- beta X- ray fiber diffraction pattern. The core structure of the fibril is stabilized primarily by interactions, particularly hydrogen bonds, involving the polypeptide main chain. The ability of polypeptide chains to form amyloid structure is not restricted to the relatively small number of proteins associated with recognized clinical disorder but is a generic feature of polypeptide chain **(Dobson, 2001 a, b).** Thus, fibrils can be formed *in vitro* by many other peptides and proteins, including well known molecules as myoglobin and also by homopolymers such as polythreonine or polylysine **(Dobson, 2001; Fändrich and Dobson, 2002)**. Even though the ability to form amyloid fibrils seems to be generic, the propensity to do so under given circumstances can vary markedly between different sequences. The relative aggregation rate for a wide variety of protein and peptides depends upon its various physiochemical properties such as charge, hydrophobicity, secondary structure propensities **(Chiti *et al.*, 2003).**

**Mechanism of amyloid formation:** One of the crucial aspects of the formation of amyloid fibrils is the mechanism by which they are assembled from the precursor species. In globular proteins the polypeptide main chain is largely buried within the folded structure, and it is necessary for it to get exposed for the formation of fibrillar species. Thus the conditions which favors the formation of amyloid fibrils are the one in which the proteins involved are at least partially unfolded, for example low pH **(Kelly, 1998)** or the fragmentation of proteins, through proteolysis etc. *In vitro* studies of amyloid indicate that the formation of fibrils, by appropriately destabilized or fragmented proteins, is generally characterized by a lag phase followed by the rapid growth phase **(Harper and Lansbury, 1997)**. Such behavior is typical of nucleated process of crystallization, and similar with crystallization, the lag phase can be eliminated by addition of pre formed fibrils to fresh solutions, a process known as seeding **(Harper and Lansbury, 1997)**. The earliest species formed during aggregation resemble to small bead like structures

described as amorphous aggregates or as micelles. These early "prefibrillar aggregates" then transform into species with more distinctive morphologies, described as "protofibrils" or "protofilaments" **(Jimenez *et al.*, 2002; Bitan *et al.*, 2003; Caughey and Lansbury, 2003)**. These structures are commonly short, thin sometimes curly, fibrillar species which assemble into mature fibrils, perhaps by lateral association, accompanied by some degree of structural reorganization **(Bouchard *et al.*, 2000).**

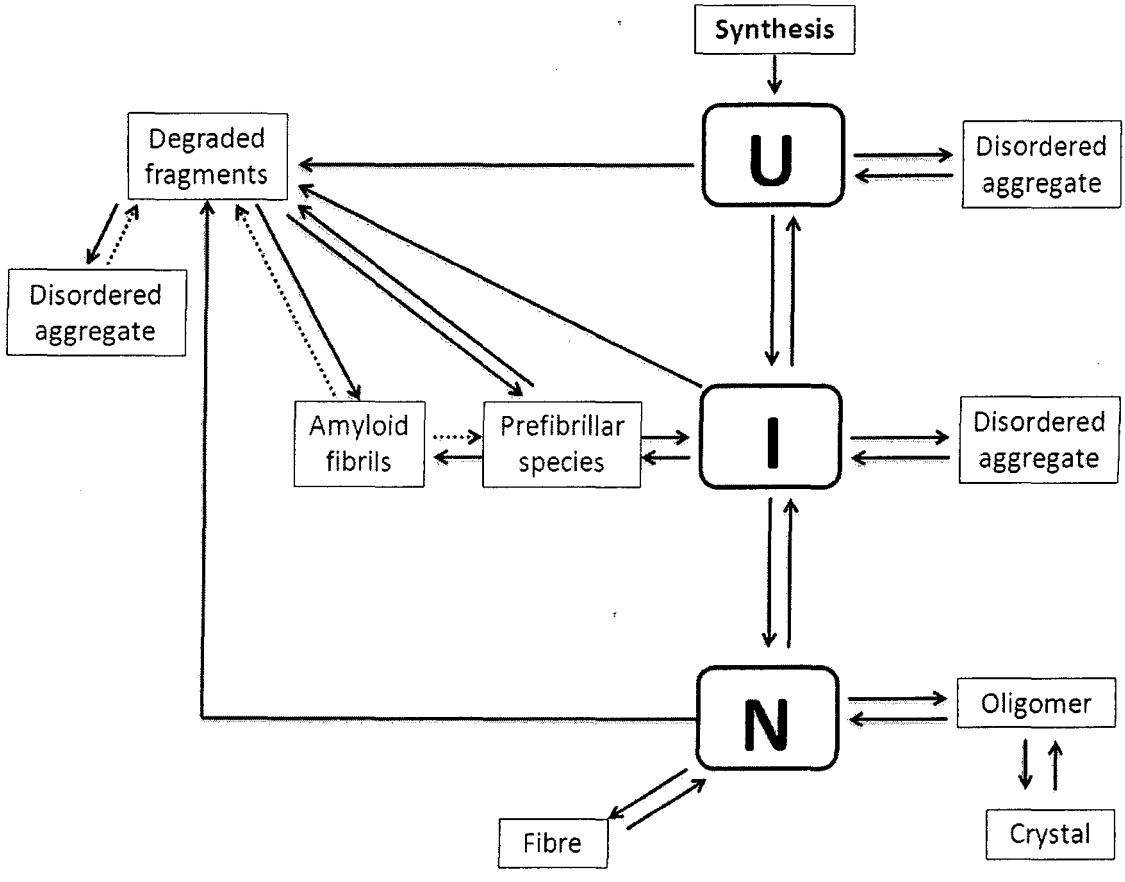



**Fig. 4:** *Schematic representation of some of the states accessible to a polypeptide chain following its synthesis on a ribosome (Adapted from* ***Dobson, 2004****).*

**Some common examples of *in vitro* aggregates**

***In vitro* aggregates: folding aggregates**

Folding (refolding) aggregates are referred to the precipitates that are formed when a protein, unfolded in high concentration of denaturant, is refolded by the dilution of the denaturant. The vast majority of proteins form aggregates under such experimental conditions, sometimes even in micromolar concentration. In some cases, aggregation formation also takes place when proteins are subjected to relatively weak native conditions (e.g. to a temperature close to that at the beginning of the thermal denaturation transition, or a concentration of denaturant close to the beginning of the unfolded transition).

An example of effect of protein concentration on aggregation during refolding is that of TEM β- lactamase (**Georgiou *et al.* 1986**). In this case, a partially folded intermediate in the equilibrium denaturation was found to correlate with aggregation: increasing the temperature increased the aggregation, whereas the presence of sucrose decreased the aggregation. This *in vitro* data closely paralleled *in vivo* observation on beta-lactamase inclusion body formation (**Przybycien *et al.*, 1994**). A partially folded intermediate in the refolding of carbonic anhydrase was found to form dimers and then higher oligomers before forming insoluble aggregates (**Cleland and Wang, 1990, 1992**). Another system where concentration dependent aggregation revealed evidence of partially folded intermediate as the source of aggregation is that of reduced lysozyme (**Goldberg *et al.*, 1991**).

***In vitro* aggregates: thermally gelled proteins**

Solutions of many proteins on heating form gel like structure due to the aggregation of proteins. Biophysical studies have shown that these gels have a large component of beta sheet structure and FTIR studies indicate the presence of band in the vicinity of $1616cm^{-1}$ (which is characteristic to anti parallel beta sheets); (**Clark *et al.*, 1981; Ismail *et al.*, 1992**). Studies have been done to show that these thermally induced aggregates arise from partially folded intermediates (**Kato and Takagi, 1988**).

***In vitro* aggregates: partially folded intermediates**

The partially folded intermediate state of proteins is highly susceptible to the aggregate formation due to the non specific interactions taking place between the exposed hydrophobic residues e.g. partially folded intermediate or '(A)' state of apomyoglobin was found to be in monomeric and aggregated form at low pH and high protein concentration and these aggregates are generally rich in beta sheet structure (**Fink *et al.*, 1997**).

**Prevention of aggregation and therapy for protein misfolding and amyloid diseases:**

It has been observed that proteins have evolved in such a way to avoid the sequences responsible for the strong propensity to aggregate. There are many short peptides that contain sequences having considerable hydrophobic residues and a high tendency for the beta sheets formation. They are most likely to form aggregates and/or amyloid like structures. The presence of polar residues in the flanking sequences increase the solubility limit and sometimes they are bulky enough to cause steric hindrance thereby preventing the favored interactions that result in the lack of aggregation or amyloid formation under natural conditions (**Fink, 1998**).

*Cellular machinery to prevent protein misfolding*: Various biochemical studies in the living system have shown that they do not depend solely on the molecular chaperone and folding catalysts to regulate protein folding but they have well developed sophisticated mechanism of quality control to check, whether proteins are correctly folded, and to target for the destruction of the misfolded proteins. The best understood quality control mechanisms are present in the endoplasmic reticulum, the major folding compartment of the eukaryotic cell (**Sitia *et al.*, 2003**). The quality control mechanism involves a complex series of glycosylation and deglycosylation processes and prevents misfolded proteins from being secreted from the cell. In addition the misfolded and folded protein is recognized and targeted for the degradation through ubiquitin-proteosome pathway (**Goldberg, 2003**).

The fundamental origin of amyloid disorder, arise from the increased tendency of proteins to aggregate under circumstances such as old age. Therefore an increased understanding of protein folding and misfolding is crucial for the rational development of

the therapeutic strategies directed against these diseases. Some of the approaches have already been developed, for example, to stabilize the native states of amyloidogenic proteins, to lower the levels of aggregation prone species, and to inhibit selectively the aggregation process that results in the formation of the amyloid structure (**Dobson, 2003 a, b; Cohen and Kelly, 2003**). Besides this, cellular housekeeping mechanisms (such as molecular chaperones and targeted degradation mechanisms) are essential for the ability of all the cellular system to function effectively and to neutralize the toxic effect of any protein aggregate that can form during the normal functioning of the cell (**Bucciantini *et al.*, 2002; Stefani and Dobson, 2003**). This information can be exploited in the future to find out the generic solution for the generic form of the amyloid disease. For example natural housekeeping mechanism can be enhanced in such a way that will provide protection against all these diseases.

Under *in vitro* conditions there are several methods to prevent or decrease aggregation, ranging from ligands that stabilize the native state (**Wetlaufer and Xie, 1995; Miroy *et al.*, 1996**) to the presence of competing peptides (**Defelippis *et al.*, 1993**). Although there are, as yet no general method that can be applied to prevent aggregation, there are number of strategies that are frequently successful (**Clealnd and Wang, 1990 a, b; Buchner and Rudolph, 1991; Rudolph and Lilie, 1996; Chaudhuri *et al.*, 1996**). The most popular approach involve the use of chaperones (**Guise *et al.*, 1996; Thomas *et al.*, 1995**), immobilization (**Stempfer *et al.*, 1996**), low concentration, stabilizing agents such as arginine and polyols (**Rudolph and Lilie, 1996; Mishra *et al.*, 2005**) optimization of growth conditions, use of fusion proteins (**Georgiou *et al.*, 1994**) and antibodies etc.

## Carbonic anhydrases

The carbonic ahydrases are extremely efficient catalysts of the reversible hydration of carbon dioxide with maximum turnover numbers among the highest known for any enzyme. They have also been found to catalyze the hydrolysis of certain esters and related compounds and the hydrolysis of aldehydes. The catalytic efficiency of the enzyme toward carbon dioxide is, however, several orders of magnitude greater than towards all other substrates. Carbon dioxide plays an important role in respiration as well

as in other physiological processes, where the rapid interconversion of carbon dioxide and bicarbonate ion is essential to the organism.

Historical background

The enzyme was first discovered by Meldrum and Roughton (**Meldrum and Roughton, 1933**), who gave it the name carbonic anhydrase. A method for the purification of the enzyme and a detailed study of its properties, including its inhibition by low concentrations of cyanide, sulphide and azide was published in 1933 (**Meldrum and Roughton, 1933**). It was demonstrated that carbonic anhydrase is a Zn containing enzyme (**Keilin and Mann, 1939, 1940**). This was the first clearly defined physiological function for this metal ion. The molecular weight was determined to be 30,000 (**Petermann and Hakala, 1942**) and the Zn content of 0.2% (**Scott and Fischer, 1942**) corresponding to one metal ion per enzyme molecule. Lindskog presented a purification procedure for the bovine erythrocyte enzyme based on the high resolution offered by the modern separation methods (**Lindskog, 1960**). Many laboratories reported the purification methods for the human erythrocytes enzymes (**Nyman, 1961; Rickli *et al.*, 1964**). The present knowledge of the molecular properties and the mechanism of carbonic anhydrase are based on a large extent on the studies of mammalian erythrocyte enzymes, especially the form from human and bovine blood.

Distribution and Physiological Functions

Carbonic anhydrase is very widespread in nature and occurs in animals, plants, and certain bacteria. The distribution and physiological function in vertebrates, particularly mammals have been discussed in detail by Maren (**Maren, 1967 a, b**). In addition to their respiratory role of facilitating the transport of metabolic $CO_2$, the enzymes are involved in the transfer and accumulation of $H^+$ or $HCO_3^-$ in a wide variety of organs. Vertebrate tissues that contain carbonic anhydrase include erythrocytes, kidney, gastric mucosa, and the eye lens where high concentration of the enzymes is found. The enzymes are also present in the gills and secretory organs of various types, such as parotid and pancreatic glands, rectal and alkaline glands, and swim bladders of many species of fish.

The physiological significance of two distinct types of mammalian carbonic anhydrase is an intriguing problem. The two proteins have been shown to be governed by

two separate structural genes (**Tashian *et al.*, 1968; McIntosh, 1970; Carter and Parsons, 1970**). The two isoenzymes have been found in several tissues (**Carter and Parsons, 1970**). However, the quantitative relation between the two isoenzymes varies considerably for the two different organs suggesting that the two proteins are functionally independent enzymes. These findings also indicate that the biosynthesis of the isoenzyme can be under separate control.

Polymorphism and Nomenclature

The polymorphism of mammalian carbonic anhydrase resulted from the presence of two isoenzymes with distinctly different amino acid sequences and specific activities. Additional polymorphism may be observed due to the presence of modified form of each of the two isoenzymes. These modified forms may arise through mutational changes or result from secondary modifications occurring *in vivo* or *in vitro.*

In earlier investigations of carbonic anhydrase isoenzymes, the various forms of enzymes were designated in accordance with their chromatographic behavior or electrophoretic mobility. On this basis, a standard nomenclature was adopted for the human isoenzymes (**Nyman and Lindskog, 1964; Rickli *et al.*, 1964**). Bovine erythrocytes contain significant amounts of two electrophoretically separable forms originally designated A and B in the order of mobility (**Lindskog, 1960**). Both of these forms have high similar specific activities and therefore belong to the C group of mammalian carbonic anhydrases. Both groups possess identical amino acid compositions (**Nyman and Lindskog, 1964**). Presumably one form is the modified variant of the other form.

Classification and Composition

All the mammalian carbonic anhydrases studied so far are composed of one Zn atom and a single polypeptide chain composed of approximately 260 amino acid residues. In general enzymes are rich in basic and acidic amino acid and have noticeably high proline content. However, sulphar containing amino acids are rare in mammalian and bacterial enzymes, and therefore occurrences of intramolecular disulphide bonds are impossible in most of the cases. The total absence of cysteine in some carbonic anhydrases rules this amino acid out of consideration for its involvement in metal binding or catalytic action (**Nyman and Lindskog, 1964**).

Carbonic anhydrase (CA) is a ubiquitous enzyme found in all the animals and photosynthesizing organisms as well as in some nonphotosynthetic bacteria. One outstanding discovery in the recent year is the discovery of three evolutionary unrelated CA families which have been designated as α-, β- and γ-CA (**Hewett-Emmett and Tashian, 1996**). There are no significant sequence homologies between representatives of different CA families, but all of them are Zn enzymes. Thus these CA families seem to be beautiful examples of convergent evolution of catalytic function.

## Bovine Carbonic anhydrase as a model system

Bovine carbonic anhydrase is a single chain protein of molecular weight 30,000Da, containing Zn as a cofactor. The native protein is a compact globular structure which mainly consists of beta pleated sheets with few alpha helices. There are total 10 pairs of beta sheets out of which two pairs are parallel, while the rest are anti parallel in nature.

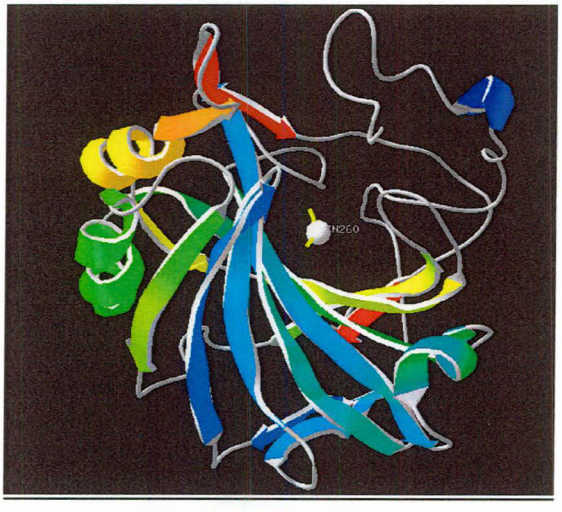


***Fig. 5:*** *Structure of Bovine Carbonic anhydrase (BCA II) (www.rcsb.org/ PDB ID: 1V9E).*

The absence of any disulphide or sulphydryl group makes it a suitable system to study the *in vitro* refolding studies of a protein which undergo successive conformational changes without any complication of subunit association or redox reshuffling. The protein is known to fully unfold in 3 M GdmCl to yield a random coil (**Wong and Tanford, 1973**) and refolds upon removal of the denaturing conditions to reform the native structure (**Yazgan and Henkens 1972; Wong and Tanford, 1973**). A Zn atom remains tightly

bound with the fully unfolded protein and facilitates the folding process (**Yazgan and Henkens, 1972; Wong and Hamlin, 1975; Ikai *et al.*, 1978**). BCA II has a well defined refolding pathway (**Stein and Henken., 1978; Doligkh *et al.*, 1984; Semistonav *et al.*, 1987**). During refolding of BCA II from its fully denatured state in 5 M GdmCl, the protein rapidly forms a compact intermediate state with exposed hydrophobic clusters. These are called first intermediate state or a molten globule state. The exposed hydrophobic clusters in the first intermediate state then collapse to form the second intermediate. This second intermediate in the refolding pathway folds to form the native protein in the overall half time of 12 min for dilution from 5 M GdmCl to 1 M GdmCl (**Stein and Henkens., 1978; Semistonov, 1987**). However the refolding of denatured BCA II depends upon the concentration of both protein and denaturant (**Wetlaufer and Xie, 1995**). It is well known that BCA II aggregates at high protein concentration (mg/ml) and low denaturant concentration (0.1-0.7M GdmCl) (**Ikai *et al.*, 1978**).

**Effect of different cosolvents/solution additives in preventing the refolding induced aggregation of BCA II and enhancing its refolding yield**

The tendency of a protein in an aqueous solution is affected by various factors such as its physiochemical properties (**Chiti *et al.*, 2002**), existence of chaperones (**Martin *et al.*, 1997**), cosolvents (**Yancey *et al.*, 1982**) and different environmental conditions (**Chi *et al.*, 2003**). Various strategies have been developed to stabilize the native form of protein (**Chi *et al.*, 2003**) or to enhance the protein refolding from the inclusion bodies *in vitro* (**Rudolph and Lilie, 1996**). Molecular chaperones are present ubiquitously in the living organisms; they protect the protein against aggregation during their synthesis and intracellular transport or in response to the various stress factors and assist in the correct folding of the proteins (**Ellis, 2001; Hartl and Hartl, 2002**). A number of small organic molecules, primarily involved in maintaining osmotic pressure in the cell (also called osmolytes), stabilize the native conformation of proteins and protect them from various kinds of stresses (**Yancey *et al.*, 1982**) in a way similar to the activity of molecular chaperones. For this reason, in many cases they are referred as chemical chaperones (**Burrows *et al.*, 2000; Diamant *et al.*, 2001; Kolter and Wendeler, 2003**). These

osmolytes are mostly natural compounds from the chemical class of sugars, polyols, amino acids, and methylamines.

The aggregation prone nature of BCA II makes it an attractive model system to study the effect of different cosolvents in preventing the aggregation and enhancing the refolding yield of the denatured BCA II at higher concentration of BCA II and lower concentration of GdmCl. Different workers have used different cosolvents depending upon their mode of action to enhance the refolding of the denatured BCA II and to inhibit its aggregation.

One such molecule is polyethylene glycol, PEG which enhances the refolding yield of bovine carbonic anhydrase by forming a non associating PEG-1$^{st}$ intermediate complex of BCA II (**Cleland and Wang, 1992; Cleland *et al.*, 1992**). However PEG-intermediate complex does not effect the folding reaction involving the formation of second intermediate. Therefore PEG does not catalyze the refolding process but it does prevent the self association of the first intermediate (**Cleland and Wang., 1992**).

Inspired by two step mechanism of GroE system, Rozema and Gellman proposed an artificial chaperone assisted refolding of BCA II by using combination of detergents and beta cyclodextrins. Cyclodextrins have a hydrophobic central cavity which can accommodate various guest molecules to form an inclusion complex. Series of detergents like CTAB, SDS and STS were used to capture the denatured/nonnative BCA II under irreversible aggregating condition (induced by heating or denaturant removal). The substrate protein cannot spontaneously refold from the detergent protein complex. Therefore in the second step, removal of detergent is facilitated by using beta cyclodextrins allowing the protein to fold into its correct conformation. Thus, addition of beta cyclodextrin to the BCA II-detergent complex prevents both competing intermolecular as well as intramolecular aggregation. (**Rozema and Gellman, 1996**).

Besides these conventional cyclodextrins, larger homologs with high degree of polymerization are also available (**Takaha *et al.*, 1996**). These high molecular weight cyclodextrins, referred to as *cycloamylose* assume single helical V-amylose conformation with an anhydrophillic channel like cavity (**Gessler *et al.*, 1999**). Cycloamylose can also form inclusion complex with both organic as well as inorganic molecules (**Kitamura *et al.*, 1999**). Sachiko et al in 2000 have found that these cycloamylose has the ability to

strip off variety of detergents (both ionic as well as nonionic) from the protein-detergent complex and promote their refolding e.g. in the case of BCA II and lysozyme refolding, cycloamylose show significant recovery of protein when applied on ionic detergent and promoted higher yield of the renatured protein (**Machida *et al.*, 2000**).

Wetlaufer and Xie showed that aggregation induced during refolding of denatured BCA II at comparatively higher concentration of protein and low concentration of denaturant (GdmCl) could be suppressed by using variety of surfactants such as CHAPS and alkanols like cycloheptanone (**Wetlaufer and Xie, 1995**). To prevent the hydrophobic interaction taking place between the intermediate species during the refolding of BCA II, some workers have explored the hydrophobic nature of the surface of 70S ribosome (**Singh and Rao, 2002**). The surface hydrophobicity of the 70S ribosome worked well in preventing the aggregation of BCA II in a concentration dependent manner during its refolding. More recently, arginine has been used as an effective folding additive to suppress the aggregation most possibly by preventing the hydrophobic interaction (**Chen *et al.*, 2008**). A linear polymer called PVP40 (Polyvinylpyrrolidone 40) has also been used to prevent the aggregation and to enhance the refolding yield of the denatured BCA II (**Jiang *et al.*, 2006**). PVP40 has been routinely used in the pharmaceutical industry due to water solubility and low toxicity. It has a hydrophobic side chain which binds with the first intermediate of BCA II during its refolding. This binding however does not block the aggregation pathway (due to the intramolecular association of the protein species) but it decreases the energy barrier for the first intermediate to refold into second intermediate. The rapid conversion of first intermediate into the second prevents its accumulation, thereby reducing the possibility of intramolecular association of the first intermediate which may lead to an aggregate formation.

Among the various strategies, which have been developed to prevent or reverse the aggregation accompanying refolding under *in vitro* conditions, application of native proteins have been extensively studied. One such protein is casein which shows similarity with molecular chaperones. These proteins have less ordered secondary and tertiary structure and remain in the random coil form in their native state. Besides this they have distinct hydrophobic and a hydrophilic domains (**Zhang *et al.*, 2005**). It has been reported

that special type of casein exhibit chaperone like activity and for several proteins it can prevent aggregation induced by heat, light or denaturant and can even solubilize hydrophobically aggregated proteins **(Bhattacharya and Das, 1999; Matsudomi *et al.*, 2004; Zhang *et al.*, 2005)**. It was found that casein not only helps in preventing the aggregation taking place during the refolding of the denatured BCA II but also enhances the refolding yield as much as 40% at 1 mg/ml concentration (**Khodarahmi *et al*, 2008**). When BCA II was refolded in the presence of an inert polymer of sucrose, Ficoll 70, it was found to promote the refolding of an inactive species into active species (**Monterroso and Minton, 2007**).

**Outline of the present research problem**

Although the basic information necessary for a polypeptide chain to fold into its native conformation resides in its amino acid sequences (**Anfinsen, 1973**), many proteins are unable to fold correctly during refolding process from their denatured state under *in vitro* conditions. Generally, two types of interactions mediated by hydrophobic forces are involved in the folding of globular protein: one is intramolecular hydrophobic forces which facilitate the spontaneous folding of the polypeptide chain and the other one is a competing intermolecular hydrophobic force between partially folded species that lead to protein aggregation and inactivation (**Goldberg *et al.*, 1991**). Aggregation and inactivation, being competitive processes during refolding of the denatured proteins, affect their renaturation yield and often result in the decreased productive folding. Bovine carbonic anhydrase (BCA II) is an aggregation prone system dependent upon the protein and denaturant concentration. As described earlier, there are several reports in the literature where different types of cosolvents have been used to enhance the refolding of BCA II and prevent its aggregation during refolding procedure.

Many plants, animals and microorganism have adapted themselves to many stressful conditions such as dehydration, high salt concentration and extreme of temperature and they have well developed strategy to protect the cellular proteins against such kind of stresses (**Yancey *et al.*, 1982; Borowitzka, 1985**). These organisms have intracellular accumulation of low molecular weight organic compounds that can fall under any of the following category of polyols, sugars, certain amino acids and

methylamines (**Brown and Simpson, 1972; Stewart and Lee, 1974; Yancey *et al.*, 1982**). Polyols and sugars are already known to increase the thermal stability of proteins. by favoring the native state formation (**Gorovits *et al.*, 1998**). Polyols, sugars and amino acids besides helping in the correct folding of the denatured protein are also known to prevent the aggregation of native proteins both during refolding process as well as during thermal incubation (**Rariy and Klibanov, 1997; Kaushik and Bhat, 1998, 2003; Mishra *et al.*, 2005; Zancan and Sola-Penna, 2005; Monterroso and Minton, 2007**). Since the molecular interactions involved in the stabilization of native state are the same for the folding process as well, in the present study, BCA II has been used as a model system to understand the mechanism of solvent mediated refolding of proteins with an aim to understand the correlation between refolding and stability aspects in the presence of structure stabilizing compounds of the polyol series, sugars, glycine and its methyl derivatives to explore the refolding and aggregation aspects of BCA II at high protein and low denaturant (GdmCl) concentration. The study has been further extended to see the effect of above mentioned cosolvents in preventing the aggregation of BCA II induced during incubation of protein at high temperature. Thermal denaturation studies in the presence of these cosolvents were carried out to establish the relationship between folding and stability.

It was observed that while some members of plolyol and sugar series significantly increased the refolding yield of denatured BCA II by suppressing the aggregation induced during folding pathway, some members showed neutral while some exhibited completely antagonistic effect on the refolding yield. It was observed that sorbitol and trehalose which are known as exceptionally good stabilizers of protein structure (**Xie and Timasheff, 1997 a, b; Kaushik and Bhat, 1998, 2003**) during thermal denaturation decreased the refolding yield considerably. However, all the members of polyols, sugar and amino acid series used were found to completely suppress the temperature induced aggregation. To establish the relationship between refolding and stability aspects, thermal denaturation studies were also carried out to find out the effect of the above mentioned cosolvents on the stability of the native BCA II. Interestingly, some of the polyols which were enhancing the refolding yield were found to be destabilizer of the native state and vice versa. Glycine and its methyl derivatives, sarcosine and betaine are well known as

protein stabilizers and counteract the deleterious effects of urea (**Yancey and Somero, 1979; Hand and Somero, 1982**). During refolding studies of BCA II these amino acids resulted in contrasting effects as compared to members of polyols and sugar series. Glycine and its methyl derivatives were found to decrease the refolding yield of denatured BCA II at all the concentration but at the same time they were effective in preventing the aggregation of native BCA II at high temperature. Glycine a well known protein stabilizer (**Arakawa and Timasheff, 1983, 1985b**) considerably decreased the temperature onset of denaturation/aggregation while sarcosine and betaine showed negligible effect on the stability of native BCA II during thermal denaturation experiments.

The non-parallel nature of stability and refolding aspects indicate that while some cosolvents stabilize the native state of BCA II against thermal denaturation and aggregation, their ability to help in the refolding of the proteins depends critically on the dynamic nature of interactions of the osmolytes with the folding polypeptide chain. A balance between preferential hydration and preferential binding effects is expected to critically regulate the folding of the polypeptide chain towards productive folding.

# MATERIALS AND METHODS

## Materials

Carbonic anhydrase II (bovine), polyols (glycerol, erythritol, xylitol and sorbitol), sugars (glucose, sucrose and trehalose), amino acid glycine and its methyl derivatives sarcosine and betaine, p-nitrophenyl acetate, Tris ([hydroxymethyl] aminomethane), guanidinium hydrochloride were all purchased from Sigma Chemical Co, USA. The chemicals used were of the highest purity grade and were used without any further purification. Water from Millipore water purification system (Milli Q) was used to make all the solutions and buffers. Buffer components, di-hydrogen potassium phosphate and di-potassium hydrogen phosphate were purchased from Qualigens, India. All the incubation studies at high temperature were carried out in a water bath from Hakke. The pH of the solutions used in the studies was adjusted on a Radiometer PHM204 research pH meter by adding $H_2SO_4$, HCl and NaOH solutions. The pH standards used for calibrating the pH meter were either from Hanna instrument, Europe or Sigma Chemical Co, USA. 20 mM Tris-$SO_4$ was used for all the refolding, circular dichroism, fluorescence emission, thermal denaturation and thermal incubation studies at pH 7.5. Phosphate buffer at pH 7.5 was used for the light scattering experiments.

## Methods

### Determination of protein concentration:

The concentration of native BCA II was determined by measuring absorbance at 280 nm using extinction coefficient of 1.83 $mg^{-1}$ ml $cm^{-1}$ (**Wong and Tanford, 1973**). Protein stock was prepared in 20 mM Tris-$SO_4$ buffer at pH 7.5.

### Denaturation:

BCA II was denatured in 5 M GdmCl at 25°C, in 20 mM Tris-$SO_4$ buffer at pH 7.5 (**Wetlaufer and Xie, 1995**) and was incubated for 12-14 hours prior to further use.

### Refolding:

Denatured BCA II samples were refolded by single shot dilution method. The denatured samples were diluted 20 fold in the refolding buffer using 20 mM Tris-$SO_4$ at pH 7.5 and the samples were subjected to rapid vortexing. Extreme care was taken at this step because rapid mixing is essential for reproducible and better refolding yields. The final protein concentration varied according to the extent of aggregation both in the absence

and presence of cosolvents used. GdmCl concentration during refolding was 0.25 M. The refolded samples were kept at 25°C for one hour before being assayed for the enzymatic activity. To study the effect of different cosolvents on the refolding yield of BCA II, they were added to the refolded buffer at desired concentrations before the dilution of the denatured protein by the refolding buffer was done.

**Activity assay:**

Carbonic anhydrase possesses remarkable property of absolute specificity with respect to the reversible hydration of carbon dioxide (**White *et al.*, 1964**). Carbonic anhydrase contains zinc atom as a cofactor and this cofactor is necessary for its catalytic activity. It catalyzes the reversible hydration of carbon dioxide with high efficiency (Equation 1).

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad \text{---------- (1)}$$

The hydrase activity has been further extended to include other substrates such as acetaldehyde and related carbonyl systems (**Pocker and Meany, 1965a, b; 1967a, b**). The similarity between aldehyde hydration and certain bimolecular mechanism of ester hydrolysis involving tetrahedral intermediates (**Bender, 1951, 1953**) led to the discovery that carbonic anhydrase possess the possibility of esterase activity (**Pocker and Stone, 1965a, b**). Later on, detailed study by Pocker and Stone (**Pocker and Stone, 1967**) measured the esterase activity of carbonic anhydrase using *p*-nitrophenyl acetate as a substrate. The ester can be easily handled, hydrolyzes irreversibly at convenient rates, and allows precise spectrophotometric determinations to be performed at constant pH. The hydrolysis of p-nitrophenylacetate was followed spectrophotometrically by monitoring the appearance of p-nitrophenolate anion primarily at its peak absorbance at 400 nm (Equation 2).

$$\textbf{p-nitophenyl acetate + buffer} \xrightarrow{\text{BCAII}} \textbf{p-nitrophenolate ion} \quad \text{---------- (2)}$$

Thus, the enzymatic activity of BCA II was measured after adding 5 µg/ml BCA II to the assay sample, containing 3 mM p-nitro phenyl acetate as a substrate in 20 mM Tris-$SO_4$ buffer, pH 7.5. The formation of the product p-nitrophenolate was measured at 400 nm for 3 minutes using UV-visible spectrophotometer. The percent recovery after refolding was measured by comparing with the activity of the native protein in the same concentration of GdmCl in 20 mM Tris-$SO_4$ buffer without any additives used.

**Residual protein Concentration determination in the refolded samples**

In order to measure the residual protein concentration, all the refolded samples, in the absence and presence of different cosolvents were subjected to centrifugation at 10,000 rpm for 20 minutes on microcentrifuge (from Eppendorf) at 25°C to pellet down the aggregates formed during the renaturation step. The supernatant was taken to measure the protein concentration at 280 nm on a Cary Varian-100 Bio UV-visible spectrophotometer using a 1 cm path length cuvette. It was observed that the presence of high concentration of the cosolvents did not affect the absorbance/ extinction coefficient of the protein.

## FLUORESCENCE EMISSION SPECTRA

Any changes in the tertiary structure of the protein due to the changes in the local tryptophan environment were studied by measuring the tryptophan fluorescence emission spectra. Fluorescence measurements were carried out on a Cary-Varian Eclipse spectrofluorimeter equipped with a peltier based temperature controller unit and a temperature programmer using 1 cm path length cuvette. For the tryptophan fluorescence, an excitation wavelength of 295 nm was used, with an excitation and emission slit widths of 5 nm each. All spectra were recorded from 300 nm to 450 nm at 25°C. Denatured BCA II was refolded in 20 mM Tris-$SO_4$ buffer at pH 7.5 by rapid dilution method at 25°C for one hour. After one hour, refolded samples were removed from the water bath and centrifuged for 20 minutes to pellet down any protein aggregates. The supernatant was then taken for the fluorescence spectral measurements. The final protein concentration in the refolded samples varied according to the extent of protein aggregation, both in the absence and presence of cosolvents. Final GdmCl concentration in the refolded sample was 0.25 M. Fluorescence spectra of the native BCA II was also measured in the presence of 0.25 M GdmCl and used as a control spectrum.

## LIGHT SCATTERING MEASUREMENTS

It has been proposed that the intensity of scattered light is directly proportional to the amount of protein in the aggregated form. The intensity of the scattered light was measured on Cary Varian spectrofluorimeter equipped with a peltier based temperature

controller and temperature programmer using 1 cm path length cuvette. BCA II was heated at 65°C and the intensity of scattered light was measured with respect to time. The excitation and emission wavelength was 650 nm each. Excitation and emission slit width were kept at 5 nm. The protein concentration was 5 μg/ml in 0.05 M $PO_4$ buffer at pH 7.5. Aggregation kinetics was carried out for 30 minutes. The effect of different cosolvents in suppressing the temperature-induced aggregation was observed by adding them to the native BCA II and then by measuring the change in the intensity of the scattered light at 65°C using the above mentioned conditions.

## THERMAL INCUBATION STUDIES

Since glycine and its N-methyl derivatives scatter light in the visible range, their effect in suppressing the aggregation of native BCA II was monitored by incubating the protein in their presence at high temperature followed by measuring the absorbance of residual protein in the incubated samples. Native BCA II aggregates at 65°C. To examine the role of glycine and its derivatives in suppressing the temperature-induced aggregation, 0.1 mg/ml BCA II was incubated at 65°C both in the absence and presence of glycine and its methylamine derivatives for one hour in a water bath. After one hour, samples were removed from the water bath and centrifuged to pellet down the protein aggregates. The residual protein concentration was then measured in the supernatant solution at 280 nm as described.

## THERMAL DENATURATION STUDIES

Thermal denaturation studies were carried out by using UV-visible spectrophotometer. Changes in the absorption intensity were measured using a Cary Varian-100 Bio spectrophotometer equipped with a peltier based temperature controller using a 1 cm path length cuvette at a wavelength of 292 nm (**Lavecchia and Zugaro, 1991**). In all the cases the protein concentration was 0.05 mg/ml and the buffer used was 20 mM Tris-$SO_4$ buffer at pH 7.5. Since the protein shows aggregation during thermal denaturation, one could only evaluate the temperature onset of denaturation/aggregation ($T_{agg}$) and not the transition temperature, $T_m$ value.

## CIRCULAR DICHROISM STUDIES

Far-UV CD measurements of all the refolded samples in the absence and presence of different cosolvent additives were carried out on a JASCO J-815 CD spectropolarimeter equipped with a peltier based temperature controller. The machine was precalibrated with (+)-10-camphorsulfonic acid. All spectra were acquired at 25°C using a 1 mm path length rectangular cuvette. Spectra were recorded at a scan rate of 50 nm/min and 0.5 nm data pitch from 250 nm to 200 nm. Each spectrum was an average of five scans with an integration time of 2 sec at each wavelength and the baseline was corrected using a cuvette containing buffer or cosolvent alone. A molecular mass of 30 kDa for bovine carbonic anhydrase were used in the calculations of molar ellipticity. Due to strong optical activity in far-UV region, amino acids could not be used for CD measurements. Denatured BCA II was refolded in 20 mM Tris-$SO_4$ buffer at pH 7.5 by rapid dilution method at 25°C for one hour. After one hour, refolded samples were removed from the water bath and centrifuged for 20 minutes to pellet down the protein aggregates. The supernatant was then taken for the CD spectral measurements. The final protein concentration in the refolded samples for the CD measurements varied according to the extent of protein aggregation; both in the absence and presence of cosolvents. Final GdmCl concentration in the refolded sample was 0.25 M. CD spectrum of the native BCA II was also measured in presence of 0.25 M GdmCl and was used as a control.

# EFFECT OF POLYOLS ON THE REFOLDING AND AGGREGATION OF BCA II

## Introduction

Many organisms including plants and animals live in the extreme environment of high temperature, extreme of pH, high salt concentration, dehydration condition, etc., and have very well adapted themselves to these kinds of harsh environmental conditions. One such strategy is the intracellular accumulation of low molecular weight organic compounds such as polyols, sugars, certain amino acids and some methylamines (**Brown and Simpson, 1972; Stewart and Lee, 1974; Borowitzka, 1985; Brown, 1990; Timasheff, 1995; Popp and Smirnoff, 1995; Sato *et al.*, 1996; Hincha and Hagemann, 2004**). These compounds are often referred as osmolytes and are known to provide protection against thermal as well as chemical stress and help in retaining the biological activity (**Arakawa and Timasheff, 1982 a, b, c, 1983, 1985 a, b; Timasheff, 1992**).

Bovine carbonic anhydrase (BCA II) is a Zn containing protein composed of single polypeptide chain, devoid of any disulphide bond. It has been established by both kinetics and equilibrium studies that the refolding pathway of BCA II involves two intermediates. The first refolding intermediate often self associates to give off-pathway species which are highly prone to aggregation. The first intermediate converts into the second intermediate which is not aggregation prone. The second intermediate then rearranges to form the native structure (**Stein and Henkens, 1978; Semistonov *et al.*, 1987**).

Off-pathway folding intermediates associate to form non native aggregated species which decrease the refolding yield considerably. One of the general methods to prevent folding induced aggregation is by changing the aqueous solvent environment. This could be achieved by using a cosolvent which may prevent the association of intermediate protein structure without interfering with the formation of other intermediates or the native state. Based on the understanding of interactions between a protein and the cosolvents that could affect the protein environment during refolding and stability at high temperature, cosolvents are classified into two categories (1) cosolvents that can bind to the protein and stabilize the protein structure such as unfolded state, e.g., ethylene glycol (EG) (**Tanford *et al.*, 1962**), polyethylene glycol (PEG) (**Arakawa *et al.*, 1990; Cleland *et al.*, 1992; Cleland and Randolph, 1992**), PVP40 (**Jiang *et al.*, 2006**),

Arg.HCl and GdmCl (**Arakawa and Tsumoto, 2003; Arakawa *et al.*, 2007; Chen *et al.*, 2008**), etc (2) cosolvents which could preferentially hydrate the protein structure by increasing the chemical potential of the solvent system e.g. polyols, sugars etc (**Gekko and Timasheff, 1981a; Mishra *et al.*, 2005**).

Polyols have been extensively used to protect the functional activity of enzymes, and to provide stability to the native state of proteins ( **Jarabak, 1972; Bradbury and Jakoby, 1972; Myers and Jakoby, 1973; Lee *et al.*, 1975; Lee and Lee, 1987; del Vecchio *et al.*, 1999; Ruan *et al.*, 2003; Singh *et al.*, 2004; Meng *et al.*, 2004; Mishra *et al.*, 2007; Ortbauer and Popp, 2008**). For a vast majority of proteins, preferential hydration was found to be the main cause for their stabilization. Effect of polyols has been further extended to a number of globular proteins as well as multidomain and multimeric protein systems (**Kaushik and Bhat, 1998; Naseem and Khan, 2003; Tiwari and Bhat, 2006**). The ability of polyols to stabilize the native state of proteins was also exploited in the refolding studies. Polyols like glycerol was found to be effective in leading to the correct folding of unfolded and reduced lysozyme with a gain of substantial catalytic activity (**Rariy and Klibanov, 1997; Meng *et al.*, 2001; Zancan and Sola-Penna, 2005**) and also it acts as a chemical chaperone during the refolding of GdmCl denatured aminoacylase (**Kim *et al.*, 2006**). It has been reported in case of Rhodanese that polyols like glycerol can help in preventing irreversible aggregation induced during refolding, by stabilizing the intermediate species and thereby leading to increase in the refolding yield (**Gorovits *et al.*, 1998**). In another study, glycerol was found to induce productive refolding of GroEL-bound bacterial glutamine synthetase in ATP independent manner (**Voziyan and Fisher, 2002**). Polyols like sorbitol can induce non native α-helical structure in denatured BLG (bovine β-lacto globulin) and help in the refolding and stabilization of protein by increasing the $T_m$ (**Divsalar *et al.*, 2006**). These studies using polyols establish that besides being a good protein stabilizer, polyols can also act as efficient folding agents.

Polyols have been studied extensively aiding refolding of highly aggregation prone citrate synthase (CS) (**Mishra *et al.*, 2005**). Polyols were found effective in enhancing the refolding yield of CS in a concentration dependent manner. Among all the polyols, glycerol was the most effective which not only led to considerable recovery of

the enzymatic activity but also suppressed its aggregation at high temperature. While polyols were found to be effective in the efficient folding of denatured CS, polyethylene glycol, which successfully refolded and prevented aggregation of BCA II (**Cleland and Wang, 1990 a, 1992**) as well as of lysozyme (**Moorthy, 1997**) did not help in case of CS folding under any condition (**Mishra, Ph.D. thesis, 2001**). Thus it was concluded that cosolvent effect on the refolding and aggregation aspects of protein could vary according to protein molecule and is highly dependent upon the type of interaction taking place between the cosolvent and the folding polypeptide chain. To further extend the study of cosolvent effect on the refolding, aggregation and stability of proteins, another aggregation prone system, bovine carbonic anhydrase (BCA II) was taken as a model in order to further elucidate the role of small structure stabilizing molecules of polyol series as effective agents in folding and prevention of protein aggregation.

## Results

### Effect of protein and denaturant (GdmCl) concentration on the refolding yield of BCA II

Bovine carbonic anhydrase (BCA II) is an aggregation prone system, whose refolding from its denatured state is dependent upon both protein and denaturant concentration (**Wetlaufer and Xie, 1995**). During refolding of BCA II from its denatured state in 5 M GdmCl, the refolding yield varied according to the concentration of BCA II and GdmCl. Protein aggregation is a general phenomenon at high protein concentration and low denaturant concentration. Accordingly it was seen that at low BCA II and high GdmCl concentration, the refolding yield was very high indicating the complete recovery of the native state of BCA II from its denatured state. However, as the denatured BCA II was diluted in the refolding buffer to a much higher protein and lower GdmCl concentration, refolding yield decreased drastically. At BCA II concentration of 0.5 mg/ml and GdmCl 0.25 M, refolding percentage was only 37% (Table 1). This condition, was, thus used for all the subsequent studies to investigate the effect of polyols on the refolding yield of the protein.

**Table 1: Effect of protein and denaturant concentration on the refolding yield of BCA II at 25°C, pH 7.5**

| Initial BCA II conc. (mg/ml) | Initial GdmCl conc. (M) | Final BCA II conc. (mg/ml) | Final GdmCl conc. (M) | Refolding yield (% activity) |
|---|---|---|---|---|
| 2.5 | 5 | 0.5 | 1 | 94 |
| 5 | 5 | 0.5 | 0.5 | 61 |
| 10 | 5 | 1 | 0.5 | 55 |
| 10 | 5 | 0.5 | 0.25 | 37 |

*BCA II refolding studies were carried out at different BCA II and GdmCl concentration in 20 mM Tris-$SO_4$ buffer at 25°C, pH 7.5. Refolding yield was calculated by taking ratio of the activity of the refolded sample and of the native protein in buffer alone.*

*The data are averages of at least three independent observations with a maximum percentage error of ± 5%. It was true for all the tables showing refolding yield in this chapter.*

**Effect of polyols on the refolding yield of BCA II**

BCA II was denatured in 5 M GdmCl and the solution was kept for 12-14 hours at 25°C. Refolding was done by rapid dilution method with vigorous vortexing in 20 mM Tris-$SO_4$ buffer at pH 7.5. To observe the effect of different polyols in enhancing the refolding yield, they were introduced at a desired concentration in the refolding buffer prior to dilution. The refolded samples were then left for one hour at room temperature. After one hour, refolded samples were centrifuged to pellet down the aggregates and the supernatant was taken to measure the enzymatic activity. Refolding was measured by calculating the activity of all the refolded samples with and without polyols using p-nitrophenyl acetate as a substrate as described in Materials and Methods section in chapter 2. Percentage refolding yield was calculated by taking the ratio of refolded

samples in presence of polyols to the native BCA II refolded in the buffer alone (i.e. without using any polyol) at the same concentration of GdmCl.

The effect of different polyols on the refolding yield of denatured BCA II is summarized as below:

Effect of Ethylene glycol

Ethylene glycol (EG) is a '2' carbon compound and is soluble in water. In the present study, EG has been used from 0.1 to 8 M concentrations. During refolding of BCA II there was no significant change in the refolding yield up to 3 M ethylene glycol. Further increase in the concentration lead to gradual increase in the refolding yield. When the concentration of EG reached upto 8 M, 70% refolding yield was achieved which was 30% higher with respect to the control (Table 2). Thus, ethylene glycol, at higher concentration was found to be very effective in enhancing the folding of denatured BCA II. Higher concentrations were not possible due to solutions becoming too viscous and interfering in mixing.

**Table 2: Effect of ethylene glycol on the refolding yield of BCA II at 25°C, pH 7.5**

| Ethylene glycol (M) | Percentage refolding yield |
|---|---|
| 0.0 | 40 |
| 0.1 | 40 |
| 0.5 | 41 |
| 1.0 | 42 |
| 2.0 | 43 |
| 3.0 | 44 |
| 4.0 | 48 |
| 6.0 | 54 |
| 8.0 | 70 |

*Refolding of BCA II in the presence of ethylene glycol at 25°Cin 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Glycerol

Glycerol is a polyol containing '3' carbon atoms in its chain with three –OH groups. In the present study glycerol has been used in the concentration range of 0.1 to 8 M. It was observed that refolding yield gradually increased with increase in the concentration of glycerol. There was a 28% increase in the refolding yield in the presence of 4 M glycerol as compared to control (41%). Higher concentration (> 4 M) led to a small decrease in the refolding yield (Table 3).

**Table 3: Effect of glycerol on the refolding yield of BCA II at 25°C, pH 7.5**

| Glycerol (M) | Percentage refolding yield |
|---|---|
| 0.0 | 41 |
| 0.1 | 46 |
| 0.5 | 50 |
| 1.0 | 57 |
| 2.0 | 62 |
| 4.0 | 69 |
| 6.0 | 68 |
| 8.0 | 61 |

*Refolding of BCA II in the presence of glycerol at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Erythritol

Erythritol contains '4' carbon atoms in its chain and 4 –OH groups. In this study erythritol was used in the concentration range of 0.1 to 3 M. Concentrations higher than 3 M could not be explored due to its limited solubility at higher concentrations. It was found that there was no significant increase in the refolding yield of the denatured BCA II and it remained unaffected at almost all the concentration of erythritol (Table 4). The changes were within the limits of experimental uncertainty.

**Table 4: Effect of erythritol on the refolding yield of BCA II at 25°C, pH 7.5**

| Erythritol (M) | Percentage refolding yield |
|---|---|
| 0.0 | 43 |
| 0.1 | 44 |
| 0.5 | 46 |
| 1.0 | 50 |
| 2.0 | 50 |
| 3.0 | 43 |

*Refolding of BCA II in the presence of erythritol at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Xylitol

Xylitol has '5' carbon atoms in its chain and 5 –OH groups. In this study xylitol was used in the concentration range of 0.1 to 3 M. Concentrations higher than 3 M could not be explored due to the limited solubility of xylitol at higher concentrations. It was observed that at 1 M xylitol, refolding yield of BCA II gradually increased from 47% (control) to 60%. However, further increase in xylitol concentration lead to a gradual decrease in the refolding yield of BCA II (Table 5).

**Table 5: Effect of xylitol on the refolding yield of BCA II at 25°C, pH 7.5**

| Xylitol (M) | Percentage refolding yield |
|---|---|
| 0.0 | 47 |
| 0.1 | 51 |
| 0.5 | 59 |
| 1.0 | 60 |
| 2.0 | 54 |
| 3.0 | 37 |

*Refolding of BCA II in the presence of xylitol at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Sorbitol

Sorbitol contains '6' carbon atoms with 6 –OH groups. Sorbitol was used in the concentration range of 0.1 to 3 M. Concentrations beyond 3 M were not possible due to limited solubility of sorbitol at higher concentrations. While lower concentrations of sorbitol did not affect the refolding yield, there was a drastic decrease at 2 and 3 M concentration. At 3 M sorbitol, refolding yield was only 17% indicating a considerable (23%) decrease in the refolding yield as compared to control (Table 6).

**Table 6: Effect of sorbitol on the refolding yield of BCA II at 25°C, pH 7.5**

| Sorbitol (M) | Percentage refolding yield |
|---|---|
| 0.0 | 40 |
| 0.1 | 40 |
| 0.5 | 40 |
| 1.0 | 43 |
| 2.0 | 29 |
| 3.0 | 17 |

*Refolding of BCA II in the presence of sorbitol at 25°Cin 20 mM Tris $SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

**Comparison of the refolding yield of BCA II in the presence of polyols**

In the refolding studies it was found that the refolding yield of the control sample (i.e. without adding any polyol) was ~40% although it varied slightly (Table 7). Among all the polyols, xylitol was the most effective, which at 1 M concentration, increased the refolding yield from 40% (control) to 61%. While further increase in erythritol and xylitol concentration decreased the refolding yield, there was a gradual increase in the refolding yield at higher concentration of ethylene glycol and glycerol. On increasing the concentration (which was not possible in case of erythritol, xylitol, and sorbitol due to their limited solubility) it was found that 8 M ethylene glycol and 4 M glycerol enhanced the refolding yield from 40% (in control) to ~70%. Except for ethylene glycol, there was a gradual decrease in the refolding yield at higher concentration of all other polyols. Erythritol, however, did not show any effect on the refolding yield. Decrease in the

refolding yield was much more pronounced in the case of sorbitol at concentration > 2 M. Taken together the refolding data indicate that while at the lower concentration of all the polyols, folding remained unaffected, it increased to a moderate extent in case of ethylene glycol and glycerol when their concentration was further increased. At the same time, there was a sharp decrease in the refolding in presence of polyols with comparatively longer carbon chain like xylitol and sorbitol with erythritol being ineffective (Table 7, Fig. 1).

**Table 7: Refolding yield of BCA II in the presence of different polyols at 25°C, pH 7.5**

| Concentration of polyols (M) | % Refolding yield | | | | |
|---|---|---|---|---|---|
| | Ethylene glycol | Glycerol | Erythritol | Xylitol | Sorbitol |
| 0.0 | 40 | 41 | 43 | 47 | 40 |
| 0.1 | 40 | 46 | 45 | 51 | 41 |
| 0.5 | 42 | 50 | 46 | 60 | 41 |
| 1.0 | 42 | 57 | 50 | 61 | 44 |
| 2.0 | 44 | 62 | 50 | 55 | 29 |
| 3.0 | 44 | 57 | 44 | 38 | 17 |
| 4.0 | 48 | 69 | - | - | - |
| 6.0 | 55 | 68 | - | - | - |
| 8.0 | 71 | 61 | - | - | - |

*Percentage refolding yield of BCA II in the absence and presence of different polyols at 25°C in 20 mM Tris- $SO_4$ buffer at pH 7.5. Final refolding condition was 0.5 mg/ml BCA II and 0.25 M GdmCl.*

*The data are averages of at least three independent observations with a maximum percentage error of ± 5%.*

A comparative representation of the effect of different polyols on the refolding yield of BCA II is shown in figure 1.

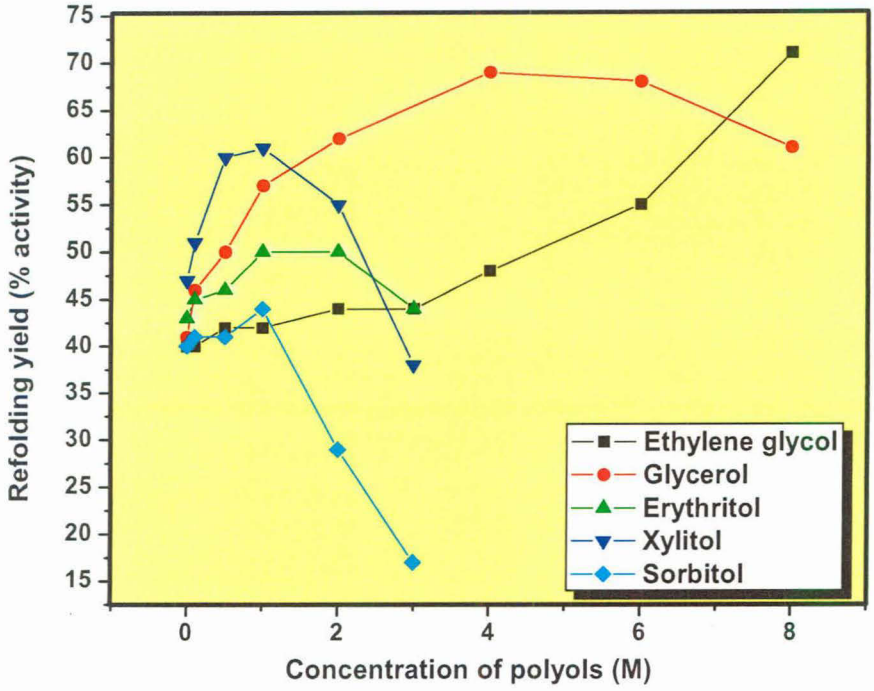



***Fig. 1:*** *Graphical representation of the effect of different concentrations of polyols on the refolding yield of BCA II.*

**Effect of polyols on the residual protein concentration of the refolded samples of BCA II**

When the protein is rapidly diluted in the refolding buffer at high protein and low denaturant (GdmCl) concentration, it forms aggregates which are visible by the naked eye in the form of white aggregates. Polyols were found not only effective in enhancing the refolding yield of the denatured BCA II but also helped in reducing the aggregation which is induced during the refolding step. The residual protein concentration (after centrifugation) in the refolded samples at 280 nm, during spontaneous refolding, without using any polyol, was ~0.2 mg/ml (Table 8). It increased to 0.46 mg/ml in case of 6 M glycerol, and 0.39 mg/ml at 8 M ethylene glycol. While erythritol did not affect the residual protein concentration there was a decrease in the residual protein at higher concentrations of xylitol. On the other hand there was a very sharp decline in the protein

concentration at sorbitol concentrations >2 M (Table 8, Fig. 2). These results paralleled the effect of polyols on the refolding yield presented in Table 7 and suggest that the decrease in the protein refolding yield is prominently due to the aggregation of proteins.

**Table 8: Residual protein concentration of BCA II in the presence of different polyols at 25°C, pH 7.5**

| Polyol Conc. (M) | Residual BCA II concentration in the presence of polyols (mg/ml) | | | | |
|---|---|---|---|---|---|
| | Ethylene glycol | Glycerol | Erythritol | Xylitol | Sorbitol |
| 0 | 0.21 | 0.19 | 0.20 | 0.20 | 0.20 |
| 0.1 | 0.20 | 0.22 | 0.21 | 0.22 | 0.20 |
| 0.5 | 0.20 | 0.22 | 0.22 | 0.25 | 0.21 |
| 1 | 0.21 | 0.24 | 0.25 | 0.24 | 0.21 |
| 2 | 0.21 | 0.28 | 0.23 | 0.22 | 0.14 |
| 3 | 0.22 | - | 0.23 | 0.13 | 0.08 |
| 4 | 0.23 | 0.36 | - | - | - |
| 6 | 0.28 | 0.46 | - | - | - |
| 8 | 0.39 | 0.43 | - | - | - |

A comparative representation of the effect of different polyols on the residual protein concentration in the refolded samples is shown in figure 2.

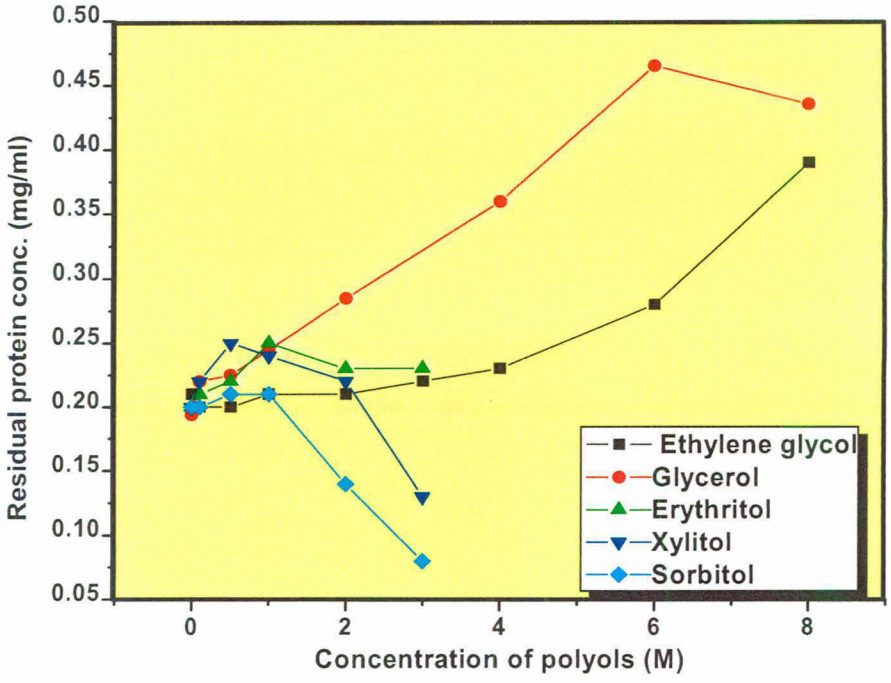
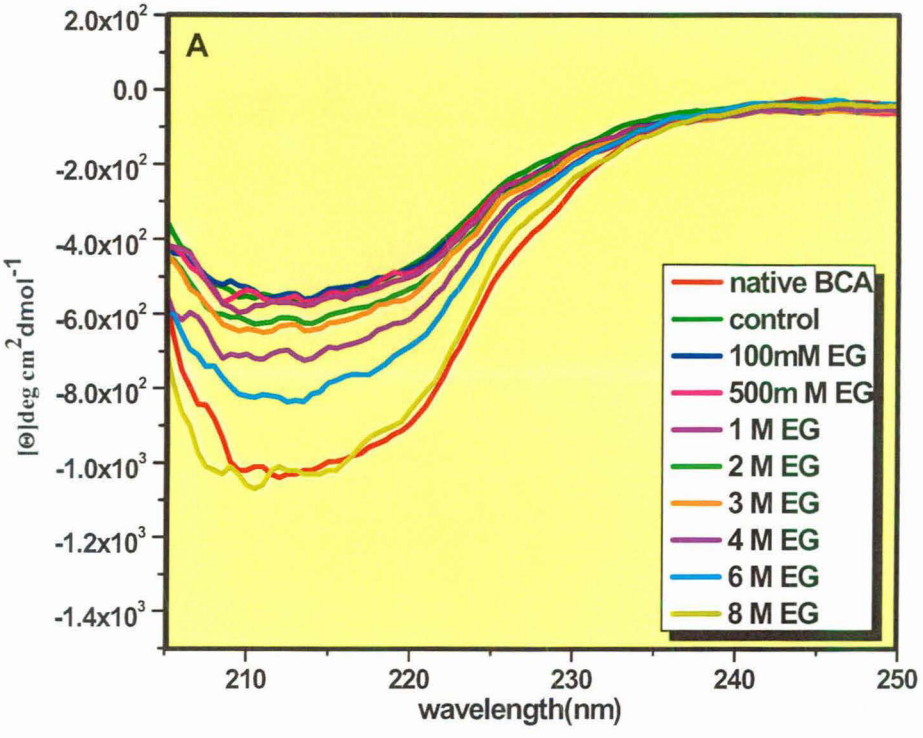


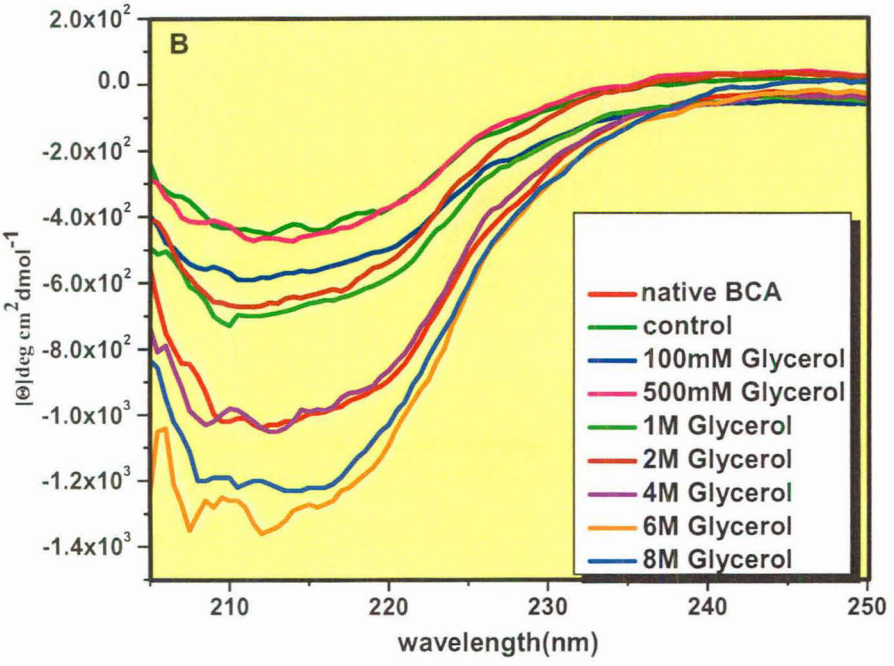

*Fig. 2: Graphical representation of the effect of polyols on the residual concentration of BCA II in the refolded samples.*

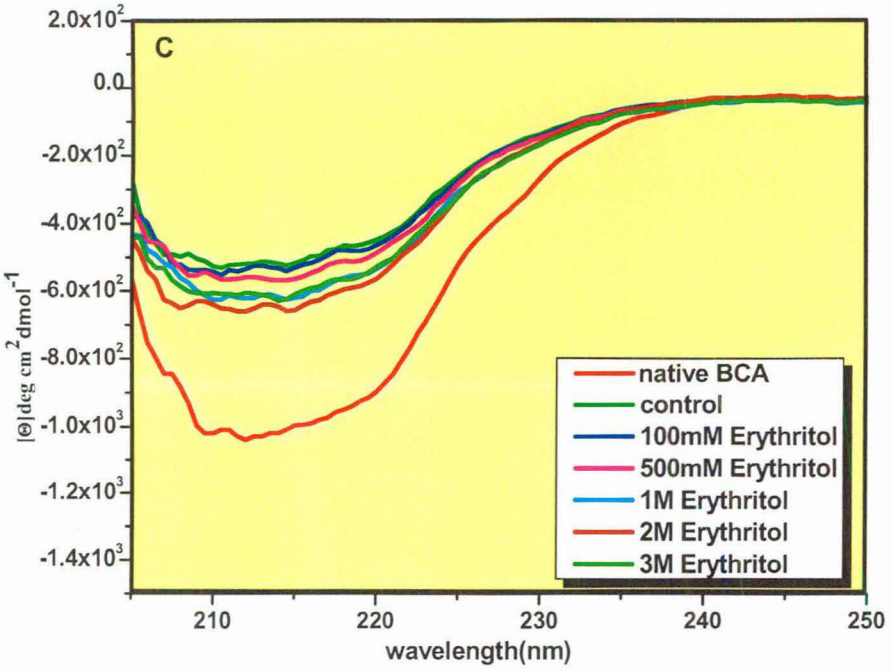
**Effect of polyols on the secondary structure of the refolded samples of BCA II**

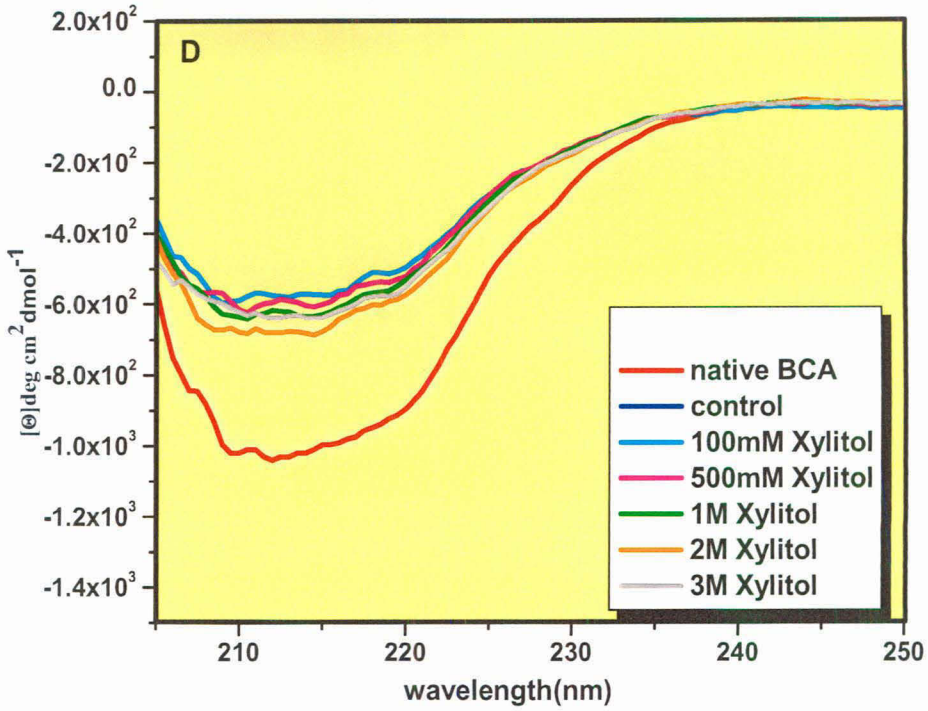
To explore the effect of polyols on the conformational state of BCA II during refolding, circular dichroism studies were carried out using CD spectroscopy. BCA II was refolded in the presence of different polyols, refolded samples were kept for 1 hour and then centrifuged to pellet down the aggregates. The supernatant was taken and far-UV CD spectra were recorded from 200 nm to 250 nm to see the effect of polyols on the secondary structure of the refolded samples. The native BCA is known to comprise primarily of beta sheets (**Kannan *et al.*, 1972; Liljas *et al.*, 1972**). The spectra of the native BCA II shows negative ellipticity at nearly 215 nm indicating substantial amount of beta sheet structure. A far-UV CD spectrum of control samples (without any polyol) was found to have decreased value of negative ellipticity showing considerable loss in the secondary structure. In case of ethylene glycol, while lower concentrations did not affect

the secondary structure and the negative ellipticity remained close to the control value, higher EG concentration (8 M) decreased the negative ellipticity and the spectrum got closed to the native state structure (Fig. 3A). Thus, 8 M concentration of ethylene glycol which shows the maximum refolding yield was also found to be most effective in retaining the secondary structure of the refolded sample. Similarly, in case of glycerol, there was no effect on the secondary structure of the refolded samples up to 3 M concentration. However, at 4 M glycerol, far-UV CD spectra showed the negative ellipticity close to the native state of BCA II (Fig. 3B). There was no effect on the secondary structure of the refolded samples at all the concentrations of erythritol and xylitol (Fig. 3C and D). In presence of sorbitol, however, there was no change in the structure at lower concentrations of 0.1 and 0.5 M, but from 1 M onwards there was a perturbation in the secondary structure as the negative ellipticity was observed to decrease and it became less negative (Fig. 3E). 3 M concentration of sorbitol which decreased the refolding yield to a considerable value was found to be completely disrupting the secondary structure of the refolded sample with negative ellipticity values close to zero (Fig. 3E).




B
2.0x10^2
0.0
-2.0x10^2
-4.0x10^2
-6.0x10^2
-8.0x10^2
-1.0x10^3
-1.2x10^3
-1.4x10^3
[Θ]deg cm^2dmol^-1
210
220
230
240
250
wavelength(nm)
native BCA
control
100mM Glycerol
500mM Glycerol
1M Glycerol
2M Glycerol
4M Glycerol
6M Glycerol
8M Glycerol


C
2.0x10^2
0.0
-2.0x10^2
-4.0x10^2
-6.0x10^2
-8.0x10^2
-1.0x10^3
-1.2x10^3
-1.4x10^3
[Θ]deg cm^2dmol^-1
210
220
230
240
250
wavelength(nm)
native BCA
control
100mM Erythritol
500mM Erythritol
1M Erythritol
2M Erythritol
3M Erythritol



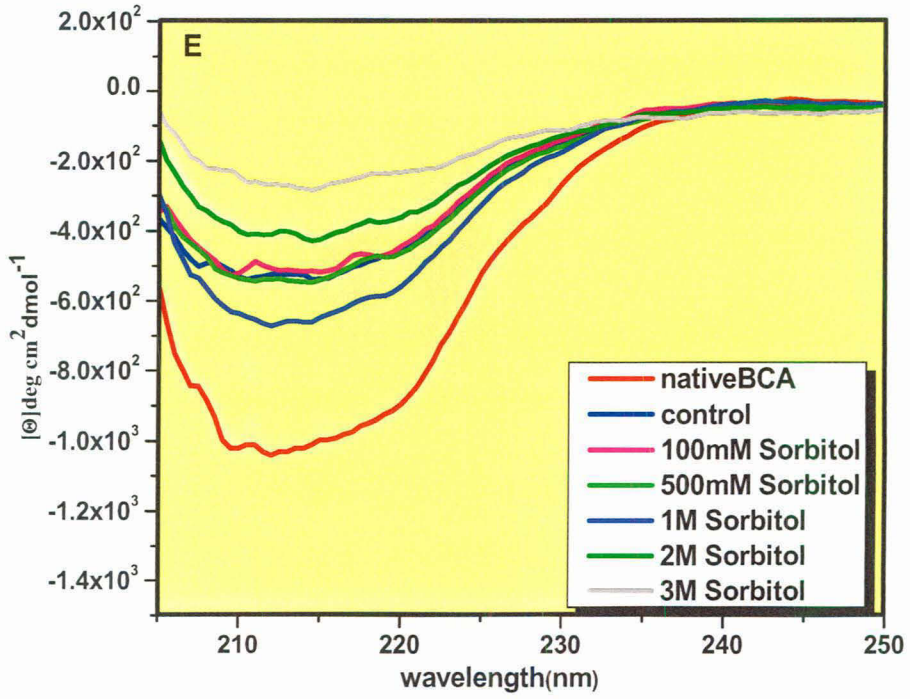
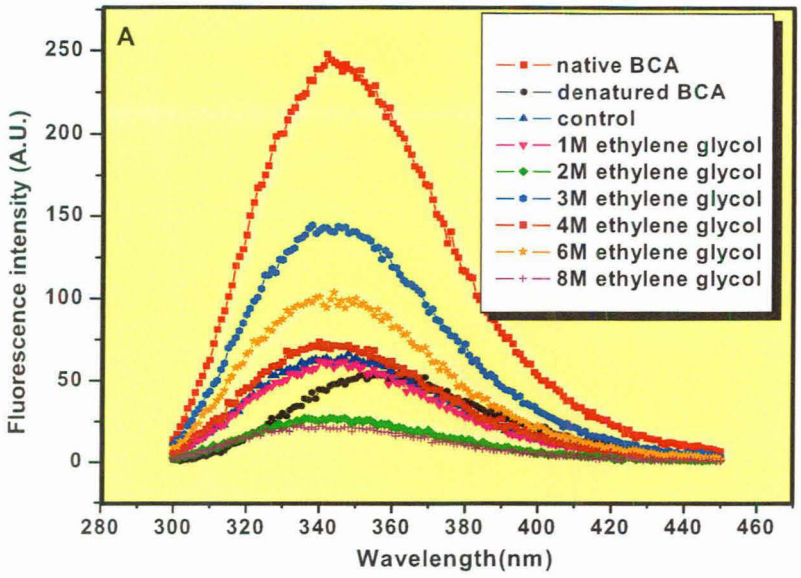
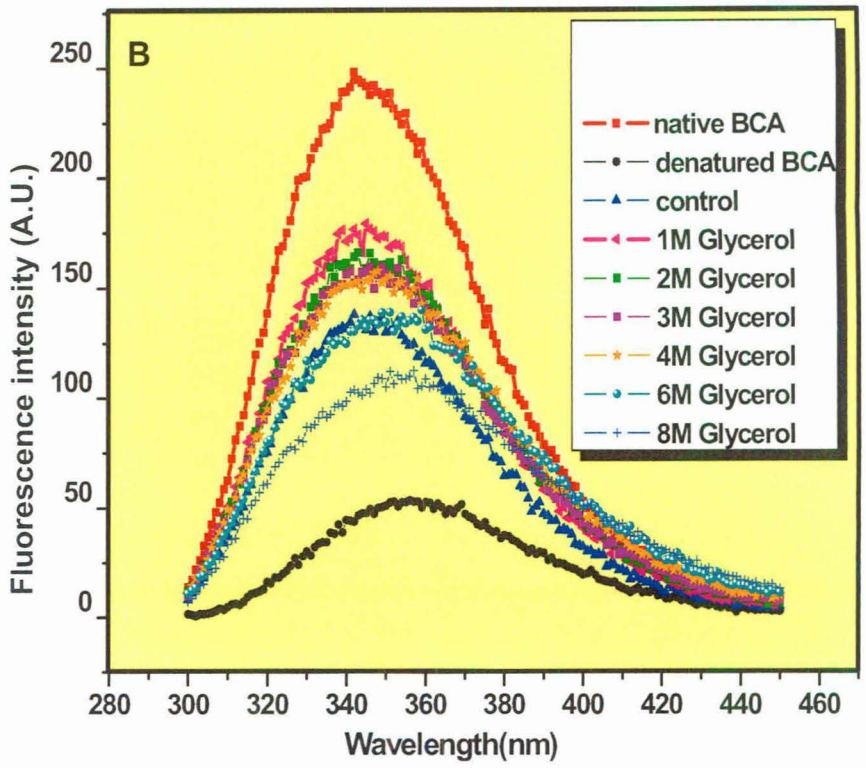



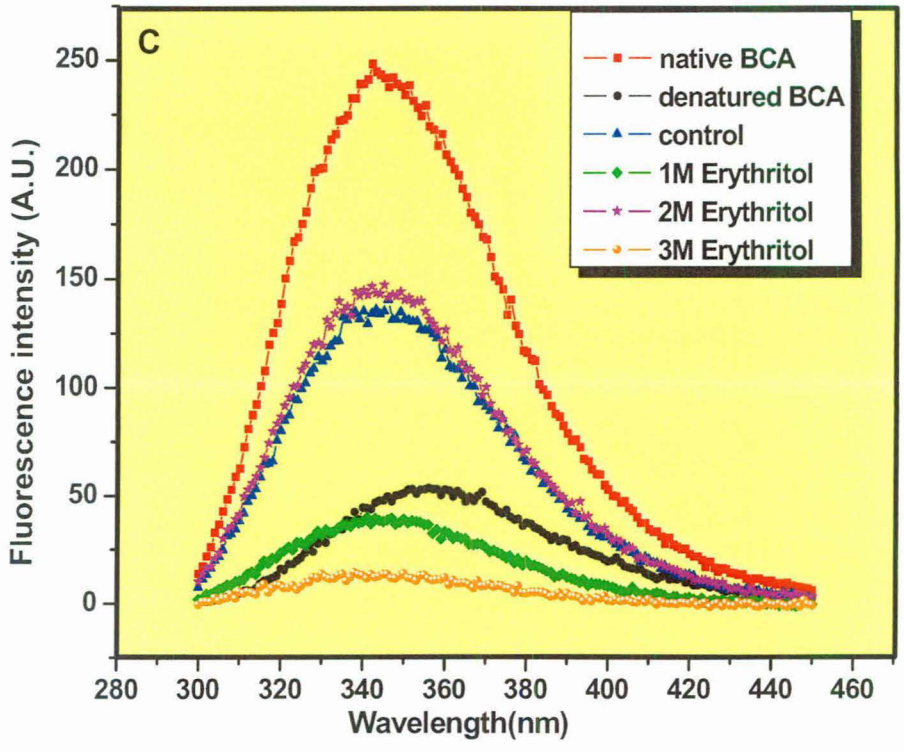

***Fig. 3:*** *Far-UV CD spectra of the refolded samples of BCA II in the presence of A) Ethylene glycol, B) Glycerol, C) Erythritol, D) Xylitol, E) Sorbitol. Native BCA II spectra and refolded BCA II spectra in the absence of polyols (control) are included in all the figures.*

**Effect of polyols on the tryptophan emission spectra of the refolded samples of BCA II**

Intrinsic fluorescence emission spectroscopy can provide information about the tryptophan and tyrosine in terms of polar and non polar environment, as well as the solvent accessesibilty of tryptophan that are sensitive to local conformational changes at the tertiary structure level (**Lakowicz, 2006**). Intrinsic emission spectra for all the refolded samples in the presence of polyols were measured to observe their effect on the local changes in the tryptophan environment. All the refolded samples, with and without polyols, were excited at 295 nm and the emission spectra was obtained in the range of 300 to 500 nm (Fig. 4A, B, C, D and E). Native BCA II gives an emission peak at 342 nm. When the protein is denatured by using 5 M GdmCl there was a sharp decrease in the fluorescence intensity followed by a significant red shift of 18 nm as compared to the native peak. During spontaneous refolding of the protein (without using any polyol) an increase in the fluorescence intensity along with a blue shift has been observed. In presence of ethylene glycol, there was a decrease in the emission intensity with increase in its concentration. At 8 M ethylene glycol, the fluorescence intensity was minimum, with a prominent blue shift (Fig. 4A). In case of glycerol also, there was an initial increase in the fluorescence intensity which was maximum at 1 M concentration with a significant blue shift. Further increase in glycerol concentration gradually decreased the fluorescence intensity (Fig. 4B). Similar trend was observed in the case of erythritol, xylitol and sorbitol (Fig. 4C, D and E)




B
native BCA
denatured BCA
control
1M Glycerol
2M Glycerol
3M Glycerol
4M Glycerol
6M Glycerol
8M Glycerol
Fluorescence intensity (A.U.)
Wavelength(nm)
0
50
100
150
200
250
280
300
320
340
360
380
400
420
440
460


C
native BCA
denatured BCA
control
1M Erythritol
2M Erythritol
3M Erythritol
Fluorescence intensity (A.U.)
Wavelength(nm)
0
50
100
150
200
250
280
300
320
340
360
380
400
420
440
460

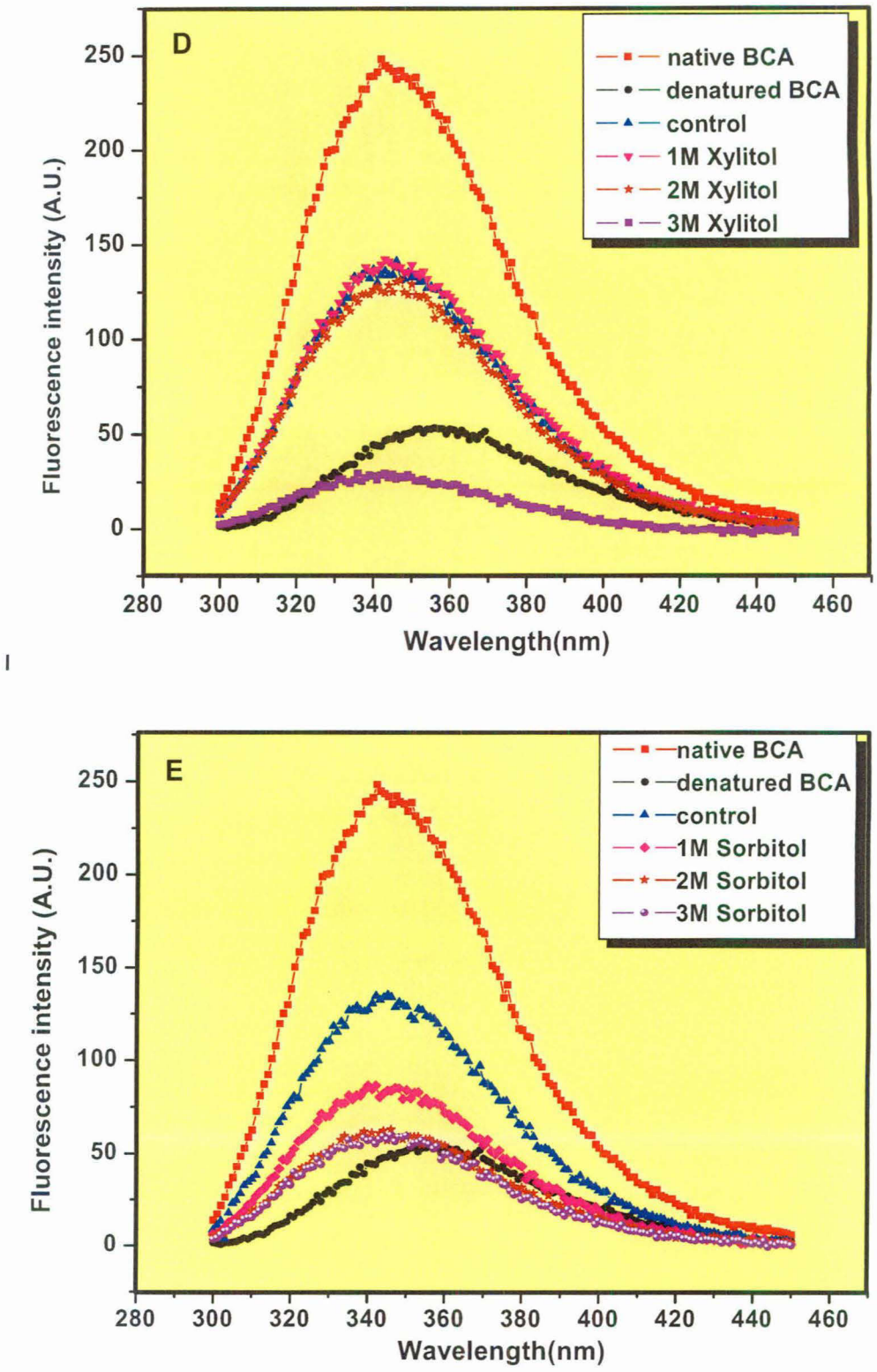
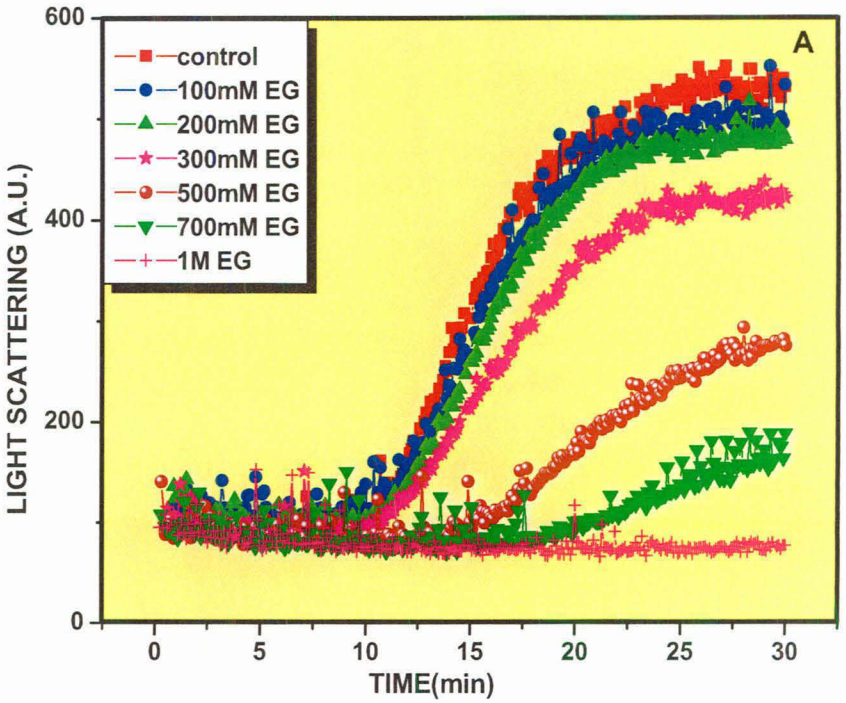


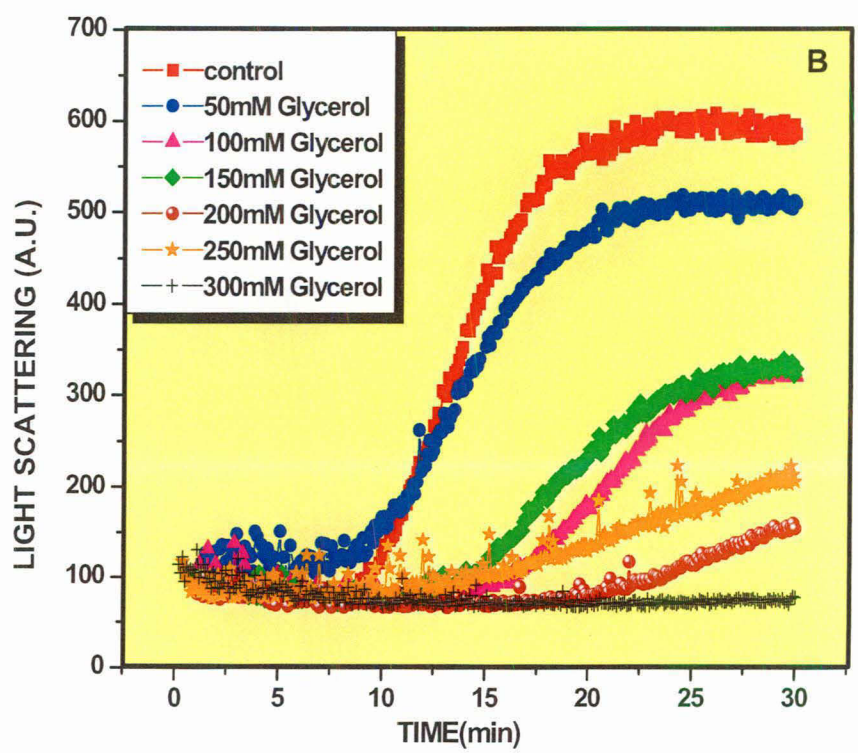

***Fig. 4:*** *Typtophan emission spectra of refolded BCA II in the presence of A) Ethylene glycol, B) Glycerol, C) Erythritol, D) Xylitol, E) Sorbitol. Native BCA II spectra (in buffer alone), denatured BCA II spectra and the refolded BCA II spectra in the absence of polyols (control) have also been included in the figures.*

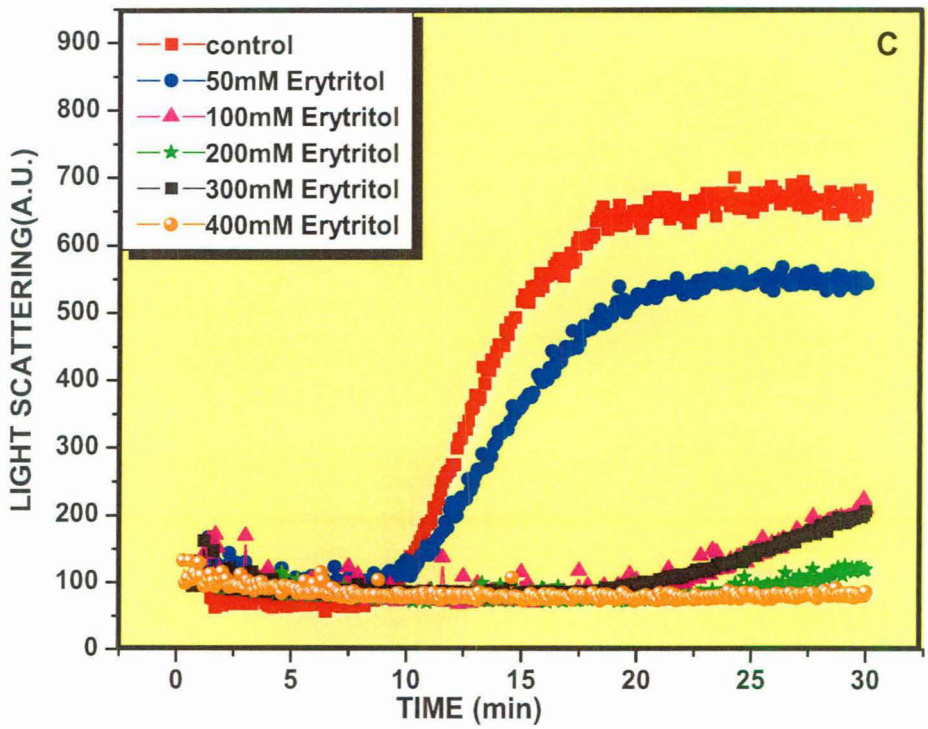
**Polyols prevent the aggregation of native BCA II at 65°C.**

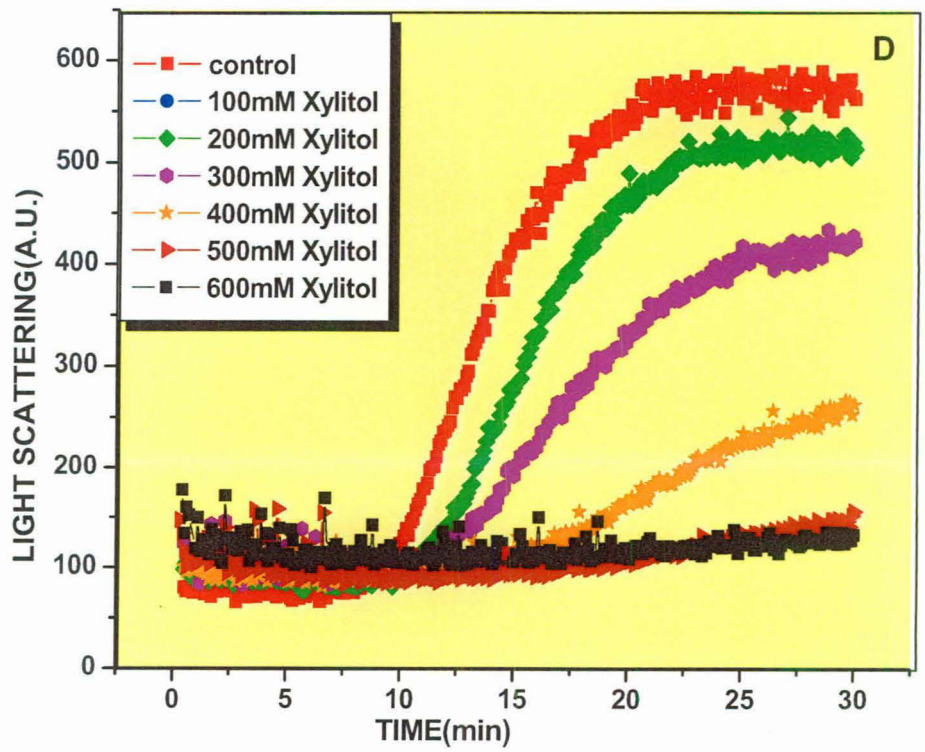
Refolding experiments clearly demonstrated that polyols were not only able to enhance the refolding yield of the denatured BCA II but were also capable of preventing the aggregation induced during the refolding procedure, especially when the concentration of protein was very high and that of the denaturant very low. Thus, the effect of polyols was further explored in preventing the aggregation that could be induced due to the incubation of the protein at high temperature. To study the aggregation kinetics of native BCA II, light scattering experiments were carried out using a spectrofluorimeter. In this experiment, when the protein is heated at a high temperature (temperature above its $T_m$) it rapidly forms aggregates and the size of the aggregates is directly proportional to the light scattered by the solution. Thus, when native BCA II was incubated at 65°C, the intensity of the scattered light started increasing within 10 minutes after incubation, showing the formation of aggregated species. The intensity rapidly reached an optimum value after which no further changes were observed following a typical sigmoidal curve. It was found that the polyols, in general, were capable of completely inhibiting the protein aggregation at higher temperature. There was a gradual decrease in the scattered intensity of light with the increasing concentration of all the polyols.

In the case of ethylene glycol, upto 200 mM concentration no significant change in the scattered intensity was observed but as the concentration was further increased considerable inhibition of aggregation was observed, with 1 M EG completely suppressing the aggregate formation (Fig. 5A). Glycerol was much more effective than EG and there was a complete inhibition of native BCA II aggregation at 300 mM concentration (Fig. 5B). Similar trend was observed for the remaining three polyols also. While erythritol and sorbitol suppressed the aggregate formation at 400 mM concentration, xylitol was able to prevent the aggregation only at 600 mM concentration (Fig. 5C, D and E).


A
control
100mM EG
200mM EG
300mM EG
500mM EG
700mM EG
1M EG
LIGHT SCATTERING (A.U.)
TIME(min)
0
200
400
600
0
5
10
15
20
25
30


B
control
50mM Glycerol
100mM Glycerol
150mM Glycerol
200mM Glycerol
250mM Glycerol
300mM Glycerol
LIGHT SCATTERING (A.U.)
TIME(min)
0
100
200
300
400
500
600
700
0
5
10
15
20
25
30


C
control
50mM Erytritol
100mM Erytritol
200mM Erytritol
300mM Erytritol
400mM Erytritol
LIGHT SCATTERING(A.U.)
TIME (min)
0
100
200
300
400
500
600
700
800
900
0
5
10
15
20
25
30


D
control
100mM Xylitol
200mM Xylitol
300mM Xylitol
400mM Xylitol
500mM Xylitol
600mM Xylitol
LIGHT SCATTERING(A.U.)
TIME(min)
0
100
200
300
400
500
600
0
5
10
15
20
25
30

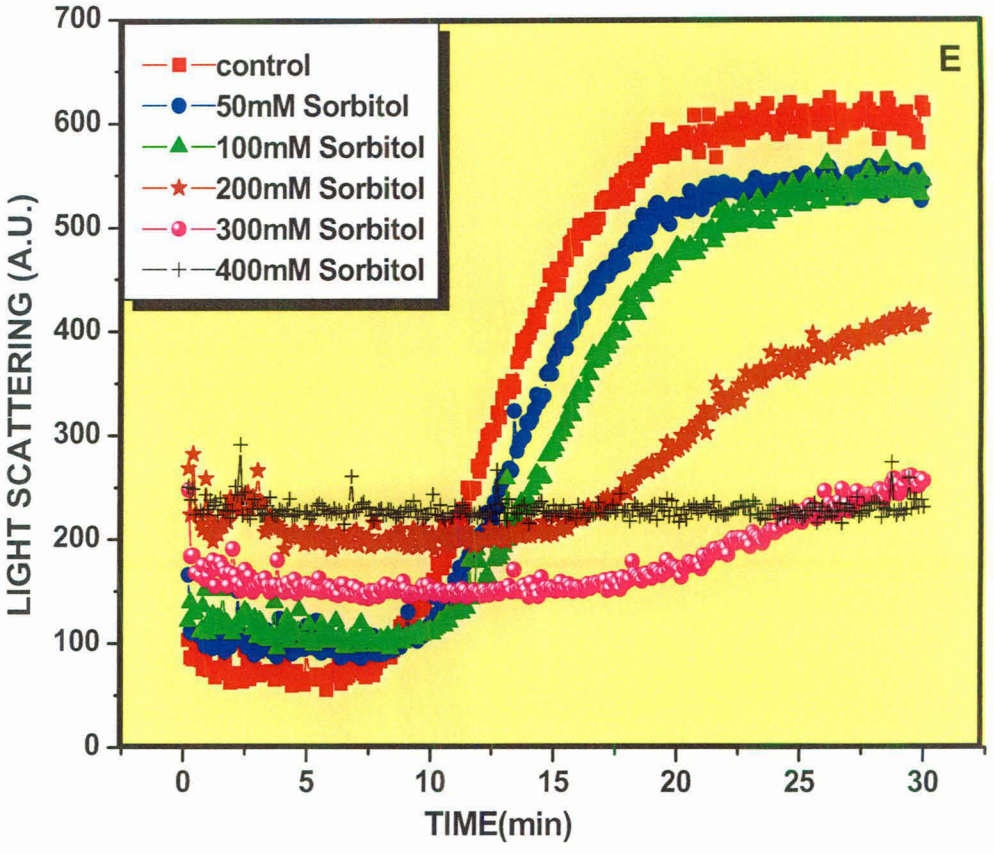
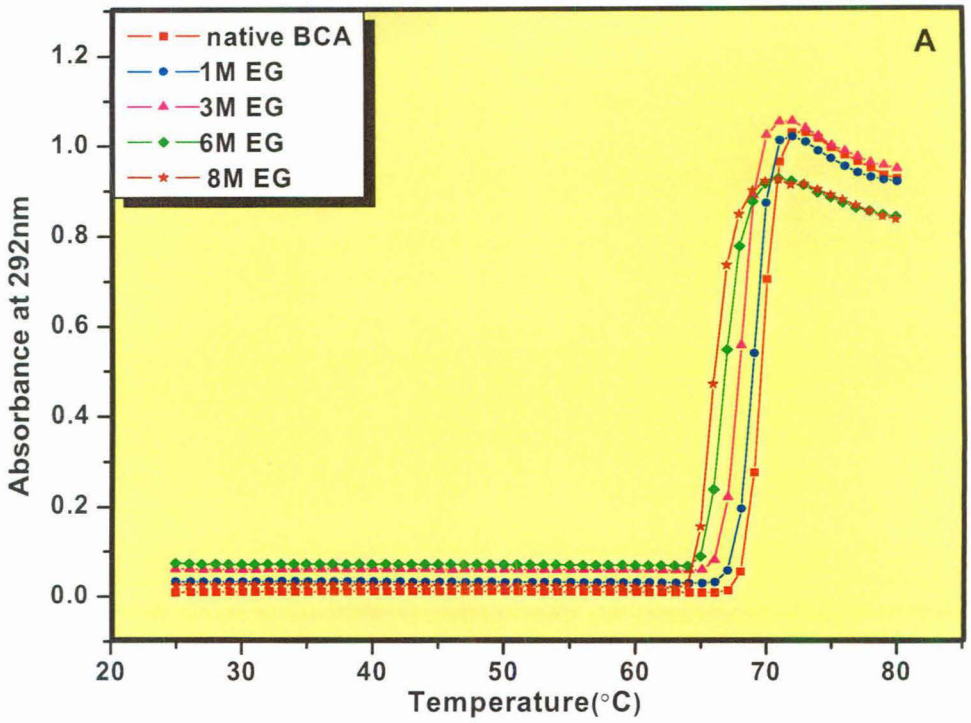


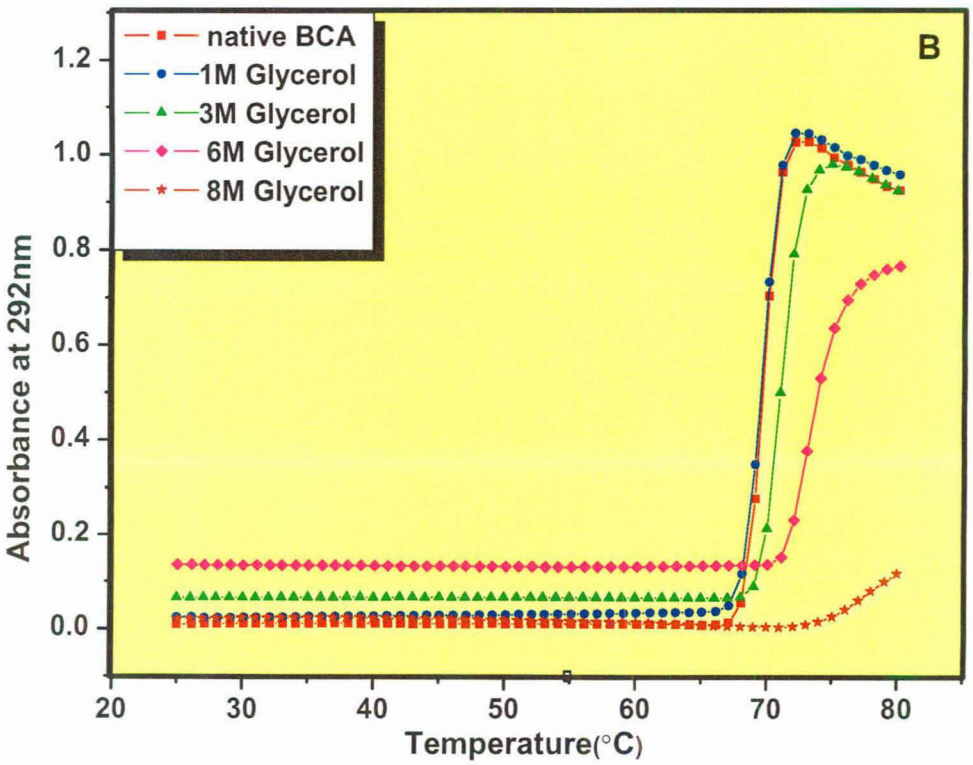

*Fig. 5: Aggregation of native BCA II monitored by light scattering in the absence and presence of different polyols at 65°C. A) Ethylene glycol, B) Glycerol, C) Erythritol, D) Xylitol, E) Sorbitol.*

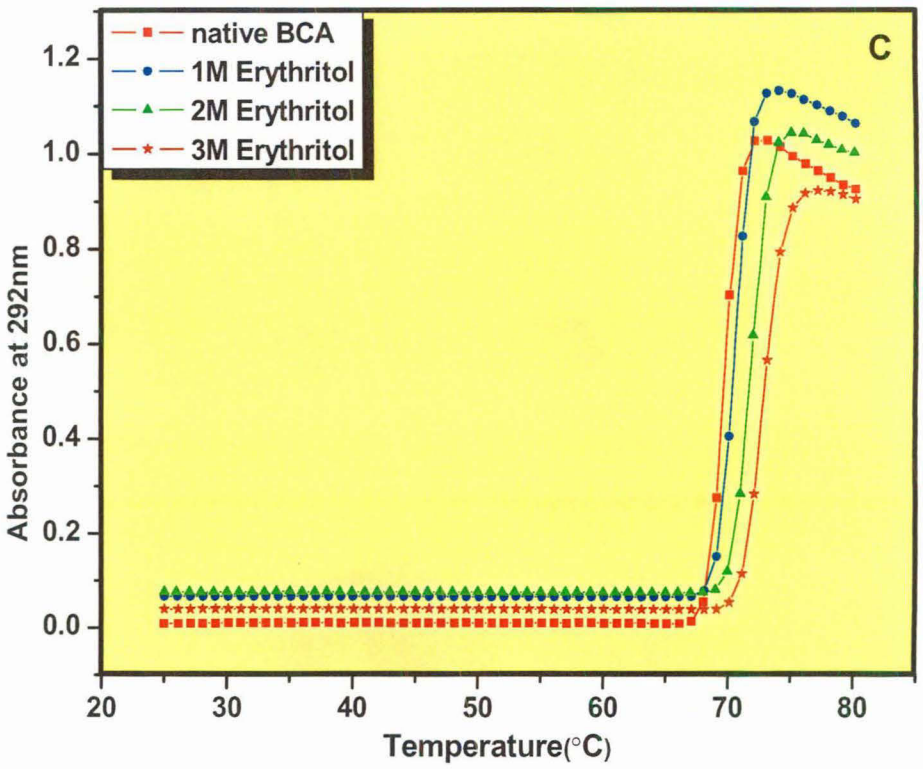
**Polyols affect the temperature onset of denaturation/aggregation of native BCA II**

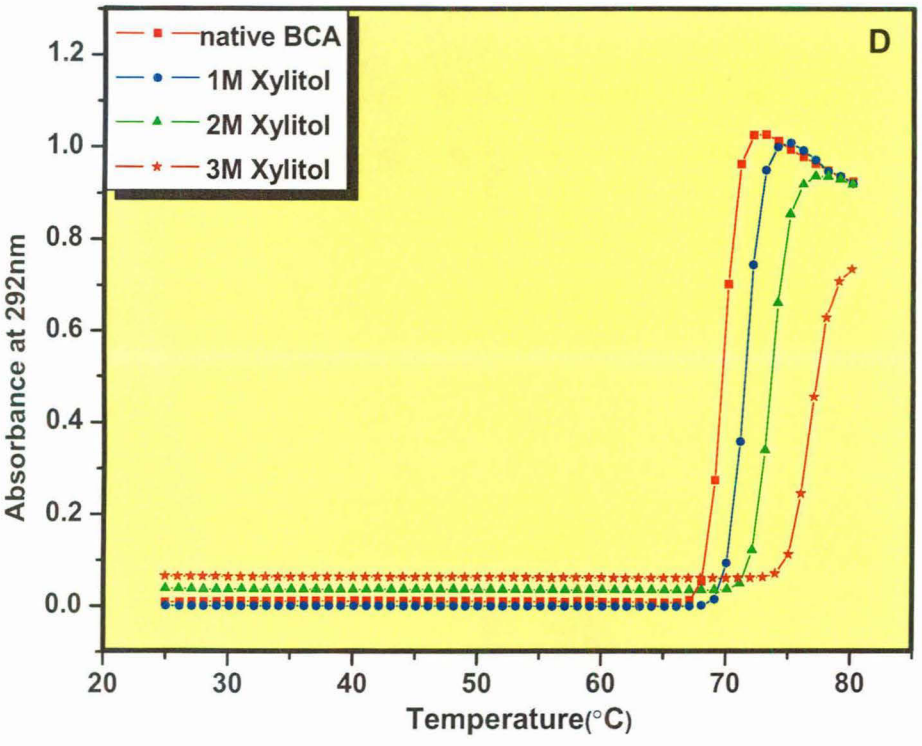
The effect of polyols on the stability of the native BCA II was explored by thermal denaturation experiment. Since BCA II aggregates rapidly at high temperatures, therefore one could only determine the temperature onset of denaturation/aggregation ($T_{agg}$) and not the $T_m$ values. It was observed that thermal denaturation results obtained were in contrast to the refolding results, as those polyols which were enhancing the refolding yield were observed to destabilize the native state and the good stabilizers of the native state of BCA II were observed to decrease the refolding yield of the denatured BCA II (Fig. 1 and Fig. 6A, B and E). Native BCA II has an apparent onset of denaturation at 68°C. In case of ethylene glycol, this value decreased gradually with increase in its concentration. At 6 and 8 M ethylene glycol, onset of denaturation/aggregation was nearly 64°C, which was 4 degree less than that of control (Fig. 6A). Though 8 M ethylene

glycol decreased the stability of the native state but at the same time it also decreased the aggregation induced at high temperature which was indicated by decreased absorption intensity at 292 nm in the post denaturation zone (68-80°C) (Fig. 6A). Glycerol, on the other hand acts as both a refolding enhancer as well as structure stabilizer (Fig. 1 and Fig. 6B). $T_{agg}$ of the native BCA II increased significantly with higher concentration of glycerol. At 3 M glycerol, $T_{agg}$ was nearly 69°C, and it increased further by 2 degree at 6 M concentration. 8 M glycerol, was found to be effective in not only increasing the $T_{agg}$ from 68°C to 74°C (6 degrees increase), but also decreased the aggregation of native BCA II considerably which was apparent by a decrease in the intensity of light absorbed at 292 nm in the post denaturation zone (Fig. 6B). In case of erythritol, while lower concentration (1 M) had negligible effect on $T_{agg}$, 2-3 M erythritol increased the $T_{agg}$ by almost 2 degrees (Fig. 6C). In case of xylitol, there was a gradual increase in the $T_{agg}$ value at all the concentrations. There was a pronounced increase in the $T_{agg}$ from 68°C in control to 74°C at 3 M xylitol (Fig. 6D). Both erythritol and xylitol at 3 M concentration also decreased the aggregation in the post denaturation zone (Fig. 6C and D). Sorbitol which considerably decreased the refolding yield of the denatured BCA II was found to be a good stabilizer of the native state. On increasing the concentration of sorbitol from 0-3 M, there was an increase in the $T_{agg}$ value upto 7 degrees. 3 M sorbitol was not only effective in increasing the temperature onset of aggregation from 68°C to 75° C but also sharply decreased the absorbance intensity showing inhibition of aggregation in the post denaturation zone (Fig. 6E).


native BCA
1M EG
3M EG
6M EG
8M EG
A
Absorbance at 292nm
Temperature(°C)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
20
30
40
50
60
70
80


native BCA
1M Glycerol
3M Glycerol
6M Glycerol
8M Glycerol
B
Absorbance at 292nm
Temperature(°C)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
20
30
40
50
60
70
80


native BCA
1M Erythritol
2M Erythritol
3M Erythritol
C
Absorbance at 292nm
Temperature(°C)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
20
30
40
50
60
70
80


native BCA
1M Xylitol
2M Xylitol
3M Xylitol
D
Absorbance at 292nm
Temperature(°C)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
20
30
40
50
60
70
80

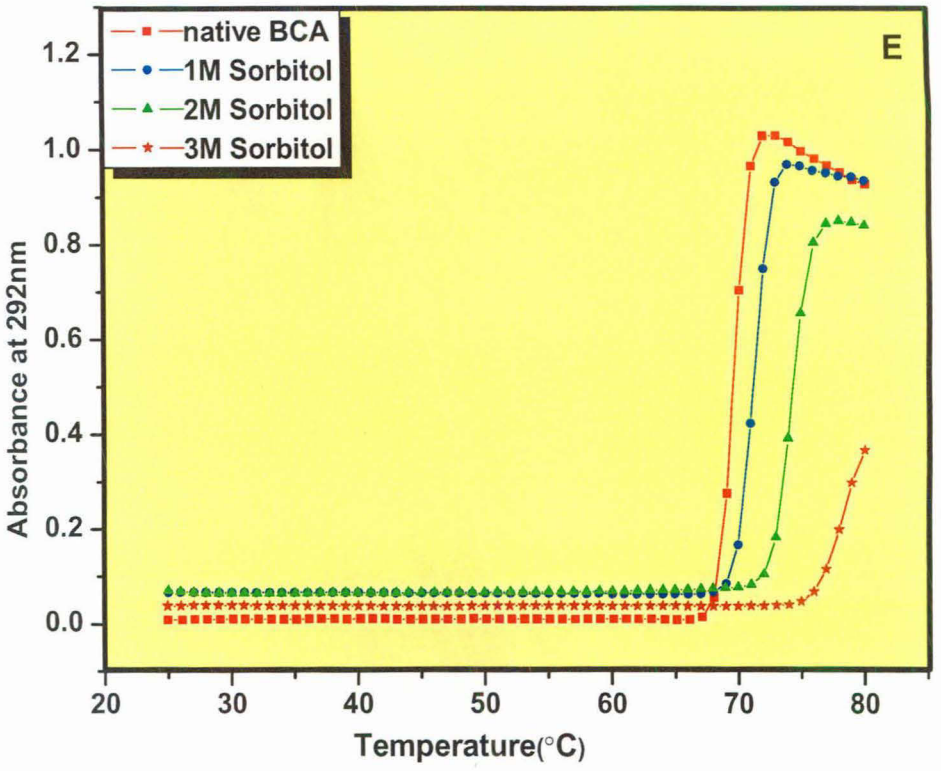



***Fig. 6:*** *Effect of different polyols on the Temperature onset of denaturation/aggregation of native BCA II. A) Ethylene glycol, B) Glycerol, C) Eryhtritol, D) Xylitol, E) Sorbitol. Temperature onset of denaturation/aggregation of native BCA II is also included in each graph.*

## Discussion

Bovine carbonic anhydrase (BCA II) is an aggregation prone system, whose refolding from its denatured state is dependent upon both protein and denaturant concentration (**Wetlaufer and Xie, 1995**). The refolding pathway of BCA II has been well studied and the folding of BCA II from its denatured state is known to involve two intermediate species (**Stein and Henkens, 1978; Semistonov *et al.*, 1987, 1990; Cleland and Wang, 1990a, 1992**). The first intermediate in the folding pathway showed molten globule like characteristics (**Doligikh *et al.*, 1984; Rodionova *et al.*, 1989**). The hydrophobic clusters present on the surface of first intermediate collapse to form the hydrophobic core in the second intermediate which then rearranges to form the native protein structure. Formation of second intermediate and then the native structure is a slow process because it requires proline isomerization to obtain the correct conformation (**Semisotnov *et al.*, 1990**). Due to the slow proline isomerization process the first

intermediate which has hydrophobic molten globule like characteristics gets enough time to be stabilized (**Ptitsyn *et al.*, 1990**). Since the first intermediate remains for longer duration of time during refolding process, there is a fair possibility for the hydrophobic clusters present on its surface to form associated species which could result in aggregate formation (**Cleland and Wang, 1990a, 1992).**

High protein and low denaturant (GdmCl), often results in a very low refolding yield (Table1) as large proportion of the protein becomes inactive due to aggregation. Refolding data of BCA II (Table1) indicate that different polyols refolded BCA II to different extents depending upon the concentration used. While lower concentrations of all the polyols did not affect the refolding much, there was an appreciable increase in the refolding yield at higher concentration of ethylene glycol and glycerol. On the other hand, while erythritol had negligible effect on refolding at all the concentration, sorbitol was found to decrease the refolding yield significantly at higher concentrations.

Ethylene glycol is known to decrease the $T_m$ of a number of proteins during thermal denaturation, and therefore acts as a protein destabilizer, though the effect is very small (**Back *et al.*, 1979**; **Gekko and Morikawa, 1981a, b**). Contrary to its destabilization effect, ethylene glycol was found to be a good folding enhancer during refolding of proteins from its denatured state. EG was found to successfully refold the denatured citrate synthase with considerable refolding yield (nearly 16% increase as compared to the control) (**Mishra *et al.*, 2005**). Similar trend has been observed in the case of BCA II with a much more pronounced effect. Among all the polyols used, ethylene glycol was most effective as it enhanced the refolding at all the concentrations. Refolding yield of BCA II increased up to 70% in presence of 8 M EG which was highest for all the polyols used. Ethylene glycol is the first member of polyol series used and has a chemical formula of $(CH_2OH)_2$. While methylene group ($-CH_2$) is hydrophobic, hydroxyl group, (-OH) provides considerable hydrophilicity to the molecule.

During refolding process, in the presence of ethylene glycol, non specific, weak interactions between the methylene groups of EG and non polar regions of the amino acid residues present on the partially folded polypeptide chain could occur. Such interactions would prevent the self association between the protein molecules which in turn could minimize the formation of off-pathway aggregation prone species. Presence of ethylene

glycol in the refolding buffer, could, therefore, block the first intermediate to form an aggregate. This could give sufficient time for it to rearrange itself into functionally active conformation of BCA II, which results in the productive folding. Refolding data is further supported by the absorbance of residual protein at 280 nm in the refolded samples in presence of ethylene glycol. It was found that higher concentration of ethylene glycol lead to minimum aggregation of the protein during rapid folding procedure (Table 8).

Glycerol has been used for a very long time for the stabilization of native conformation and activity of many proteins (**Jarabak *et al.*, 1966; Jarabak, 1972; Myers and Jakoby, 1973; del Vecchio; 1999**) as well as for the self assembly of many biological systems (**Green *et al.*, 1972; Behnke, 1975; Shifrin and Parrott, 1975**). High concentration of glycerol enhances self assembly of purified tubulin to form microtubules (**Timasheff, 1975, 1977**). The detailed mechanism of preferential hydration of proteins in the presence of glycerol and its stabilization effect has been explained in detail by Gekko and Timasheff (**Gekko and Timasheff, 1981a, and b**). According to this theory, when glycerol is added to the protein-solvent system, it excludes itself from the surface of protein molecule which in turn stabilizes the native form of proteins. Glycerol has been used as an excellent medium for protein folding (**Rariy and Klibanov, 1997**) as well as an efficient folding agent in the case of highly aggregation prone citrate synthase (**Mishra *et al.*, 2005**). In case of BCA II refolding also, glycerol was found to be a better folding aid as compared to all other polyols used. Glycerol was able to refold the denatured BCA II by resulting in significant refolding yield (comparable to 8 M ethylene glycol) at concentration as low as 4 M. It is possible that glycerol, like ethylene glycol enhances the refolding of denatured BCA II, by binding to the off-pathway aggregation prone intermediate species. Inhibition of protein aggregation by glycerol was evident by the enhanced fraction of soluble protein in the refolded samples at high glycerol concentration (Table 8).

Though erythritol acts as a protein stabilizer but during refolding of denatured citrate synthase, it showed anomalous behavior by reducing the refolding yield at all the concentrations (**Mishra *et al.*, 2005**). However, during refolding of BCA II, erythritol had negligible effect on its refolding yield unlike the reduction in the folding yield of citrate synthase. The residual protein concentration in all the refolded samples in

presence of erythritol remained almost same indicating its inability to prevent the formation of aggregation during folding pathway. Xylitol is also a stabilizer of proteins (**Kaushik and Bhat, 1998**). Refolding studies on citrate synthase showed that xylitol enhanced protein folding at lower concentration but at higher concentration it lead to a considerable decrease in the refolding yield (**Mishra *et al.*, 2005**). Similar results were observed for BCA II as well. Xylitol is known to stabilize the molten globule state of cytochrome c (**Kamiyama *et al.*, 1999**). It is therefore possible that at higher concentrations xylitol may stabilize the off-pathway intermediate states formed during the folding of BCA II, which in turn decrease the refolding yield.

Sorbitol enhances the stability of many globular proteins by increasing their $T_m$ during thermal denaturation (**Kaushik and Bhat, 1998**). Among the polyols studied, sorbitol is the most effective stabilizer of protein conformation as compared to other polyols used. When present in the protein- solvent mixture, sorbitol is known to increase the surface tension of water by large extends, and excludes itself from the immediate vicinity of protein molecules which in turn leads to preferential hydration of proteins and thereby stabilization of their native states (**Kaushik and Bhat, 1998**) Sorbitol besides being the best stabilizer among all the polyols is also reported to protect the protein against urea denaturation (**Tiwari and Bhat, 2006**). On similar lines to the stabilization of native state of proteins during thermal denaturation, sorbitol is also reported to stabilize the acid unfolded molten globule state of cytochrome c (**Majumder *et al.*, 2001**).

Extensive work done on the refolding of citrate synthase (**Mishra *et al.*, 2005**) has led to the findings that sorbitol can significantly enhance the refolding yield of the denatured citrate synthase in a concentration dependent manner. However, the stabilization effect of sorbitol is dependent much upon the nature and type of protein and it can act differently for different proteins (**Kaushik and Bhat, 1998**). Based on the detailed analysis of preferential hydration mechanism in various solvent systems (**Arakawa *et al.*, 1990 b**), cosolvents have been classified into two categories (1) those in which preferential hydration is independent of solution conditions (pH, cosolvent concentration etc) (2) those in which it varies with conditions. Those cosolvents which belong to the first class stabilize the structure of globular proteins, whereas the others do

not always do so. Sorbitol is one such example of cosolvent whose action of preferential hydration is dependent upon a delicate balance of various weak interactions and it can change according to the solvent conditions (**Xie and Timasheff, 1997a, b**). Sorbitol was found to preferentially hydrate the thermal denatured state of ribonuclease A, causing greater extend of stabilization as compared to the native state (**Xie and Timasheff, 1997 a**).

In case of BCA II refolding from its GdmCl denatured state sorbitol showed decreased folding yield. At 3 M sorbitol refolding yield was as low as 17% in comparison to the control (Table 1). Out of the two intermediates, involved during the folding of BCA II from its denatured state, the first intermediate has considerable hydrophobicity due to the presence of many non polar amino acid residues on its surface (which normally remain buried in the interior of the protein in the native state) (**Doligikh *et al.*, 1984; Semisotnov *et al.*, 1987; Rodionova *et al.*, 1989**). Being a hydrophilic molecule sorbitol, like glycerol (**Gekko and Timasheff, 1981a**) should have affinity for the polar amino acids present on the protein surface. But due to the presence of non- polar amino acids on the surface of folding intermediates, sorbitol molecules could compel to orient themselves away from the vicinity of the protein molecules. All these events could lead to the preferential hydration of the intermediate states leading to their stabilization. Intermediates on being stabilized, would easily form many intramolecular hydrophobic interactions leading to protein aggregation. This is very well supported by the decreased residual concentration of the protein in the refolded sample in the presence of high concentration of sorbitol. Concentration of proteins in the refolded sample in presence of 3 M sorbitol was only 0.08 mg/ml compared to the control (0.2 mg/ml) as most of the protein aggregated on its pathway to folding. Thus, it appears that polyols like ethylene glycol and glycerol could enhance the refolding of the denatured BCA II by preventing the self association of several aggregation prone, intermediate species in the refolding pathway. Xylitol and sorbitol, on the other hand, at higher concentration could stabilize the same aggregation prone, off-pathway species and therefore decrease the refolding yield of the denatured protein.

Biological activity of a protein depends upon the intact three dimensional structure of the protein. Polyols like ethylene glycol and glycerol, which enhanced the

refolding yield of denatured BCA II, by suppressing the aggregation, were also found to protect the structural perturbation of polypeptide chain against the denaturant, by maintaining the integrity of the protein conformation close to its native state as observed by CD measurements (Fig. 3). BCA II, being predominantly beta pleated sheet protein, gives characteristic negative ellipticity at 215nm in the far-UV CD region. When denatured BCA II was refolded in presence of 8 M EG and 6 M glycerol, there was significant increase in the intensity of negative ellipticity as compared to the control samples (where no polyol was used) (Fig. 3A). These spectra almost overlapped the native spectra which illustrates that higher concentrations of ethylene glycol and glycerol were able to retain the secondary structure of the refolded BCA II (Fig. 3A and B). While xylitol and erythritol had negligible effect on the structure, there was a significant decrease in the negative ellipticity of the refolded sample at higher concentration of sorbitol, suggesting that sorbitol can favor non native aggregation prone intermediate species over native state (Fig. 3C, D and E).

BCA II contains seven tryptophan residues in the polypeptide chain. BCA II has one exposed Trp residue in its native state (**Liljas *et al.*, 1972**) and six exposed Trp residues in the first intermediate state (**Jonasson *et al.*, 1997**). Changes in the intrinsic Trp fluorescence of protein is highly sensitive to the change in its immediate environment, and hence could be used as a tool to monitor the effect of different polyols on the refolding of GdmCl denatured BCA II. Tryptophan emission of native BCA II shows emission maxima at 342 nm. The usual red shift in the emission peak of denatured BCA II occurs due to the change in the tryptophan environment from non polar to polar. The solvent mediated quenching, resulting from the tryptophan exposure upon unfolding, led to an overall decrease in the intensity of emission. Refolding of denatured BCA II, in the presence of all the polyols showed a blue shift in the emission spectra, as compared to the denatured state and it became more prominent at higher concentration of ethylene glycol and glycerol indicating the restructuring of the polypeptide chain to attain the native conformation (Fig. 4A, B, C, D and E).

In the light scattering experiments it was found that the intensity of the scattered light decreased with the gradual increase in the concentration of all the polyols. The maximum suppression effect was seen in glycerol which is also known to be a good

protein stabilizer. It was able to completely inhibit the protein aggregation at 300 mM concentration (Fig. 5B). It was followed by sorbitol erythritol, xylitol and ethylene glycol (Fig. 5E, C, D and A respectively). When protein is incubated at high temperatures its hydrophobic surface gets exposed, making it prone to aggregation due to intramolecular association of the protein molecules. In the case of BCA II, compounds such as ANS (**Kundu and Guptasarma, 1999**) have been used to prevent the aggregation of the protein at elevated temperature. It has been suggested that the binding of hydrophobic ANS to BCA II non polar groups could prevent protein- protein interactions and hence protein aggregations. It is proposed that polyols known to be preferentially excluded from globular protein could bind to the exposed hydrophobic patches of BCA II molecules at higher temperature and thereby suppress the aggregation by preventing the self association of protein molecules through hydrophobic interactions.

Polyols are known to stabilize proteins by changing the solvent properties or by altering the water structure around them (**Kaushik and Bhat, 1998**). Several globular proteins are known to be preferentially hydrated in polyol-water mixtures through a delicate balance between the repulsion from the non polar region and attraction toward polar regions of the protein surface mediated via solvent water. It is also known that strengthening of hydrophobic interactions in the presence of polyols can make proteins more stable (**Back *et al.*, 1979**). In the thermal denaturation experiment, the temperature onset of aggregation was found to be nearly 68°C in case of native BCA II without adding any polyol (Fig. 6A-E). Ethylene glycol, already known to be a mild protein destabilizer, was found to decrease the temperature onset of aggregation from 68°C to nearly 64°C at 8 M concentration. Destabilization mechanism of ethylene glycol is also well defined. Ethylene glycol at higher concentration may preferentially bind with the exposed non polar surface of protein molecule which can destabilize the native form of the protein. Decrease in the $T_m$ of proteins has also been observed in the presence of PEG, depending upon size and concentration based on a similar mechanism (**Lee and Lee, 1987**). As already proposed, glycerol, erythritol, xylitol and sorbitol provide stability to native BCA II against thermal denaturation due to the mechanism of preferential hydration of the protein (**Gekko and Timasheff, 1981 a, b; Kaushik and Bhat, 1998**).

Erythritol was found effective in stabilizing the native state of BCA II against thermal denaturation as well as aggregation induced due to incubation of protein at high temperature (Fig. 5C and 6C). The preferential exclusion of erythritol like other polyols could stabilize the native protein against thermal denaturation. Erythritol could prevent the self association of hydrophobic regions present on the unfolded polypeptide chain during incubation at high temperature and therefore aggregation is inhibited (Fig. 5C). However, when GdmCl denatured BCA II was refolded in the presence of erythritol there was negligible effect on the refolding yield as well as residual protein concentration of the refolded samples (Table 4). The above result illustrates that erythritol behaved differently during folding of polypeptide chain from its denatured state. There is a fair possibility that erythritol was unable to block the off-pathway folding intermediates formed during refolding process. Thus, formation of aggregation and low refolding yield of BCA II remained unaffected even at high concentration of erythritol (Fig. 1 and 2).

In conclusion, refolding studies of BCA II in the presence of different polyols reveals that different polyols refold the denatured BCA II to different extents and that the folding of protein is not only concentration dependent but also depends on the type of polyol used. Polyols were found to be efficient inhibitors of protein aggregation occurring either during refolding from GdmCl denatured state or by incubation at high temperature. However, the effect of a polyol can be different for the two aspects of folding and aggregation even for the same protein. At the same time, refolding and stability results of BCA II in the presence of different polyols, put together, suggest that a good protein folding agent may not necessarily act as a good protein stabilizer and vice versa. Thus, for the polyol mediated folding of proteins, the two aspects of protein folding and stability do not bear any direct correlation. For efficient folding, a balance needs to be striked which includes the stabilizing effect, the solubility effect, and the binding/ exclusion effect of the polyols with respect to the folding of polypeptide chain that is in dynamic contact with the surrounding water and the cosolvent additive.

# EFFECT OF SUGARS ON THE REFOLDING AND AGGREGATION OF BCA II

## Introduction

In nature, many plants, animals and micro organisms have been found to be well adapted against various extremities of temperature, pH, salt, and desiccation, etc. These organisms are known to accumulate a number of organic compounds that provide protection against the above mentioned harsh environmental conditions and maintain the osmotic pressure in the organism's cell (**Yancey *et al.*, 1982; Sato *et al.*, 1996**). These molecules are mostly natural compounds and belong to the chemical class of sugars, polyols, methylamines and amino-acids. These small organic molecules are known as organic osmolytes, and they protect proteins not only against thermal denaturation but also prevent the loss of their functional activity (**Lee and Timasheff, 1981; Arakawa and Timasheff, 1982 a, b, 1983, 1985; Santoro *et al.*, 1992; Sola-Penna *et al.*, 1997**). Due to the similarity of their action with the molecular chaperones they are also termed as 'chemical chaperones' (**Burrows *et al.*, 2000; Kolter and Wendeler, 2003**). Also, the ability of these osmolytes to provide protection against denaturation is believed to be both generic and independent of the evolutionary history of the proteins (**Yancey *et al.*, 1982; Hochachka and Somero, 1984; Wang and Bolen, 1996**).

Bovine carbonic anhydrase (BCA II) is a Zn containing protein composed of single polypeptide chain which is devoid of any disulphide bond and this system is described in detail in chapter 3. Refolding pathway of BCA II is also well characterized and is known to involve two folding intermediates (**Stein and Henkens, 1978; Semistonov *et al.*, 1987**). One of the general methods to prevent folding induced aggregation is by changing the aqueous solvent environment. This could be achieved by using a cosolvent which may prevent the association of intermediate protein structure without interfering with the formation of other intermediates or the native state.

Sugars are known from a very long time to protect proteins against thermal and chemical denaturation (**Ball *et al.*, 1943; Simpson and Kauzmann, 1953; Taylor *et al.*, 1995; Melo *et al.*, 2001; Kaushik and Bhat, 2003; Kim *et al.*, 2003; Sola-Penna and Fernandes, 2005; Ortbauer and Popp, 2008**) and belong to the compatible osmolytes category which provide protection without affecting the biological activity of the macromolecules. Sugars have been found to increase the transition temperature of proteins in aqueous solutions (**Gerlsma, 1970; Gerlesma and Sturr, 1972; Arakawa**

**and Timasheff, 1982 a, b**). Structure stabilization mechanism of sugars has been well studied and it has been established that sugars stabilize the protein structure mainly by being preferentially excluded from the vicinity of protein surface which in turn leads to the preferential hydration of the protein molecule (**Back *et al.*, 1979; Lee and Timasheff, 1981; Timasheff, 1993; Lin and Timasheff, 1996**). Though the mechanism of stabilization of proteins by sugars has been thoroughly investigated, its mechanistic role in the folding-unfolding mechanism of proteins and prevention of aggregate formation during refolding step is not so well explored. Sucrose was reported to be effective in slowing the rate of unfolding of 33 kDa protein from photosystem II during pressure induced denaturation reaction (**Ruan *et al.*, 2003**). Sugars like glucose and sucrose were shown to act as a chemical chaperone during protein folding and in some cases they also slowed down the rate of denaturation (**Kim et al, 2006; Divsalar *et al.*, 2006; Monterroso and Minton, 2007**). Trehalose is another widely used sugar in several biotechnological applications such as stabilization of biological membranes, pharmaceutical preparations, for the organ transplantation (**Schiraldi *et al.*, 2002**) as well as in the stabilization of protein structure during heat shock, desiccation, osmotic shock, freezing and freeze drying (**Attfield, 1987; Hottiger *et al.*, 1987; Wiemken 1990, Sola-Penna and Fernandes, 1994, 1996**). Role of trehalose as a stress protectant as well as stabilization of proteins at high temperature has been attributed to its ability to suppress the aggregation of denatured proteins (**Singer and Lindquist, 1998**). Stabilization effect of trehalose is also a well studied aspect. Trehalose not only helps in the stabilization of proteins against high temperature but it also plays role as a stress protectant and has the ability to suppress the aggregation of denatured proteins (**Sola-Penna and Fernandes, 1998; Zancan and Sola-Penaa, 2005, Attanasio *et al.*, 2007, Jain and Roy, 2009**). Besides being a protein stabilizer, trehalose is also known to prevent the protein aggregation and amyloid formation under different conditions (**Arora *et al.*, 2004; Liu *et al.*, 2005; Davis *et al.*, 2006; Sharp *et al.*, 2006; Jain and Roy, 2008, 2009**).

Keeping above observations in mind, in the present chapter, members of sugar series glucose, sucrose and trehalose with varying concentration have been used in an attempt to enhance the refolding of the denatured BCA II as well as to see their effect in preventing the aggregation induced due to incubation at higher temperature. The study

also aims to establish relationship between refolding and stability by carrying out thermal denaturation experiment of native BCA II in the presence of these sugars and employing a number of spectroscopic tools.

## Results

### Effect of sugars on the refolding yield of BCA II

Bovine carbonic anhydrase was denatured in 5 M GdmCl and the solution was kept for 12-14 hours at 25°C. Refolding was done by rapid dilution method with vigorous vortexing in 20 mM Tris-$SO_4$ buffer at pH 7.5. To observe the effect of different sugars in enhancing the refolding yield, they were introduced at the desired concentration in the refolding buffer prior to dilution. The refolded samples were then left for one hour at room temperature. Refolding was measured by calculating the activity of all the refolded samples with and without sugars using p-nitrophenyl acetate as a substrate. The percentage refolding yield was calculated by taking the ratio of refolded samples in presence of sugar to the native BCA II refolded in the buffer alone (i.e. without using any sugar) at the same concentration of GdmCl. The effect of different sugars on the refolding yield of denatured BCA is summarized as below:

Effect of Glucose

Glucose is a known to stabilize many proteins against thermal stress by increasing their transition temperature (**Arakawa and Timasheff, 1982 b**). Not only this, glucose also protects many macromolecules from degradation and helps in maintaining their biological activity (**Gerlesma, 1968, 1970; Gerlesma and Sturr, 1972, 1974**). Thus it was assumed that glucose can also help in the refolding of proteins from their denatured state. Keeping this in mind, different concentration of glucose from 0.1-3.0 M were used to study the refolding of denatured BCA II. It was found that there was a gradual increase in the refolding yield with increase in the concentration of glucose. At 2 M glucose there was nearly 13% increase in the refolding yield as compared to the control (where no glucose was used) Concentrations higher than 3 M could not be explored due to the limited solubility of glucose at higher concentrations (Table 1).

**Table 1: Effect of glucose on the refolding yield of BCA II at 25°C**

| Glucose (M) | Percentage refolding yield |
|---|---|
| 0.0 | 37 |
| 0.1 | 39 |
| 0.5 | 47 |
| 1.0 | 49 |
| 2.0 | 50 |
| 3.0 | 46 |

*BCA II refolding in the presence of glucose at 25°Cin 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Sucrose

Sucrose is the next member of the sugar series and is made up of two glucose molecules. Sucrose is also known to protect the proteins against thermal denaturation in many cases (**Back *et al.*, 1979**). Being a good protein stabilizer, sucrose was used in the refolding experiment to enhance the folding of GdmCl denatured BCA II. It was observed that there was a gradual increase in the refolding yield of BCA II with increase in the concentration of sucrose. At 1 M sucrose, refolding yield was 60% which was highest for all the concentration used and there was nearly 17% increase as compared to the control (when no sucrose was used). Further increase in the sucrose concentration lead to decrease in the refolding yield. Concentrations higher than 2 M could not be explored due to the limited solubility of sucrose at higher concentrations (Table 2).

**Table 2: Effect of sucrose on the refolding yield of BCA II at 25°C**

| Sucrose(M) | Percentage refolding yield |
|---|---|
| 0.0 | 43 |
| 0.1 | 46 |
| 0.5 | 57 |
| 1.0 | 60 |
| 1.5 | 54 |
| 2.0 | 43 |

*BCA II refolding in the presence of sucrose at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

Effect of Trehalose

Among various sugars, trehalose was found to be the most efficient stabilizer which can provide protection to numerous biological macromolecules against dehydration and desiccation (**Sampedro *et al.*, 1998; Sun and Davidson, 1998**). Trehalose acts as an exceptional stabilizer against thermal inactivation and it provides thermal stability to many proteins (**Sola-Penna and Fernandes, 1998; Kaushik and Bhat, 2003**). In the present studies effect of trehalose was further explored in the refolding of GdmCl denatured BCA II. The refolding results of BCA II in the presence of trehalose were found to contradict its stabilization effect. It was observed that there was a continuous decrease in the refolding yield of BCA II with increase in trehalose concentration. At 1.5 M trehalose there was nearly 28% reduction in the refolding of denatured BCA II with respect to control (where no trehalose was used) as the percentage refolding yield was only 9%. Concentrations higher than 1.5 M could not be explored due to the limited solubility of trehalose at higher concentration (Table 3).

**Table 3: Effect of trehalose on the refolding yield of BCA II at 25°C**

| Trehalose (M) | Percentage refolding yield |
|---|---|
| 0.0 | 37 |
| 0.1 | 31 |
| 0.5 | 21 |
| 1.0 | 13 |
| 1.5 | 9 |

*BCA II refolding in the presence of trehalose at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was: 0.5 mg/ml BCA II and 0.25 M GdmCl.*

**Comparison of the refolding yield of the denatured BCA II in the presence of different sugars**

Due to the exceptional ability of sugars to stabilize numerous proteins against thermal inactivation, in the present study, their role was explored in the refolding of BCA II system. BCA II was denatured in 5 M GdmCl and refolding was done in the presence of different sugars- glucose, sucrose and trehalose by gradually increasing their concentration. At 1 M concentration, sucrose was the most effective among the three sugars and it enhanced the refolding yield upto 60% as compared to the control (refolded samples without using any sucrose) where the refolding yield was only 37%, showing an effective 22% increase (Table 4). Sucrose was closely followed by glucose which at 1 M concentration shows 49% refolding yield, which was a 12% increase from the control (Table 4). In case of glucose and sucrose, further increase in the concentration, lead to a gradual decrease in the refolding yield. On the other hand, trehalose was found to decrease the refolding yield at all the concentrations. There was a drastic decrease of 28% in the refolding yield of denatured BCA II at 1.5 M trehalose as compared to control (Table 4). A comparative representation of the effect of different sugars on the refolding yield of denatured BCA II is shown in figure 1.

**Table 4: Refolding yield of BCA II in the presence of different sugars at 25°C, pH 7.5**

| Concentration of sugars (M) | % Refolding yield | | |
|---|---|---|---|
| | Glucose | Sucrose | Trehalose |
| 0.0 | 37 | 43 | 37 |
| 0.1 | 39 | 46 | 31 |
| 0.5 | 47 | 57 | 21 |
| 1.0 | 49 | 60 | 13 |
| 1.5 | - | 54 | 9 |
| 2.0 | 50 | 43 | - |
| 3.0 | 46 | - | - |

*Percentage refolding yield of BCA II in the absence and presence of different sugars at 25°C in 20 mM Tris-$SO_4$ buffer at pH 7.5. Final refolding condition was 0.5 mg/ml BCA II and 0.25 M GdmCl.*
*The data are averages of at least three independent observations with a maximum percentage error of ± 5%.*

A comparative representation of the effect of different sugars on the refolding yield of BCA II is shown in Fig. 1.

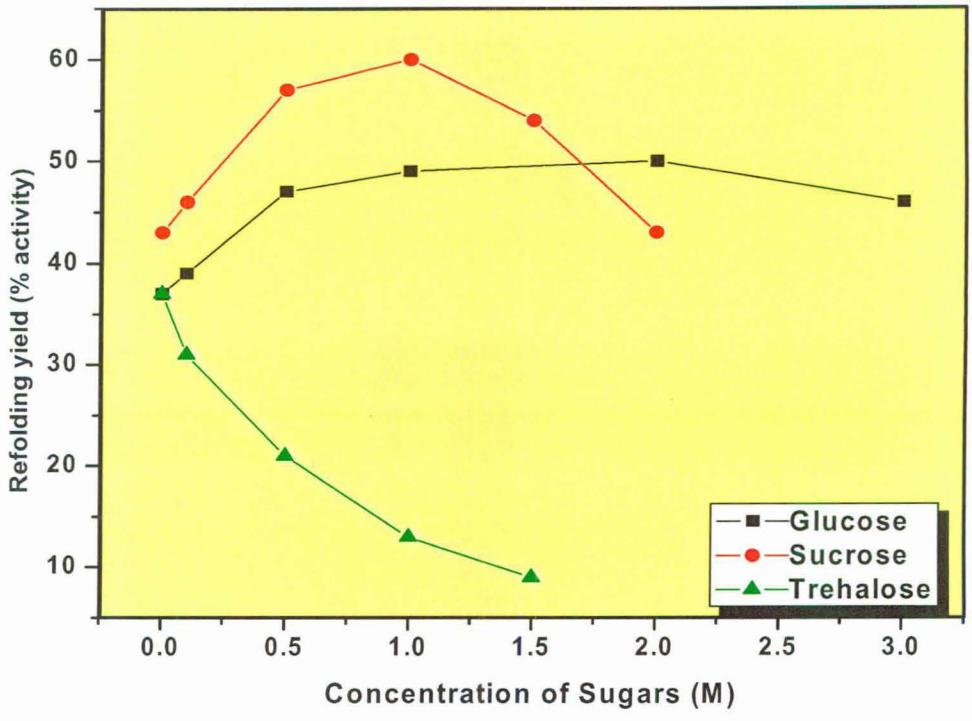



***Fig. 1:*** *Graphical representation of the effect of different concentration of sugars on the refolding yield of BCA II.*

**Effect of sugars on the residual protein concentration of the refolded samples of BCA II**

When the protein is rapidly diluted in the refolding buffer at high protein and low denaturant (GdmCl) concentration it forms aggregates which are visible by the naked eye in the form of white aggregates. Sugars like glucose and sucrose were found effective in reducing the aggregation induced during refolding step whereas trehalose was found to increase the extent of aggregation during folding process. The residual protein concentration in the refolded samples at 280 nm, during spontaneous refolding, without using any sugar, was nearly 0.2 mg/ml. In case of glucose while lower concentrations were ineffective in preventing the folding induced aggregation, residual concentration of BCA II was found to increase at 2 and 3 M glucose. Sucrose, on the other hand was able to increase the residual protein concentration in the refolded samples at all the

concentrations except for 2 M where there was a slight decrease. Contrary to the effect shown by the above two sugars, trehalose was found to promote the aggregation during folding process. Residual concentrations of BCA II in the refolded samples were found to decrease with increase in the concentration of trehalose and it became drastically low (0.002 mg/ml) at 1.5 M. Thus during refolding process, it was found that sucrose was the most effective sugar in retaining the residual concentration of BCA II in the refolded samples by minimizing aggregation. At 1 M sucrose concentration, residual protein concentration was 0.38 mg/ml which was highest among all the three sugars. These results paralleled the effect of sugars on the refolding yield observed in Table 1 and suggest that decrease in the protein folding yield is prominently due the formation of many non native, aggregated species during the folding pathway (Table 5), (Fig. 2).

**Table 5: Residual concentration of BCA II in the refolded samples in presence of different sugars at 25°C, pH 7.5**

| Sugar conc. (M) | Residual BCA II concentration in the presence of sugars (mg/ml) | | |
|---|---|---|---|
| | Glucose | Sucrose | Trehalose |
| 0.0 | 0.21 | 0.22 | 0.20 |
| 0.1 | 0.21 | 0.26 | 0.11 |
| 0.5 | 0.24 | 0.32 | 0.07 |
| 1.0 | 0.27 | 0.38 | 0.025 |
| 1.5 | - | - | 0.002 |
| 2.0 | 0.26 | 0.35 | - |
| 3.0 | 0.29 | - | - |

A comparative representation of the effect of different sugars on the residual concentration of BCA II is shown in figure 2.

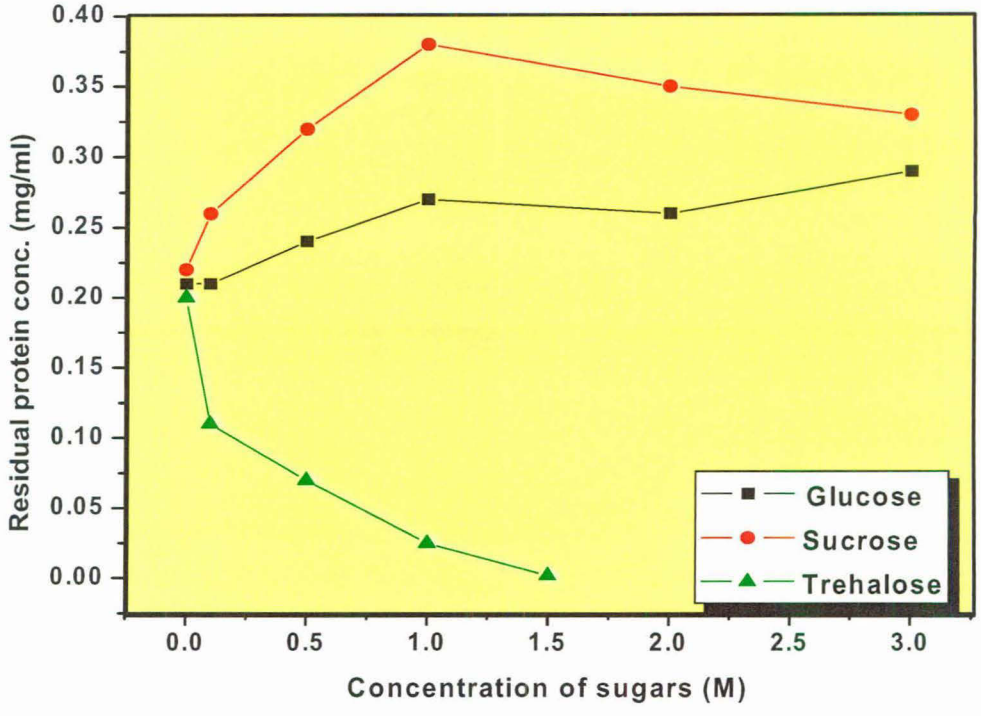
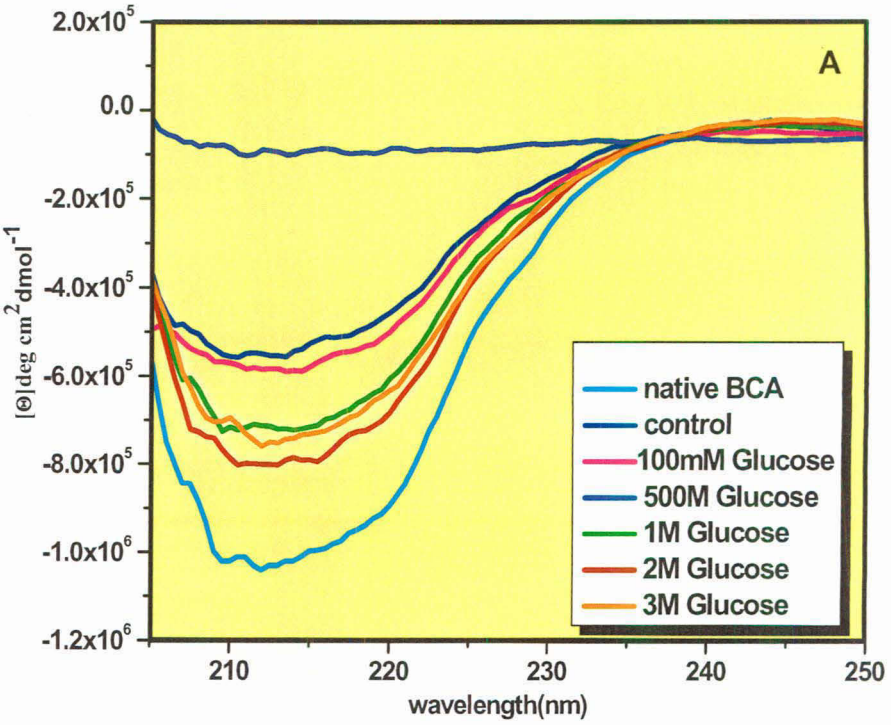


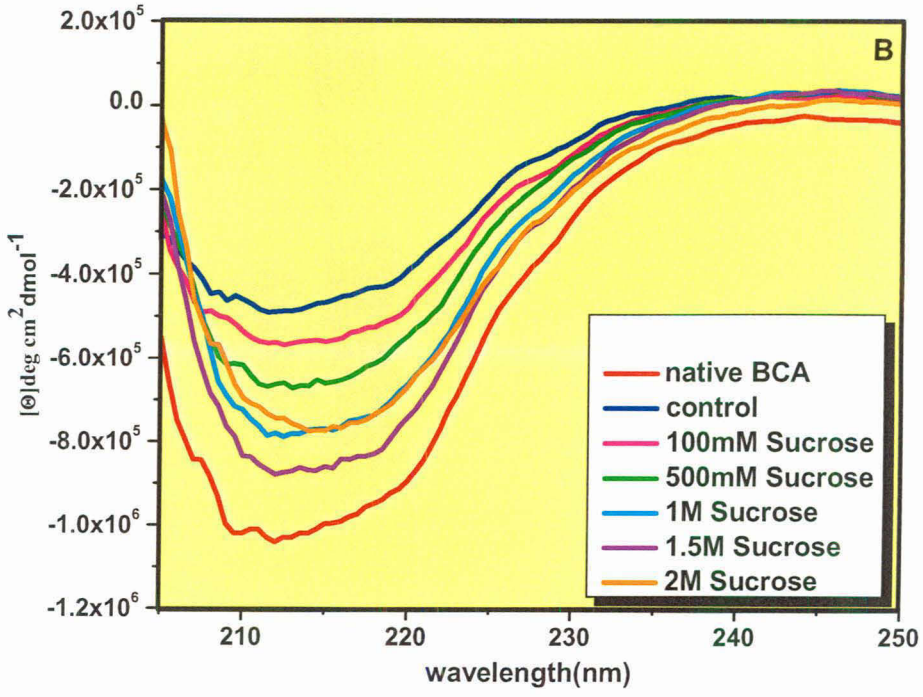

*Fig. 2: Graphical representation of the effect of sugars on the residual concentration of BCA II in the refolded samples.*

**Effect of sugars on the secondary structure of the refolded samples of BCA II**

To explore the effect of sugars on the conformational state of BCA II during refolding, circular dichroism studies were carried out using CD spectroscopy. Far-UV CD spectra were recorded from 200 nm to 250 nm to see the effect of sugars on the secondary structure of the refolded samples. The native BCA II is known to constitute primarily of beta sheets (**Kannan *et al.*, 1972; Liljas *et al.*, 1972**). The spectrum of native BCA II gives negative ellipticity at nearly 215 nm indicating substantial amount of beta sheet structure. A far-UV CD spectrum of control samples (without any sugar) was found to have decrease value of negative ellipticity showing considerable loss in the secondary structure (Fig. 3A, B and C). During refolding of denatured BCA II in the presence of

glucose it was observed that lower concentration of glucose did not affect the secondary structure much and the value of negative ellipticity of the peak (215 nm) remained closed to the control, however, glucose at higher concentration was found to retain the secondary structure of the refolded samples much more efficiently. The negative ellipticity of the peak (215 nm) in the refolded samples decreased considerably at 2 M glucose and the far-UV CD spectrum gets closed to the native BCA II (Fig. 3A). In case of sucrose, it was observed that 2 M sucrose was not only able to enhance the refolding of denatured BCA II (Table 1) but was also very effective in retaining the secondary structure of the refolded samples. Far-UV CD spectrum at 2 M sucrose reached near to the native BCA II which showed that sucrose could prevent the structural perturbation caused by the denaturant (Fig. 3B). Trehalose, the next member of the series and a very good protein stabilizer was found to behave in a completely antagonistic manner as compared to glucose and sucrose. Far-UV CD spectrum in the presence of trehalose showed considerable perturbation in the structure of refolded BCA II and there was a complete loss of ellipticity at all the concentrations of trehalose (Fig. 3C). These results paralleled the refolding data where trehalose showed considerable decrease in the refolding yield (Table 1).


A
2.0x10^5
0.0
-2.0x10^5
-4.0x10^5
-6.0x10^5
-8.0x10^5
-1.0x10^6
-1.2x10^6
[Θ]deg cm^2dmol^-1
210
220
230
240
250
wavelength(nm)
native BCA
control
100mM Glucose
500M Glucose
1M Glucose
2M Glucose
3M Glucose


B
2.0x10^5
0.0
-2.0x10^5
-4.0x10^5
-6.0x10^5
-8.0x10^5
-1.0x10^6
-1.2x10^6
[Θ]deg cm^2dmol^-1
210
220
230
240
250
wavelength(nm)
native BCA
control
100mM Sucrose
500mM Sucrose
1M Sucrose
1.5M Sucrose
2M Sucrose

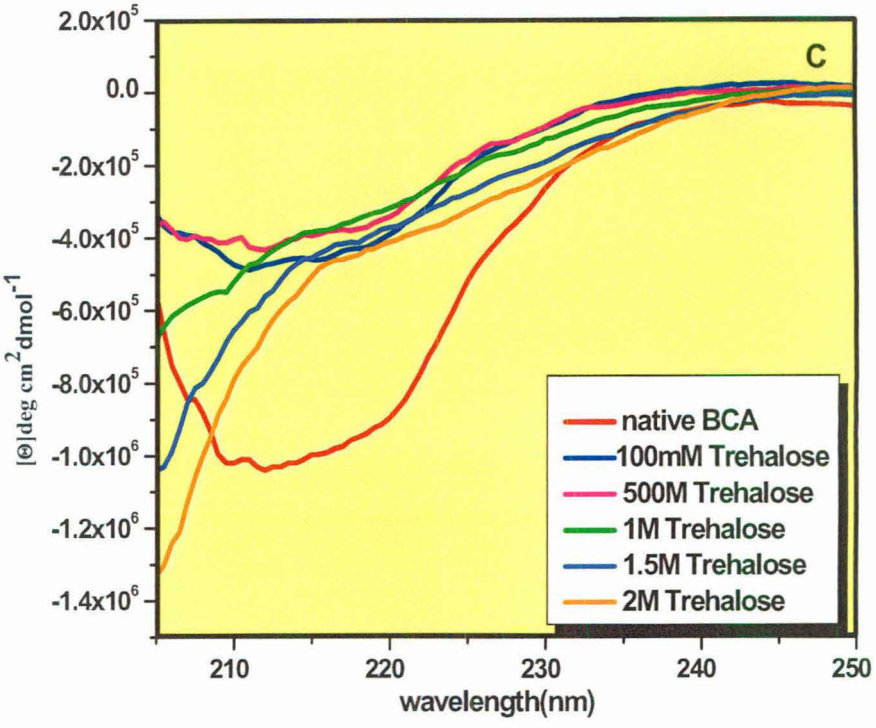
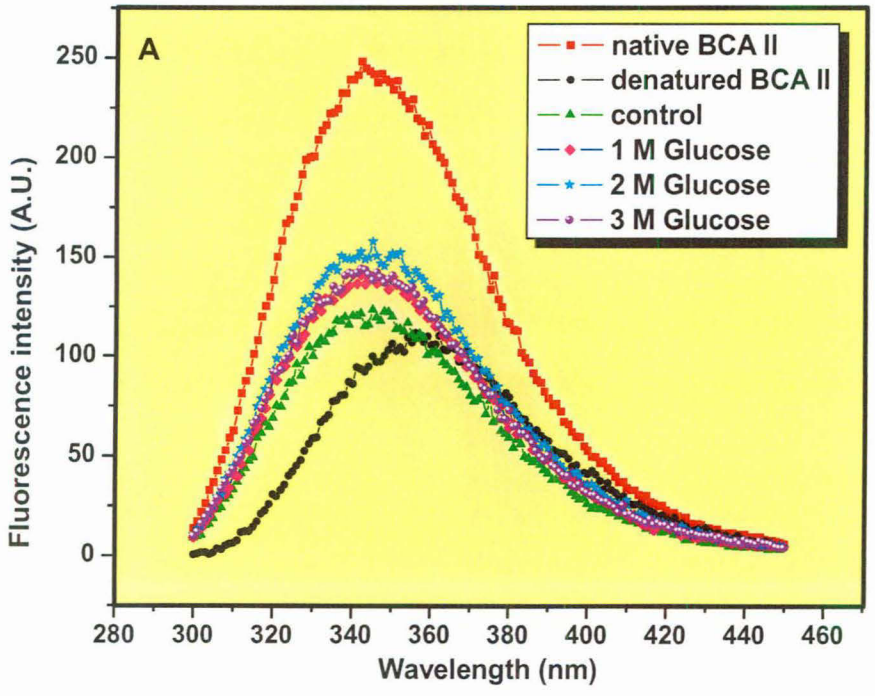



*Fig. 3: Far-UV CD spectra of the refolded samples of BCA II in the presence of A) Glucose, B) Sucrose, C) Trehalose. Native BCA II spectra (in buffer alone) and refolded BCA II spectra in the absence of polyols (control) is also included in all the panels.*

**Effect of sugars on the tryptophan emission spectra of the refolded samples of BCA II**

Intrinsic fluorescence emission spectroscopy can provide information about the tryptophan and tyrosine in terms of polar and non polar environment, as well as the solvent accessesibilty of tryptophan that are sensitive to local conformational changes at the tertiary structure level (**Lakowicz, 2006**). Intrinsic emission spectra for all the refolded samples in the presence of sugars were measured to observe their effect on the local changes in the tryptophan environment. All the refolded samples, with and without sugars, were excited at 295 nm and the emission spectra was obtained in the range of 300 to 500 nm (Fig. 4A, B and C). Native BCA II gives an emission peak at 342 nm. When the protein is denatured by using 5 M GdmCl there was a sharp decrease in the fluorescence intensity followed by a significant red shift as compared to the native peak. During spontaneous refolding of the protein (without using any sugar) an increase in the

fluorescence intensity along with a blue shift was observed. Fluorescence intensity of the refolded samples was found to increase gradually with increase in the concentration of both glucose and sucrose, and 2 M concentration of both the sugars increased the fluorescence intensity to its maximum. The increase in the fluorescence intensity of the refolded samples in the presence of these two sugars was also accompanied by the blue shift in the emission spectra (Fig. 4A and B). Trehalose which decreased the refolding yield (Fig. 1) was also found to decrease the emission intensity in the refolded samples at all the concentrations. There was a significant red shift in the refolded samples at all the concentrations of trehalose as compared to control and there was decrease in the sharpness of the peak with increased concentration of trehalose (Fig. 4C).



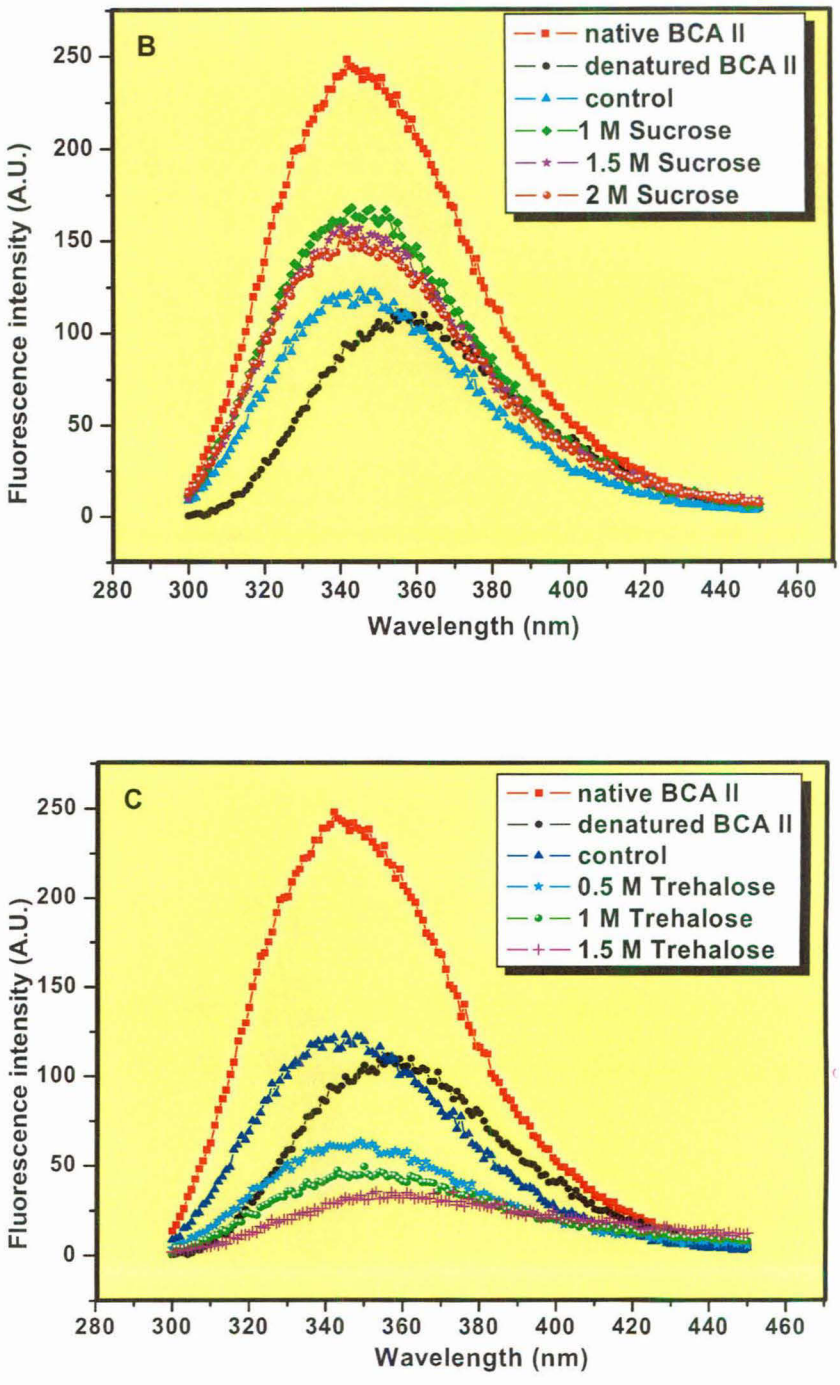
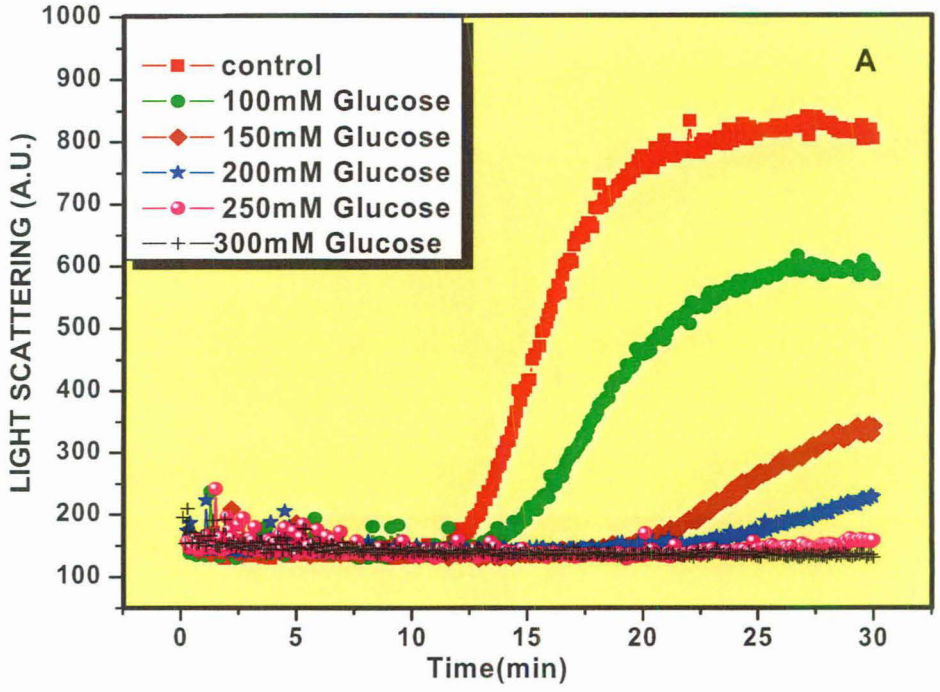


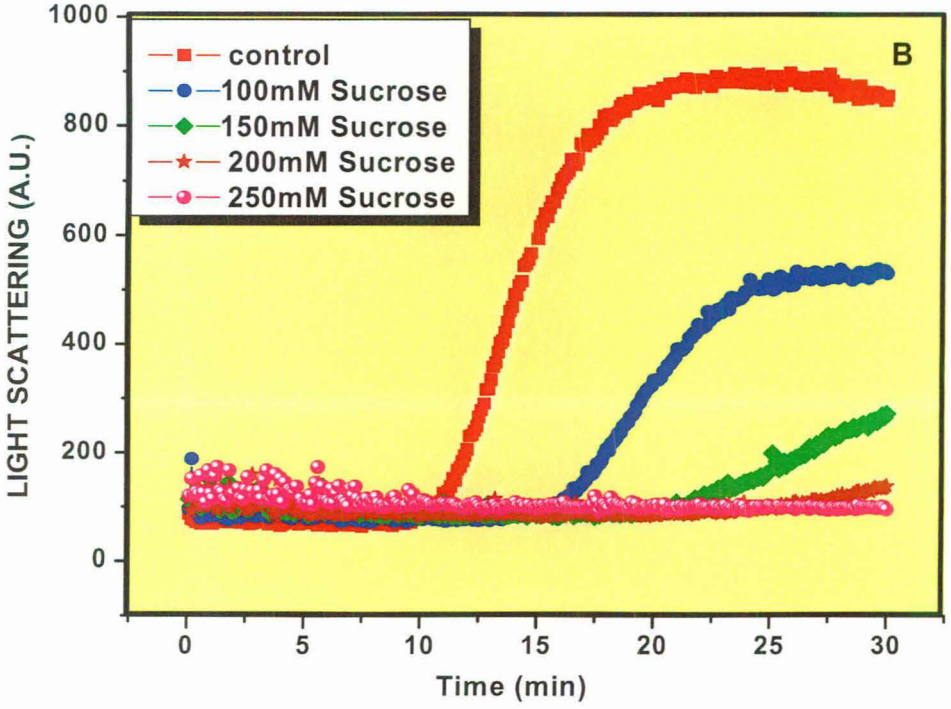

***Fig. 4:*** *Typtophan emission spectra of refolded BCA II in the presence of A) Glucose, B) Sucrose, C) Trehalose. Native BCA II spectra (in buffer alone), denatured BCA II spectra and the refolded BCA II spectra in the absence of sugars (control) have also been included in the figures.*

**Sugars prevent the aggregation of native BCA II at 65°C**

Refolding experiments clearly demonstrated that sugars, like glucose and sucrose were able to enhance the refolding yield of the denatured BCA II as well as prevented the aggregation induced during the refolding procedure, when the concentration of protein was very high and that of the denaturant very low (Fig. 1 and 2). Thus the effect of sugars was further explored in preventing the aggregation of native BCA II that could be induced due to the incubation of protein at high temperature. To study the aggregation kinetics of native BCA II, light scattering experiment were carried out on spectrofluorimeter. In this experiment when the protein is heated at high temperature (temperature above than $T_m$) it rapidly forms aggregates and the size of the aggregate is directly proportional to the light scattered by the solution. Thus when native BCA II was incubated at 65°C, the intensity of the light started increasing within 10 minutes after incubation, showing the formation of aggregated species. The intensity reached to an optimum value after which no further changes were observed following a typical sigmoidal curve. It was observed that on gradual increase in the concentration of all the sugars, onset of aggregation in the native BC II was delayed (Fig. 5A and B).

Among all the three sugars, sucrose was found to be the most effective which completely inhibited the aggregate formation at concentration as low as 250 mM (Fig. 5B). Effect of sucrose was closely followed by glucose, which was able to completely suppress the aggregation at 300 mM concentration (Fig. 5A). Contrary to the effect shown by the above two sugars, trehalose showed some anomalous behavior. It was observed that trehalose prolonged the onset of aggregation at higher temperature (65°C). Aggregation of native BCA II usually starts within 10 minutes of incubation at 65°C but in the presence of trehalose this time was further increased. At 300 mM concentration of trehalose the onset of aggregation could be delayed upto 25 minutes, however, there was a constant increase in the intensity of scattered light with increase in trehalose concentration (Fig. 5C).


A
control
100mM Glucose
150mM Glucose
200mM Glucose
250mM Glucose
300mM Glucose
LIGHT SCATTERING (A.U.)
Time(min)
1000
900
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30


B
control
100mM Sucrose
150mM Sucrose
200mM Sucrose
250mM Sucrose
LIGHT SCATTERING (A.U.)
Time (min)
1000
800
600
400
200
0
0
5
10
15
20
25
30

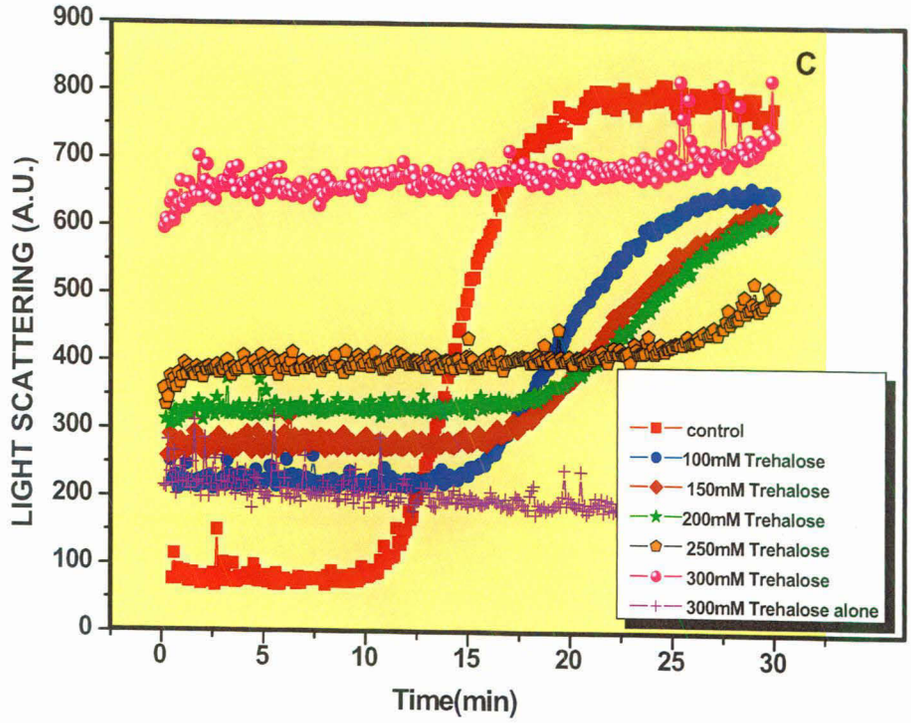
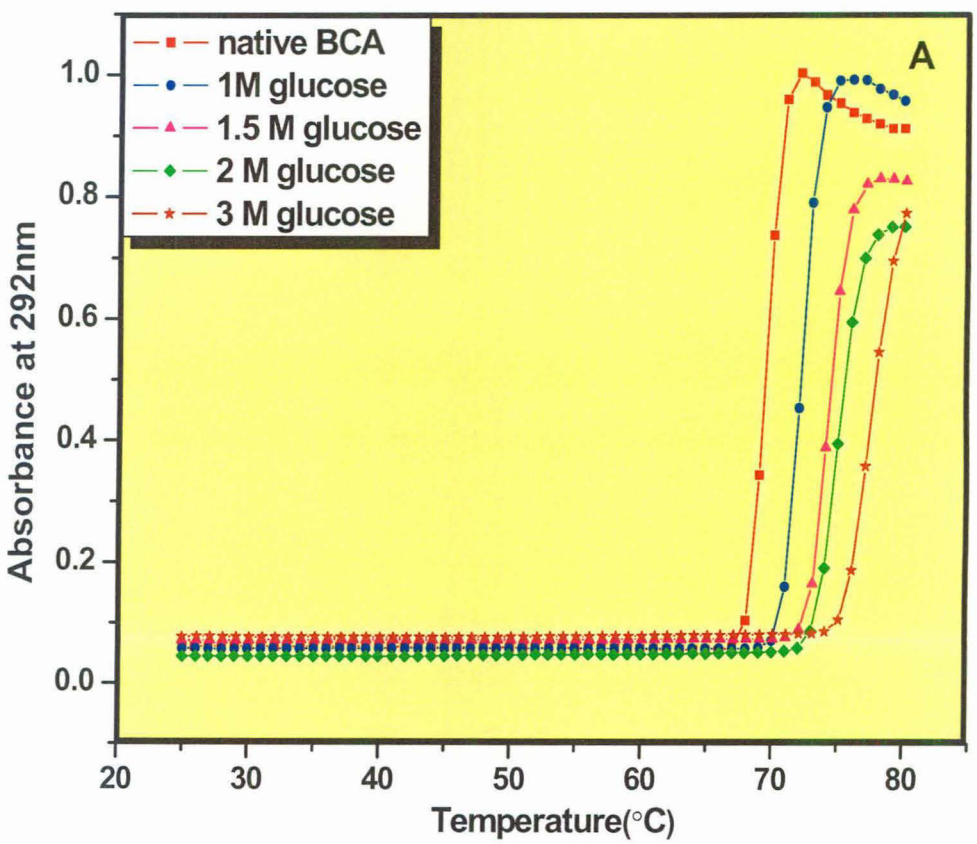



*Fig. 5: Aggregation of native BCA II monitored by light scattering in the absence and presence of different sugars at 65°C. A) Glucose, B) Sucrose, C) Trehalose.*

**Sugars affect the temperature onset of denaturation/aggregation of native BCA II**

The effect of sugars on the stability of the native BCA II was explored by thermal denaturation experiment. Since BCA II aggregates rapidly at high temperature therefore one could only determine the temperature of onset of denaturation/aggregation ($T_{agg}$) and not the actual $T_m$ value. Native BCA II has temperature onset of denaturation at nearly 68° C. Glucose and sucrose stabilized the native form of BCA II against thermal denaturation in a concentration dependent manner. By gradual increase in the concentration of glucose, $T_{agg}$ also increased. 3 M glucose increased the temperature onset of denaturation by almost 6 degrees (Fig. 6A). Sucrose was observed to be more efficient than glucose in stabilizing the native form of BCA II. Sucrose increased the $T_{agg}$ by 6 degree at 2 M concentration (Fig. 6B). Higher concentration of glucose and sucrose also decreased the aggregation of native BCA II which was evident by the decrease in the absorbance intensity at 292 nm in the post denaturation zone (68-80°C). Trehalose is the

most studied sugar due to its exceptional stabilizing effect. During thermal denaturation of native BCA II trehalose was found to destabilize the protein as there was a decrease in the $T_{agg}$ at higher concentration of trehalose. There was nearly 2 degree decrease in the $T_{agg}$ at 1 M trehalose. Further increase in trehalose concentration (upto1.5 M) did not increase the temperature onset of denaturation as compared to the native BCA II and the $T_{agg}$ remained close to 68°C. It was observed that 1.5 M trehalose decreased the temperature induced aggregation which was evident by a sharp decrease in the absorption intensity in the post denaturation zone (Fig. 6C).



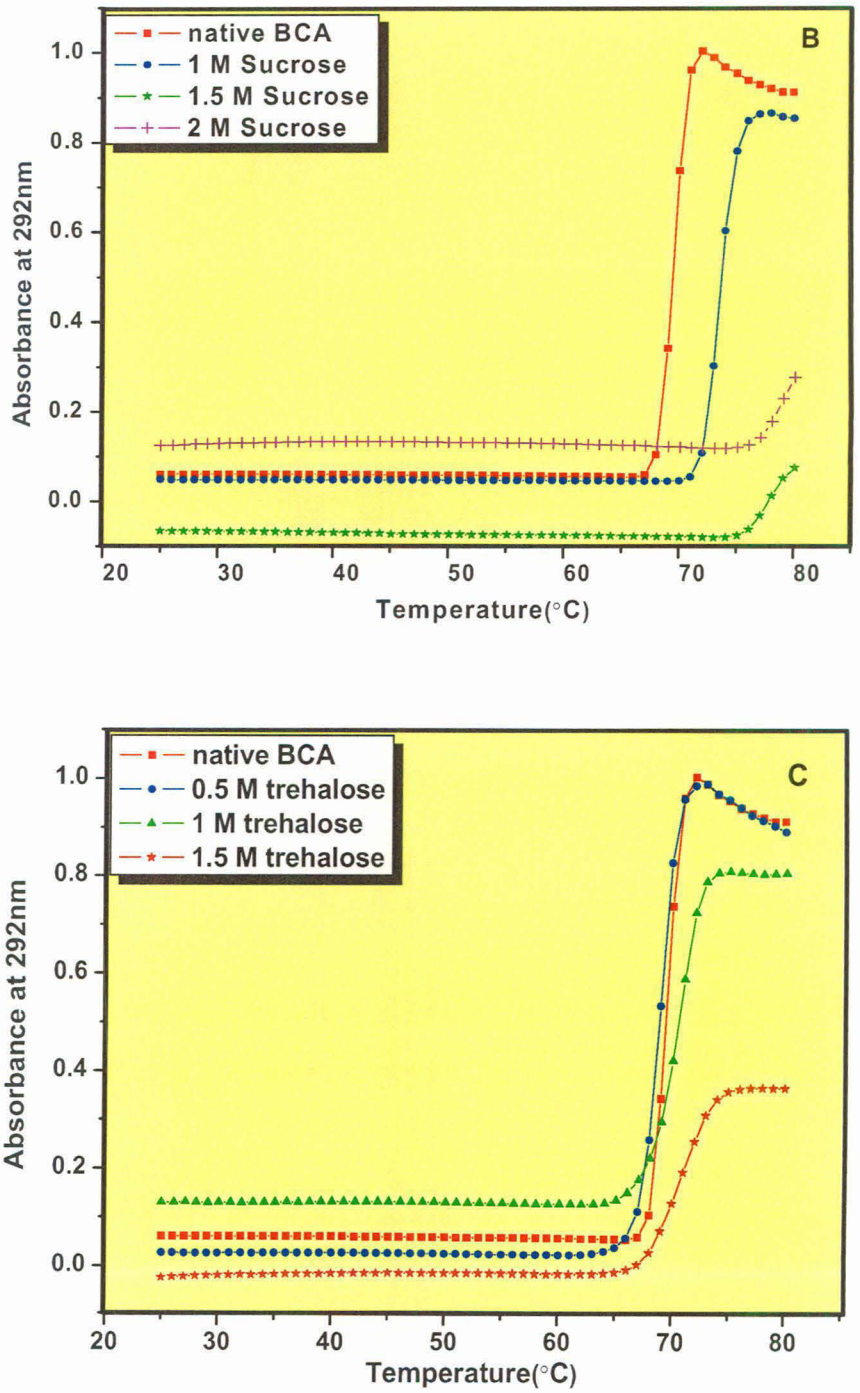



***Fig. 6:*** *Effect of sugars on the temperature onset of denaturation/aggregation of native BCA II. A) Glucose, B) Sucrose, C) Trehalose. Temperature onset of denaturation/aggregation of native BCA II (in the buffer alone) is also included in each panel.*

## Discussion

Bovine carbonic anhydrase (BCA II) is an aggregation prone system, whose refolding from its denatured state is dependent upon both protein and denaturant concentration (**Wetlaufer and Xie, 1995**). High protein and low denaturant (GdmCl), often results in a very low refolding yield (**Ikai *et al.*, 1978**) as large proportion of the protein becomes inactive due to aggregation. These aggregated species significantly reduce the yield of active protein. Refolding of BCA II has been well studied and is known to involve two intermediate species in the folding pathway (**Stein and Henkens, 1978**). The first intermediate has a molten globule like structure with hydrophobic regions on its surface (**Doligikh *et al.*, 1984; Semisotnov *et al.*, 1987; Rodionova *et al.*, 1989 Cleland and Wang, 1990a**). It was proposed that the first intermediate often self associates during refolding step and proceeds to form large aggregates. However, the other intermediate on the refolding pathway does not associate at equilibrium or during refolding (**Cleland and Wang, 1990b**).

Refolding data of BCA II (Table 1) in the presence of different sugars indicate that while lower concentrations of glucose and sucrose did not affect the refolding much, there was an appreciable increase in the refolding yield at higher concentration of both the sugars. On the other hand, trehalose which is known to be an exceptionally good protein stabilizer (**Kaushik and Bhat, 2003**) against thermal inactivation was found to decrease the refolding yield significantly at higher concentration. Glucose is one of the well studied sugars which provide thermal stabilization to many proteins (**Back *et al.*, 1979; Arakawa and Timasheff, 1982; Divsalar *et al.*, 2006; Ortbauer and Popp, 2008**). Glucose and sucrose have also been reported to act as a chemical chaperones in the refolding of proteins (**Kim *et al.*, 2006; Monterroso and Minton, 2007**). During BCA II refolding, 2 M glucose increased the refolding yield upto 50%, while sucrose was able to lead to similar refolding yield at 1 M concentration. As discussed, folding of BCA II polypeptide chain from its denatured state involves aggregation prone intermediate species. Interaction of glucose and sucrose with the first intermediate in the folding pathway could prevent the formation of non specific association of hydrophobic clusters present on the surface of intermediate species and thereby prevent the aggregation induced during folding pathway. This is very well demonstrated by glucose and sucrose

which were able to restore the enzymatic activity of BCA II against GdmCl denaturation and lead to enhanced refolding yield in a concentration dependent manner (Table 4, Fig. 2). There was also an increase in the residual protein concentration in the refolded samples in the presence of both glucose and sucrose indicating the inhibition of off-pathway aggregated species during refolding.

It was found that the addition of sugars to the acid unfolded state of cytochrome c give rise to species identical to the A state which is a collapsed conformation formed when salt is added to the unfolded sate. This species has characteristics similar to the molten globule (folding intermediate) whose stability increased both with the size and concentration of sugars (**Davis *et al.*, 1998**). Sugars were found to increase the steric repulsion between the solution components which favors the smaller 'A' state over larger unfolded state (**Davis *et al.*, 1998**). Similar effect was shown by trehalose during unfolding of cutinase enzyme where it favored a more compact off-pathway intermediate species as compared to less compact state because of its reduced surface area (**Melo *et al.*, 2003**). As discussed, folding pathway of BCA II involves the formation of off-pathway intermediate species which are prone to aggregate. It is well known that the formation of non native aggregated species decrease the refolding yield. Presence of trehalose as a cosolvent in the refolding buffer further decreased the refolding yield. In case of BCA II there was a considerable decrease in the refolding yield of denatured protein with increase in trehalose concentration. At 1.5 M trehalose percentage refolding yield of denatured BCA II was as low as 9% compared to control where it was 37%.

Refolding data was well supported by the residual protein concentration in the refolded samples which also showed significant decrease at higher concentrations of trehalsoe (Table 4 and 5). There is, thus a possibility that trehalose could stabilize more compact intermediates species formed during folding pathway as compared to the less compact unfolded species. The stabilization effect was found to be concentration dependent as it increased with trehalose concentration. Thus increased stability of intermediate species during folding of polypeptide chain could give rise to increase in intramolecular hydrophobic interactions. This self association would then lead to aggregate formation and thereby decreased folding yield.

Biological activity of a protein depends upon its intact three dimensional structure. Sugars like glucose and sucrose, which enhanced the refolding yield of denatured BCA II, by suppressing the aggregation (Fig. 1), were also found to protect the structural perturbation of polypeptide chain against the denaturant, by maintaining the integrity of the protein conformation close to its native state as observed by CD measurements (Fig. 3A, B and C). The far-UV CD spectrum characterizes the secondary structure of proteins due to the peptide bond absorption (**Kelly and Price, 1997**). Native BCA II, being predominantly beta pleated sheet protein, gives characteristic negative ellipticity at 215nm in the far-UV CD region. During spontaneous folding of BCA II without using any sugar, the intensity of the negative ellipticity decreased indicating the loss in the secondary structure of the refolded samples. While lower concentrations of glucose and sucrose were not able to bring considerable change in the secondary structure, higher concentrations (2 M glucose and 1.5 M sucrose) showed substantial increase in the negative ellipticity indicating the gain in secondary structure and their far-UV CD spectra was found close to that of the native BCA II. Contrary to the effect shown by the above two sugars (glucose and sucrose) in retaining the secondary structure of the refolded samples, trehalose effect was different. Trehalose at all the concentration was found to perturb the structure of BCA II during its refolding. As discussed above, folding pathway of BCA II involves intermediate species which are highly prone to aggregation. Stabilization of these intermediate species by trehalose molecules could favor non productive folding which result in aggregate formation. This effect was well supported by the far-UV CD spectra of the refolded samples in the presence of trehalose which showed the complete loss of secondary structure at all trehalose concentration (Fig. 3C).

BCA II contains seven tryptophan residues in the polypeptide chain. It has one exposed Trp residue in its native state (**Liljas *et al.*, 1972**) and six exposed Trp residues in the first intermediate state (**Jonasson *et al.*, 1997**). Changes in the intrinsic Trp fluorescence of protein are highly sensitive to the change in its immediate environment, and could be used as a tool to monitor the effect of different sugars on the refolding of GdmCl denatured BCA II. Tryptophan emission of native BCA II shows emission maxima at 342 nm. The usual red shift in the emission peak of denatured BCA II occurs

due the change in the tryptophan environment from non polar to polar. The solvent mediated quenching, resulting from the tryptophan exposure upon unfolding, led to an overall decrease in the intensity of emission (Fig. 4A, B and C). Refolding of denatured BCA II, in the presence of glucose and sucrose showed a blue shift in the emission spectra, as compared to the denatured state and it became more prominent at higher concentrations indicating the restructuring of the polypeptide chain to attain the native conformation. Refolded samples in the presence of trehalose on the other hand showed decrease in the emission intensity at all the concentrations. Stabilization of the intermediate states by trehalose during refolding might have resulted in the formation of aggregated species due to which there was a drastic decrease in the residual protein concentration in the refolded samples (Fig. 2). This result is further supported by the decreased fluorescence intensity of the emission spectra. Change in the tryptophan environment of the intermediate species from polar to non polar may cause gradual red shift in the refolded samples as the concentration of trehalose is further increased (Fig. 4C).

When a globular protein is incubated at high temperatures, its hydrophobic surfaces get exposed, making it prone to aggregation due to intramolecular association of the protein molecules. In the case of BCA II, hydrophobic compounds such as ANS have been used to prevent the aggregation of the protein at elevated temperature (**Kundu and Guptasarma, 1999**). It is proposed that sugars known to be preferentially excluded from globular protein could bind to the exposed hydrophobic patches of BCA II molecules at higher temperature and thereby suppress the aggregation by preventing the self association of protein molecules. Both glucose and sucrose were effective in preventing the aggregation of native BCA II during incubation at higher temperature, sucrose being more effective than glucose (Fig. 5A and B). Trehalose on the other hand was able to delay the onset of aggregation, but at the same time it increased the intensity of scattered light in a concentration dependent manner (Fig. 5C). This could occur due to some non specific interaction of protein molecules with trehalose and the formation of some higher order oligomeric species that could scatter light (Fig. 5C).

Thermal denaturation experiments revealed that glucose and sucrose besides being a good folding aid also increased the temperature onset of denaturation and

inhibited the aggregation of native BCA II at high temperatures during the denaturation process (Fig. 6A and B). The protein structure stabilization mechanism of glucose against thermal inactivation has been well established (**Back *et al.*, 1979; Arakawa and Timasheff, 1982; Timasheff, 1993**). Back *et al.*, in 1979 proposed that sugars like glucose and sucrose stabilize proteins against thermal denaturation due to their effect on the structure of water molecule which in turn determine the strength of hydrophobic interactions (**Back *et al.*, 1979).** Equilibrium dialysis experiment done by Timasheff and workers demonstrated that in a three component system of protein, glucose and water, there are present proportionally more water molecules and less glucose molecules on the surface of protein than in the bulk (**Arakawa and Timasheff, 1982 b**). Addition of sugar to a protein solution results in the net increase in the free energy that is proportional to the amount of protein surface area that must be hydrated preferentially. Since the unfolded state has greater surface area exposed than that of the folded state, therefore increase in the chemical potential is more for the denatured state which is therefore more destabilized as compared to the native state (**Arakawa and Timasheff, 1982 b**). The reason for the preferential exclusion could also be attributed to the unfavorable interactions taking place between the side chain of the polypeptide backbone and the osmolyte (**Liu and Bolen, 1995; Wang and Bolen, 1997; Bolen and Baskakov, 2001**). Sucrose at high concentrations was found to stabilize the brain tubulin in solution (**Frigon and Lee, 1972**). The stabilization property of sucrose was attributed to the lessened hydrogen bond rupturing capacity of the medium (**Gerlsma, 1968**). Lee and Timasheff proposed a general mechanism for the sucrose mediated stability of proteins against thermal denaturation (**Lee and Timasheff, 1981**). Addition of sucrose to the protein-solvent system increases the free energy of the system as well as surface tension of the water. These events led to the exclusion of the sucrose molecules from protein domain thereby leading to the stabilization of its native state (**Lee and Timasheff, 1981).** Sucrose has been recently reported to favor the conversion of unfolded state into more compact structure when the unfolded state was exposed to the native conditions (**Chen *et al.*, 2006**).

Thermal denaturation data revealed that trehalose did not affect the temperature onset of aggregation much and even at higher trehalose concentration (1.5 M) apparent

temperature onset of denaturation/aggregation was almost close to that of the native BCA II. However, there was a considerable decrease in the aggregation of native BCA II which was evident by the decrease in absorption intensity in the post denaturation zone (Fig. 6C). Trehalose has been found to play some important role in the survival of many plants and insects in harsh environmental conditions (**Arguelles, 2000; Benaroudj *et al.*, 2001; Schiraldi *et al.*, 2002; Ganea and Harding, 2005**). Trehalose is chemically uncreative and shows strong stability due to very low energy of the glycoside oxygen bond joining the two hexose rings. Trehalose has been reported as a very good stabilizer of proteins against thermal inactivation as well as inactivation caused by denaturant such as GdmCl (**Sola-Penna *et al.*, 1997; Sola-Penna and Fernandes, 1998; Kaushik and Bhat, 2003; Zancan and Sola-Penna, 2005**). The stabilization mechanism of trehalose against thermal inactivation of proteins has been well explained. Timasheff and coworkers proposed the detailed mechanism of protein (RNase A) stabilization by trehalose on the basis of preferential hydration theory. It was found that during protein unfolding there was an increase preferential exclusion of trehalose from the immediate surroundings of protein molecule which was more for the denatured state than the native state. This in turn destabilizes the unfolded state and stabilizes the native state (**Xie and Timasheff, 1997b**). Although the unfavorable interaction between peptide backbone and trehalose could help in the protein stability, the stabilization effect of trehalose could also be attributed to the change in the structure and properties of solvent water (**Kaushik and Bhat, 2003**).

Taken together the above data indicate that sugars like glucose and sucrose act as an efficient protein folding aid and at the same time they also help in preventing protein aggregation induced either by incubation at higher temperatures or during its folding. However, stabilization of the folding intermediates considerably decreased the refolding yield in the presence of trehalose, which is otherwise known to be an excellent stabilizer of proteins against thermal inactivation. At the same time all the sugars were found to be effective in their effect of protecting the protein against thermal denaturation. The results suggest that the effect of sugars on the stability of a protein may not always go parallel with their effect on refolding of the protein. As suggested earlier for the polyol effect on the protein stability and refolding, stabilizing sugars also do not necessarily lead to the

enhanced folding yield. This effect is much dependent upon the chemical nature of the sugar as well as its concentration. A subtle balance between the preferential exclusion and binding effects with the native state, the unfolded state, and the intermediate states together control the trajectory of protein folding either towards on-pathway direction or towards the stabilization of off-pathway species which are prone to aggregation.

# EFFECT OF GLYCINE AND ITS METHYL DERIVATIVES ON THE REFOLDING AND AGGREGATION OF BCA II

## Introduction

In nature, different organisms have developed elaborate strategies to cope up with the adverse environmental conditions such as extremes of temperature, pressure, desiccation, pH, salinity, etc. (**Popp *et al.*, 1997; Fields, 2001; Hoekstra *et al.*, 2001; Santos and Costa, 2002**). One such strategy is the accumulation of small organic compounds inside the cell which helps in preserving the stability of cellular proteins as well as maintaining the osmotic pressure of the cell (**Yancey and Somero, 1979; Yancey *et al.*, 1982; Sola-Penna *et al.*, 1995, Record and Courtenay, 1998; Yancey, 2005; Zancan *et al.*, 2007, Lambert and Draper, 2007; Burg and Ferraris, 2008**). Beside this, osmolytes are also known to play an important role in protein misfolding and aggregation. However, the mechanism of action of osmolytes could be different for different proteins because they are reported to not only inhibit (**Kim *et al.*, 2001; Arora *et al.*, 2004; Ignatova and Gierasch, 2006**) but also promote protein aggregation (**Yang *et al.*, 1999; Uversky *et al.*, 2001; Scaramozzino *et al.*, 2006**).

The osmoregulatory compounds belong to the diverse group of chemicals namely polyhydric alcohols, free amino acids, amino acid derivatives and methyl amines (**Yancey *et al.*, 1982; Burg and Ferraris, 2007; Venkatesu *et al.*, 2007, 2009**). Studies performed by various groups (**Brown and Simpson, 1972; Borowitzka and Brown, 1974; and Yancey *et al.*, 1982**) have classified the above mentioned osmolytes into two groups of '*compatible*' and '*counteracting*' types depending upon their effect on the functional activity of proteins. Compatible osmolytes like polyols and amino acids like proline and glycine, etc., tend to stabilize and provide protection to protein structure against stresses such as extremes of temperature, high salt and dehydration (**Yancey *et al.*, 1982**), and stabilize proteins without affecting its functional activity (**Bowlus and Somero, 1979; Pollard and Wyn Jones, 1979; Karuppiah and Sharma, 1995; Wang and Bolen, 1996; Neelon *et al.*, 2005; Kolp *et al.*, 2006**). On the other hand, counteracting osmolytes e.g. methylamines such as trimethylamine-N-oxide (TMAO), betaine (glycine betaine) and sarcosine are those which have the ability to protect the intracellular proteins of organisms against the deleterious and denaturing effect of urea (**Bowlus and Somero, 1979, Yancey and Somero, 1979; Lin and Timasheff, 1994; Baskakov, *et al.*, 1998; Burg *et al.*, 1999; Palmar *et al.*, 2000; Zou *et al.*, 2002;**

**Holthauzen and Bolen, 2007; Venkatesu *et al.*, 2007, 2009**). Contrary to the compatible osmolytes which do not affect the functional activity of proteins, counteracting osmolytes could change the protein function that are opposite to the effect urea has on the protein function (**Somero, 1986**). There exist a number of examples of body organs and animals that have high urea concentration, e.g., mammalian kidney cells with betaine and glycerophosphocholine as counteracting osmolytes and cartilaginous fishes and coelacanth, which use trimethylamine-N-oxide (TMAO) as a counteracting osmolyte (**Yancey and Somero, 1980; Yancey, 1985; Bagnasco *et al.*, 1986; Garcia-Perez and Burg, 1990).**

Counteracting osmolytes, besides having thermo protective ability, also play important role in preventing protein misfolding and aggregation (**Bourot *et al.*, 2000; Kim *et al.*, 2001; Ou *et al.*, 2002; Russo *et al.*, 2003; Arora *et al.*, 2004; Ignatova and Gierasch, 2007**). Keeping above findings in mind, the amino acid series glycine and its methylamine derivatives sarcosine and betaine with 2 and 3 methyl groups respectively were chosen to explore their effect on the refolding of denatured BCA II. Besides, the role of the above mentioned amino acid series was also examined in preventing the aggregation of BCA II during the folding pathway or during incubation of protein at high temperature. The studies were further extended to elucidate the effect of these osmolytes on the stability of native BCA II during thermal denaturation.

Bovine carbonic anhydrase (BCA II) is a Zn containing protein composed of single polypeptide chain, and this system has been explained in detail in chapter 3. It has been observed that while glycine and its N-methyl derivatives were effective in preventing the aggregation of BCA II, both during chemical and thermal denaturation, they had either negligible effect (sarcosine) or adverse effect (glycine and betaine) on the folding yield of dentured BCA II. Glycine and betaine, being efficient protein structure stabilizers against thermal denaturation were observed to act as poor folding agents decreasing the folding yield significantly.

## Results

**Effect of glycine and its methyl derivatives on the refolding yield of BCA II**

BCA II was denatured in 5 M GdmCl and the solution was kept for 12-14 hours at 25°C. Refolding was done by rapid dilution method with vigorous vortexing in 20 mM Tris-$SO_4$ buffer at pH 7.5. To observe the effect of glycine and its methyl derivatives sarcosine and betaine in enhancing the refolding yield, they were introduced at a desired concentration in the refolding buffer prior to dilution. The refolded samples were then left for one hour at room temperature. Refolded samples were centrifuged to pellet down the aggregates and the supernatant was taken to measure the enzymatic activity. Refolding was measured by calculating the activity of all the refolded samples with and without cosolvent, using p-nitrophenyl acetate as a substrate as described in the Materials and Methods section in chapter 2. Percentage refolding yield was calculated by taking the ratio of refolded samples in presence of cosolvent to the native BCA II refolded in the buffer alone (i.e. without using any cosolvent) at the same concentration of GdmCl.

The effect of glycine, sarcosine and betaine on the refolding yield of denatured BCA II is summarized as below:

In the present study glycine was used in a concentration range of 0.1 M to 2 M. Concentrations higher than 2 M could not be used due to limited solubility of glycine. It was found that glycine at all the concentrations was decreasing the refolding yield of the denatured BCA II. Refolding yield decreased considerably (~30%) even at concentration of 100 mM. The refolding yield further decreased as the concentration of glycine increased (Table 1). Sarcosine is the N-methyl derivative of glycine and is known as a protein stabilizer against thermal denaturation (**Arakawa and Timasheff, 1985 b; Ibarra-Molero *et al.*, 2000**). In the present study sarcosine was used from 0.1 M to 3 M concentration. It was observed that the refolding yield was nearly unaffected upto 0.5 M sarcosine following which it showed a small decrease (Table 1). Betaine is a trimethyl derivative of glycine and is known to act as a counteracting osmolyte as well as a protein stabilizer **(Arakawa and Timasheff, 1983; Hand and Somero, 1982)**. In the present study betaine was used in a concentration range of 0.1 to 3 M. It was observed that upto 1 M concentrations of betaine there was no appreciable effect on the refolding yield after

which the yield decreased drastically with only 17% refolding yield obtained at 3 M betaine (Table 1).

**Table 1: Refolding yield of BCA II in the presence of glycine and its methyl derivatives sarcosine and betaine at 25°C, pH 7.5**

| Concentration of amino acids (M) | % Refolding yield | | |
|---|---|---|---|
| | Glycine | Sarcosine | Betaine |
| 0.0 | 43 | 47 | 45 |
| 0.1 | 29 | 50 | 50 |
| 0.5 | 17 | 42 | 44 |
| 1.0 | 20 | 34 | 40 |
| 1.5 | 19 | - | - |
| 2.0 | 21 | 31 | 25 |
| 3.0 | - | 35 | 17 |

*Percent refolding yields are measured in terms of percent activity of the refolded samples compared to the native control under identical conditions. Final refolding condition was 0.5 mg/ml BCA II and 0.25 M GdmCl.*

*The data are averages of at least three independent observations with a maximum percentage error of ± 5%.*

A comparative graphical representation of the data for glycine, sarcosine and betaine is shown in Fig. 1.

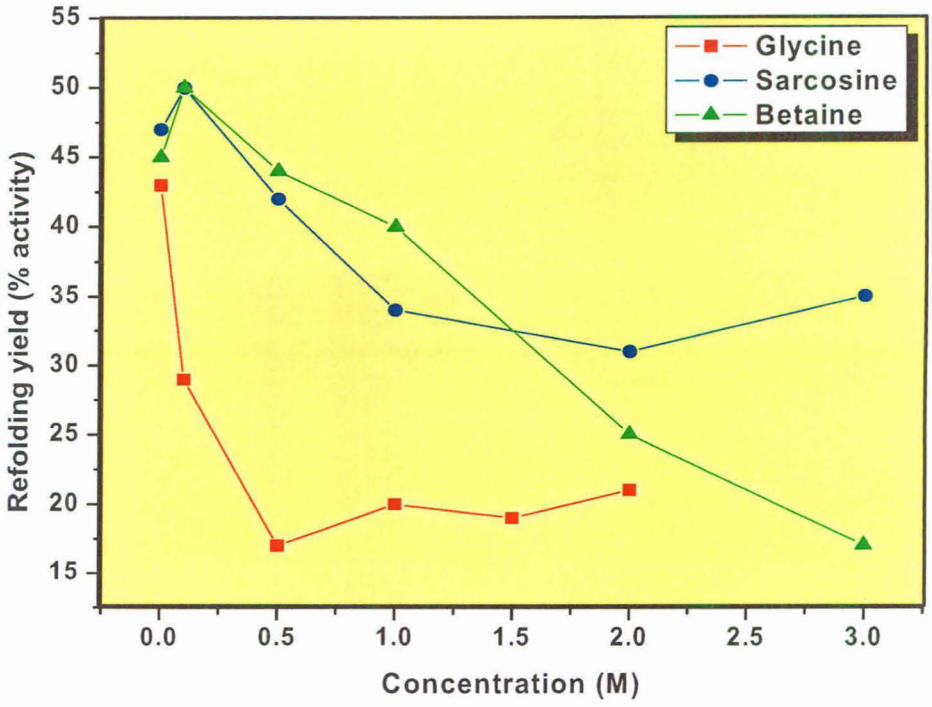



***Fig. 1:*** *Effect of glycine and its methyl derivatives on the refolding yield of BCA II at 25°C, pH 7.5.*

**Effect of glycine and its methyl derivatives on the residual protein concentration of the refolded samples of BCA II**

When protein is rapidly diluted in the refolding buffer at high protein and low denaturant (GdmCl) concentration, it forms aggregates which are visible by the naked eye in the form of white precipitates. The residual protein concentration measured after centrifugation of the refolded samples at 280 nm, during spontaneous refolding, without using glycine or its derivatives was ~0.2 mg/ml (Table 2). The residual protein concentration in the refolded samples decreased gradually in the presence of glycine and sarcosine in a concentration dependent manner. There was a drastic decrease in the residual protein concentration at 2 M glycine with a value of 0.02 mg/ml, the lowest in the series (Table 2). Among the three cosolvents used, betaine was found to be the most effective osmolyte in retaining the concentration of active protein in the refolded

samples. At 1 M concentration, betaine showed maximum residual protein concentration in the refolded samples which was nearly 0.17 mg/ml (Table 2, Fig. 2). However, on further increasing the betaine concentration there was a decrease in the residual protein content. These results paralleled the effect of glycine and its derivatives on the refolding yield presented in Table 1 and suggest that the decrease in the protein refolding yield is predominantly due to protein aggregation.

**Table 2: Residual concentration of BCA II in the refolded samples in presence of glycine and its methyl derivatives sarcosine and betaine at 25°C, pH 7.5**

| Concentration of amino acids (M) | Residual BCA II concentration (mg/ml) | | |
|---|---|---|---|
| | Glycine | Sarcosine | Betaine |
| 0.0 | 0.18 | 0.20 | 0.21 |
| 0.1 | 0.18 | 0.21 | 0.21 |
| 0.5 | 0.11 | 0.15 | 0.20 |
| 1.0 | 0.06 | 0.10 | 0.17 |
| 1.5 | 0.05 | - | - |
| 2.0 | 0.02 | 0.06 | 0.14 |
| 3.0 | - | 0.08 | 0.10 |

A comparative representation of the effect of glycine and its methyl derivatives on the residual protein concentration of BCA II is shown in Fig. 2.

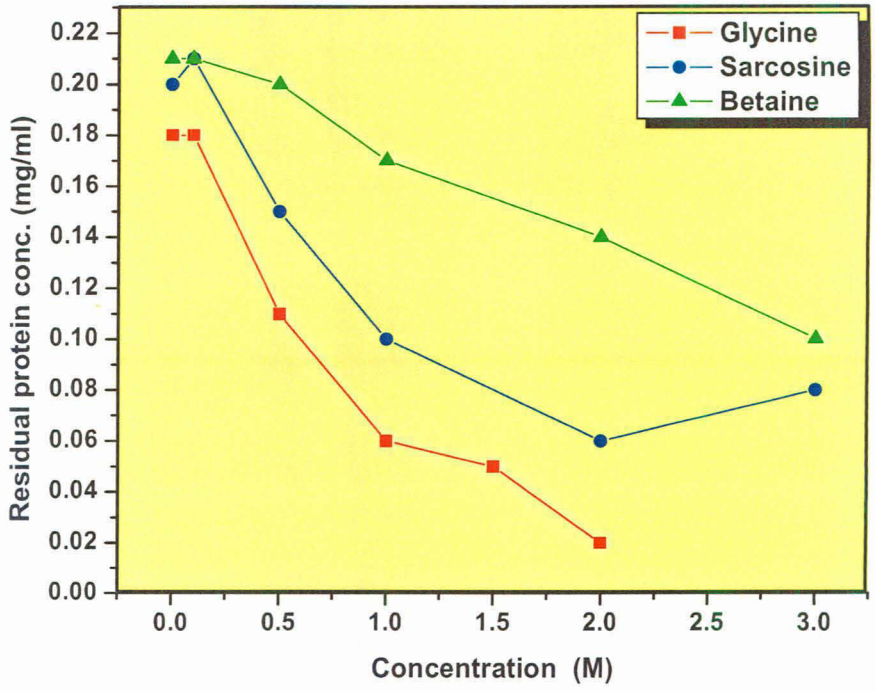
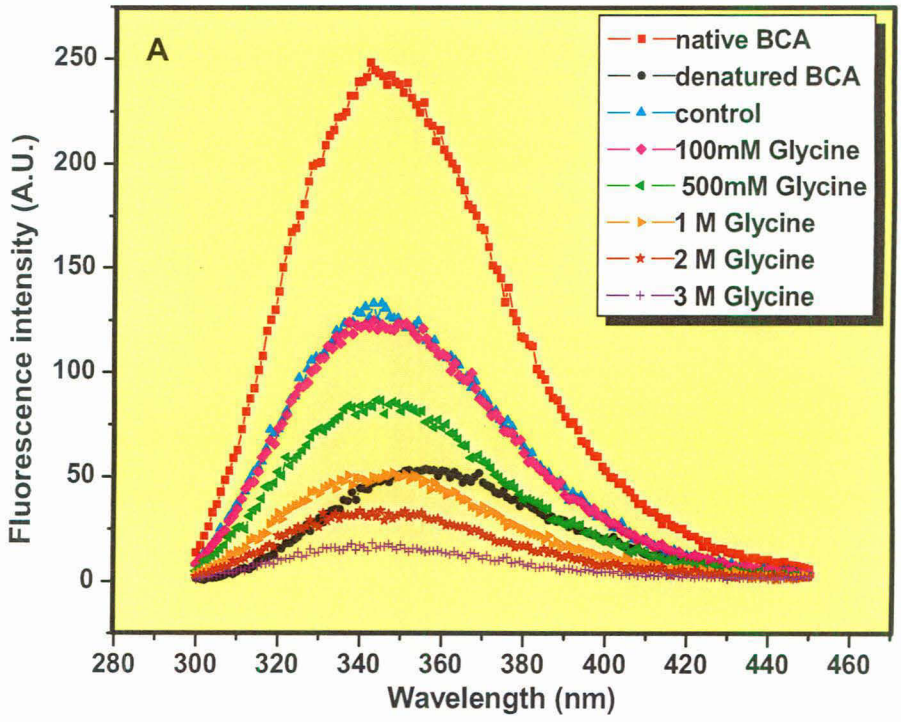



***Fig. 2:*** *Effect of glycine and its methyl derivatives on the residual concentration of BCA II in the refolded samples at 25°C, pH 7.5.*

**Effect of glycine and its methyl derivatives on the tryptophan emission spectra of the refolded samples of BCA II**

Intrinsic fluorescence emission spectroscopy can provide information about the tryptophan and tyrosine in terms of being in the polar and non polar environment, as well as the solvent accessibility of tryptophan that is sensitive to local conformational changes at the tertiary structure level (**Lakowicz, 2006**). Intrinsic emission spectra for all the refolded samples in the presence of glycine and its methyl derivatives were measured to observe their effect on the local changes in the tryptophan environment. All the refolded samples, with and without cosolvents, were excited at 295 nm and the emission spectra was obtained in the range of 300 to 500 nm (Fig. 3A, B and C). Native BCA II shows an emission peak at 342 nm. When the protein was denatured using 5 M GdmCl, there was a

sharp decrease in the fluorescence intensity followed by a significant red shift of 18 nm as compared to the native peak. During spontaneous refolding of the protein (without using glycine or its derivatives) an increase in the fluorescence intensity along with a small blue shift was observed as compared to the native BCA II under similar buffer conditions. When glycine and sarcosine were present in the refolding buffer, there was a constant decrease in the intensity of the emission peak. At 2 M glycine and 3 M sarcosine, the sharpness of the peak was lost and there was a broadening of the emission spectra (Fig. 3A and 3B).



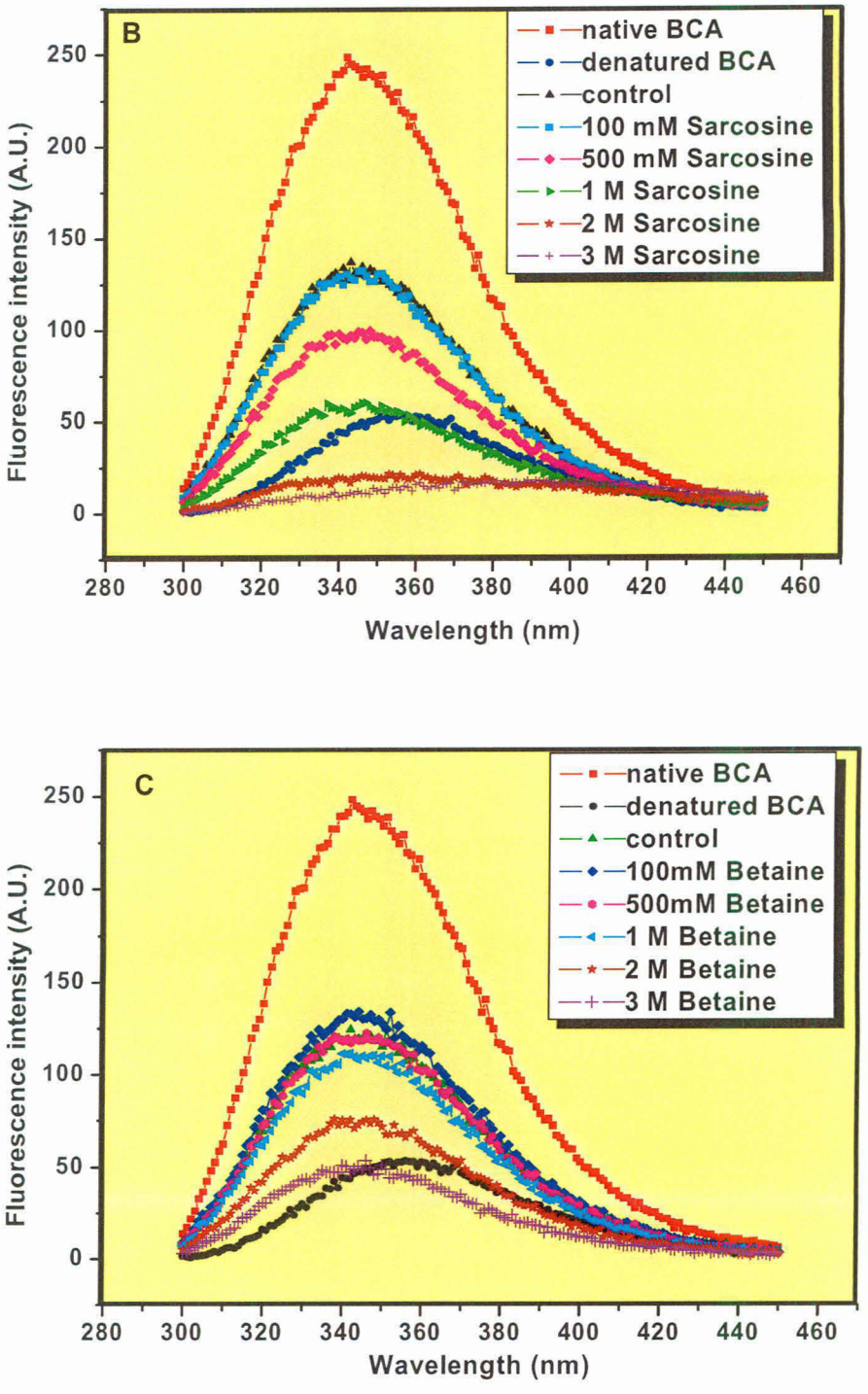



***Fig. 3:*** *Typtophan emission spectra of refolded BCA II at 25°C, pH 7.5 in the presence of A) Glycine, B) Sarcosine, C) Betaine. Native, denatured and the refolded BCA II spectra in the absence of glycine and its derivatives (control) have also been included in the figure.*

**Effect of glycine and its methyl derivatives on the aggregation of native BCA II at 65°C**

Native BCA II was incubated in the absence and presence of glycine, sarcosine and betaine at 65°C for one hour to observe the effect of these osmolytes in preventing the heat induced aggregation of native BCA II, if any. After one hour incubation, samples were taken out from the water bath and centrifuged to pellet down the protein aggregates. Concentration of the residual protein was then measured at 280 nm as discussed in Material and Methods section, chapter 2. Incubation of native BCA II at 65°C leads to its aggregation which was visible in the form of white precipitates. Formation of the precipitates led to considerable decrease in the protein concentration. Native BCA II concentration without incubation was 0.13 mg/ml which decreased to 0.05 mg/ml in the control samples where no osmolyte was used (Table 3). Residual protein concentration of the incubated samples increased gradually with increase in osmolyte concentration indicating that all of them were effective to various extents in preventing BCA II aggregation at high temperature (Table 3). Among the three, sarcosine at 3 M concentration was most effective in suppressing the aggregate formation, showing nearly 76% recovery of the native BCA II. Sarcosine was closely followed by 3 M betaine and 2 M glycine which show 53% and 42% recovery of native BCA II respectively (Table 3, Fig. 4).

**Table 3: Residual concentration of BCA II in the incubated samples at 65°C, pH 7.5**

| Concentration of amino acids (M) | Residual BCA II concentration (mg/ml) | | |
|---|---|---|---|
| | Glycine | Sarcosine | Betaine |
| 0.0 | 0.050 | 0.050 | 0.050 |
| 0.1 | 0.045 | 0.062 | 0.047 |
| 0.5 | 0.042 | 0.56 | 0.053 |
| 1.0 | 0.046 | 0.061 | 0.064 |
| 1.5 | 0.050 | - | - |
| 2.0 | 0.055 | 0.083 | 0.065 |
| 3.0 | - | 0.100 | 0.077 |

*Native BCA II concentration at 25°C, pH 7.5 (without any amino acid used) was 0.13 mg/ml.*

A comparative representation of the effect of glycine and its derivatives on the residual protein concentration in the incubated samples at 65°C is shown in Fig. 4.

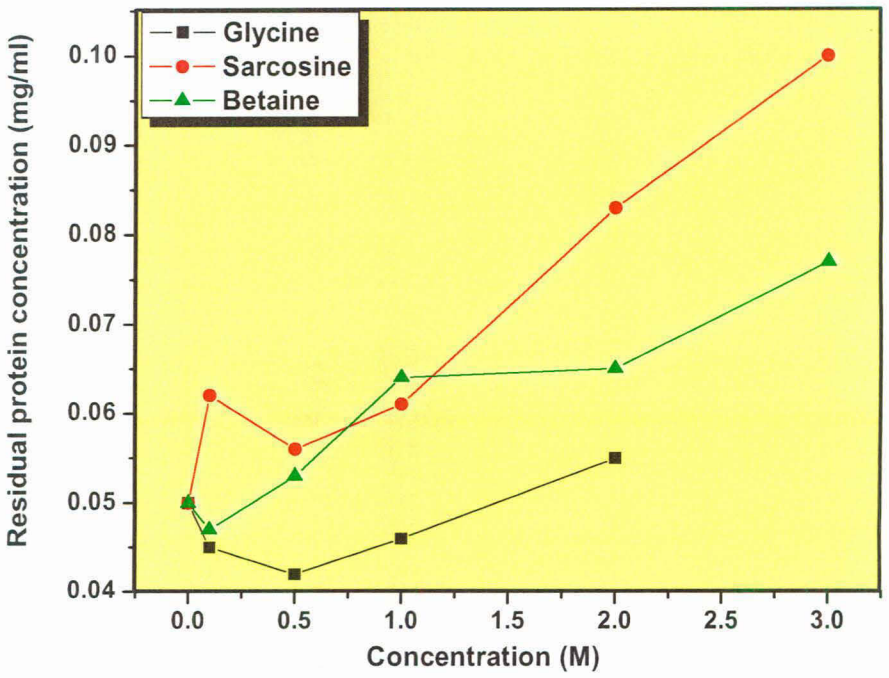
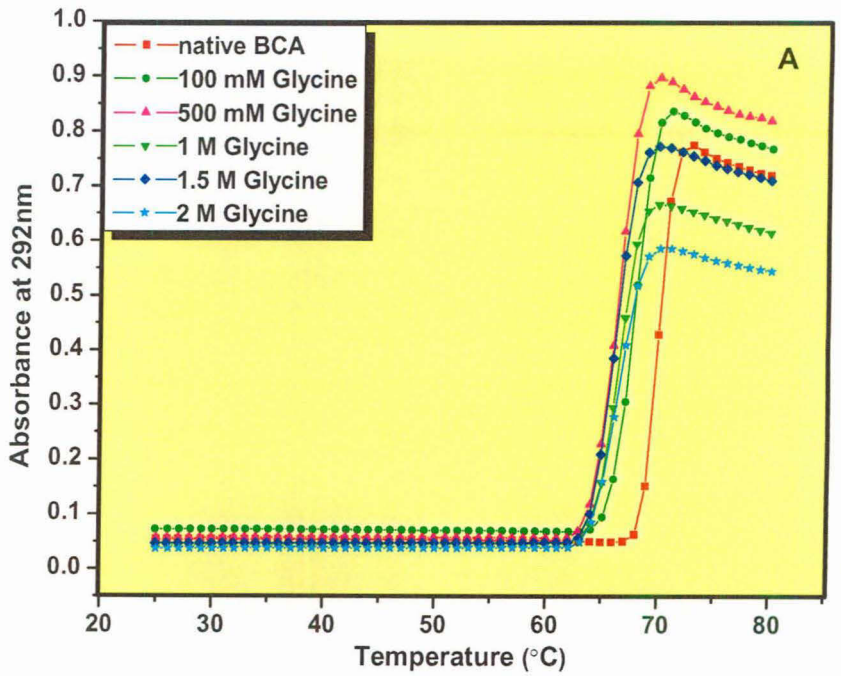



*Fig. 4: Effect of glycine and its methyl derivatives on the residual BCA II concentration after incubating the samples at 65°C, pH 7.5 for one hour.*

**Effect of glycine and its methyl derivatives on the temperature onset of denaturation/aggregation of native BCA II**

The effect of glycine and its derivatives on the stability of the native BCA II was explored by thermal denaturation experiments. Since BCA II aggregates rapidly at high temperatures, one could only determine the temperature onset of denaturation/aggregation ($T_{agg}$) and not the $T_m$ values. Native BCA II has an apparent temperature onset of denaturation at 68°C following which it aggregates rapidly as the temperature is further increased. It was found that glycine, which decreased the refolding yield at all the concentrations was also observed to decrease the stability of the native BCA II during thermal denaturation experiments (Fig. 5A). 2 M glycine lead to a significant decrease of 6 degrees in $T_{agg}$ compared to control, i.e., native BCA II in the buffer alone. Glycine was also able to inhibit the aggregation of native BCA II at all the

concentrations which was evident by the decreased absorbance at 292 nm observed in the post denaturation zone (68-80°C) (Fig. 5A). While sarcosine and betaine showed negligible affect on $T_{agg}$ at all the concentrations, they were much more effective in preventing the aggregation of native BCA II as evident from the decreased absorbance in the post denaturation region (Fig. 5B and 5C).



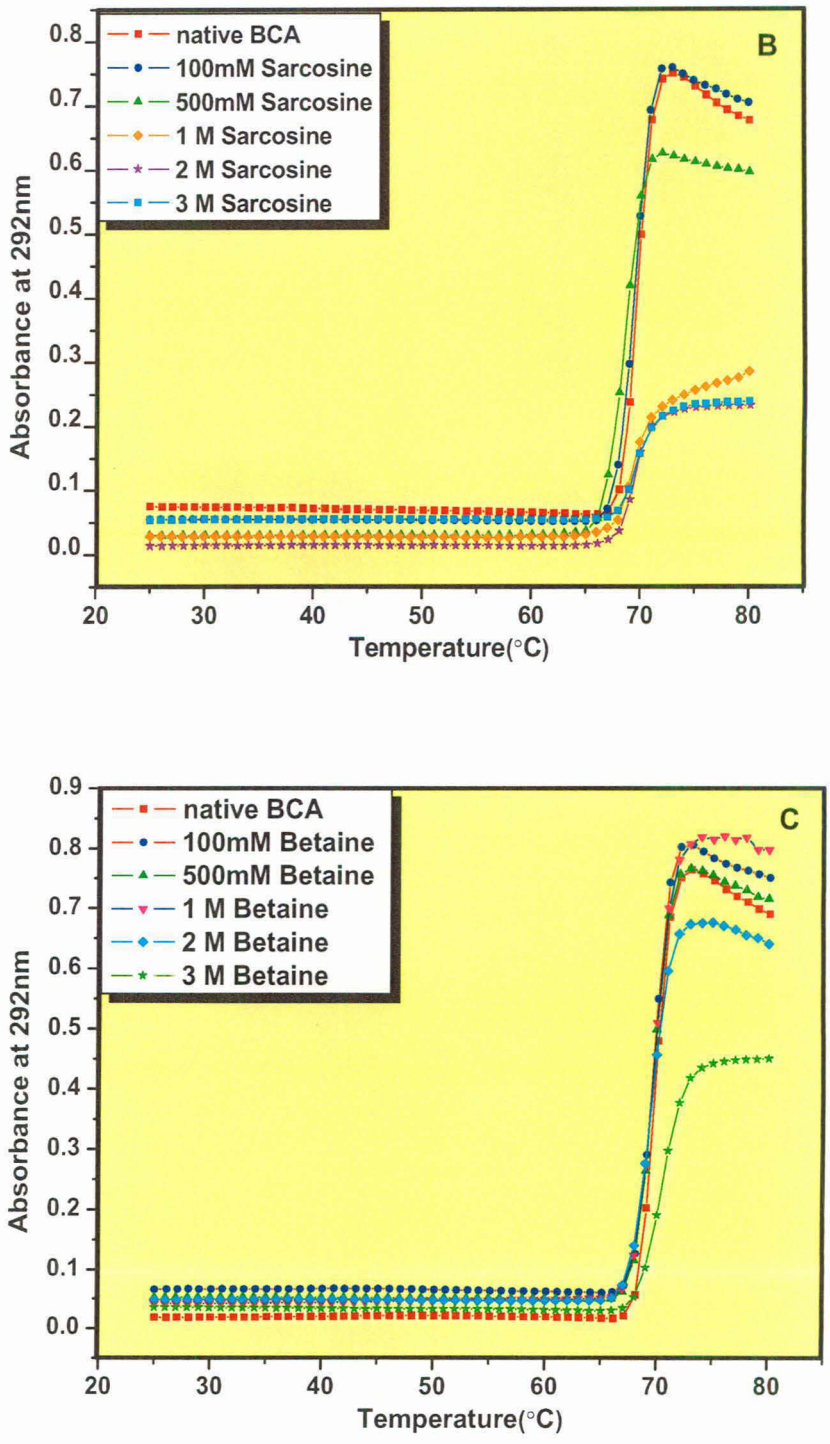



***Fig. 5:*** *Effect of glycine and its methyl derivatives on the Temperature onset of denaturation/aggregation (Tagg) of native BCA II. A) Glycine, B) Sarcosine, C) Betaine. Denaturation transition curve of native BCA II in buffer alone is also included in each panel.*

## Discussion

Methylamines are known to act as counteracting osmolytes against urea denaturation and help in maintaining protein stability and function (**Yancey and Somero, 1979; Arakawa and Timasheff, 1983; Santoro *et al.*, 1992; Anjum *et al.*, 2000**). Osmolyte like betaine is an efficient osmoprotectant which helps in maintaining the osmotic balance in both eukaryotic as well as bacterial cell. During osmotic stress, betaine could accumulate up to 0.1 M inside the cell (**Record *et al.*, 1998; Courtenay *et al.*, 2000**). Betaine is known to assist the correct folding of mutant *lys A* in *E.coli* cells (**Bourot *et al.*, 2000**), prevent the irreversible aggregation and retain the enzymatic activity of many proteins like malate dehydrogenase, citrate synthase and β-galactosidase under different stress conditions (**Pollard and Wyn Jones, 1979; Caldas *et al.*, 1999**). Being a counteracting solute, along with providing stability to wide variety of proteins under different stress conditions, betaine is used extensively as a protein stabilizer in various therapeutics and biotechnological applications (**Rontein *et al.*, 2002; Robert, 2005; de Marco *et al.*, 2005; Kharbanda *et al.*, 2007**). However, in some cases like GST-GFP fluorescent fusion protein, it was found that betaine could cause misfolding of proteins and disruption of protein aggregates in a concentration dependent manner (**Natalello *et al.*, 2009**). Similarly, glycine and sarcosine are also reported to act as efficient protein stabilizers (**Arakawa and Timasheff, 1983, 1985a; Ibarra-Molero *et al.*, 2000; Takano *et al.*, 2004**).

Bovine carbonic anhydrase (BCA II) is an aggregation prone system, whose refolding from its denatured state is dependent upon both protein and denaturant concentration (**Wetlaufer and Xie, 1995**). High protein and low denaturant (GdmCl) often results in a very low refolding yield (Table 1, Chapter 3) as large proportion of the protein becomes inactive due to aggregation. Refolding data of BCA II in the presence of glycine and its methyl derivatives showed that refolding yield decreased in presence of all the three amino acids (Table 1). In presence of glycine, there was a drastic decrease in the refolding yield and at 0.1 M glycine the refolding yield was only 29% as compared to 43% in control (where no glycine was used). Refolding yield remained low even at higher concentrations of glycine (Table 1). In case of sarcosine, while lower concentrations had negligible effect on the refolding yield, sarcosine concentrations from

1 M onwards decreased the refolding yield gradually. Similarly, lower concentrations of betaine also did not affect the refolding yield but there was a sharp decrease at higher concentrations of betaine (Table 1). The refolding data was well supported by the residual protein concentration in the refolded samples. Glycine and betaine which decreased the refolding yield were also found to decrease the concentration of active protein in the refolded samples (Table 1 and 2). It could be assumed that low refolding yield of the denatured BCA II in presence of glycine and betaine could occur due to the stabilization effect of these cosolvents on the intermediate species involved in the folding step. These intermediate species have molten globule like characteristics and contain many hydrophobic regions. Stabilization of the intermediates gives enough time for the hydrophobic protein-protein interactions which eventually lead to protein aggregation and low refolding yield.

BCA II contains seven tryptophan residues in the polypeptide chain. It has one exposed Trp residue in its native state (**Liljas *et al.*, 1972**) and six exposed Trp residues in the first intermediate state (**Jonasson *et al.*, 1997**). Changes in the intrinsic Trp fluorescence of protein are highly sensitive to the change in its immediate environment and it could be used as a tool to monitor the effect of amino acid series on the refolding of GdmCl denatured BCA II. Tryptophan emission of native BCA II shows emission maxima at 342 nm. The usual red shift in the emission peak of denatured BCA II occurs due to the changes in tryptophan environment from non polar to polar. The solvent mediated quenching, resulting from the tryptophan exposure upon unfolding, led to an overall decrease in the intensity of fluorescence emission. Refolding of denatured BCA II during spontaneous folding without using glycine or its derivatives showed a blue shift in the emission spectra indicating the restructuring of the polypeptide chain to attain the native conformation. At higher concentration of glycine, sarcosine and betaine decreased in the fluorescence intensity (Fig. 3A, B and C) could be due to the constant decrease in the residual protein concentration in the refolded samples as shown in Table 2.

Incubation of native BCA II in the presence of glycine and its derivatives showed their thermo protective effect on protein against temperature induced aggregation. All the three osmolytes were found to suppress the aggregation of native BCA II at 65°C which was evident by an increase in the residual protein concentration monitored by increased

absorbance of BCA II at 280 nm (Fig. 4, Table 3). Among the three, sarcosine was found to be most effective which at 3 M concentration was able to completely suppress the protein aggregation showing nearly 80% protein recovery (Fig. 4B, Table 3). Sarcosine was closely followed by betaine which at 3 M concentration showed nearly 60% recovery of native protein. Thermo protective ability of glycine and methyl amines has been explored for a vast number of proteins (**Caldas *et al.*, 1999; Diamant *et al.*, 2001; Taneja and Ahmad, 1994; Takano *et al.*, 2004**). The possible mechanism by which osmolyte provide protein stability may be attributed to the presence of unfavourable thermodynamic force called the osmophobic effect (**Bolen and Baskakov, 2001**). Due to the unfavourable interactions between osmolyte and protein backbone, preferential exclusion of the osmolyte occurs from the protein surface leading to increased solvation of protein molecules which stabilizes the native state of the protein (**Parsegian *et al.*, 2000**). The favorable interaction between osmolyte and side chain of the polypeptide is expected to increase with increased concentration of osmolyte. This favorable interaction would cause solubilization of the polypeptide chain (**Bolen, 2004**), i.e., higher osmolyte concentration would lead to higher protein solubility and thereby inhibition of formation of large protein aggregates. That is why at higher concentration of all the three cosolvents minimum aggregation was observed. Among the three cosolvents used sarcosine and betaine were found to be more effective than glycine. This effect could arise due to the non polar nature of the methyl groups present on sarcosine and betaine which possess 2 and 3 methyl groups respectively. Thus $-CH_3$ group present in sarcosine and betaine could prevent any hydrophobic interactions between protein molecules and would be more effective in preventing aggregation as compared to glycine.

Though all the three cosolvents were effective in suppressing the protein aggregation at high temperature, their effect on $T_{agg}$ of BCA II during thermal denturation was quite surprising. While glycine was found to destabilize the native BCA II at high temperature, sarcosine and betaine had negligible effect on the $T_{agg}$. Higher concentrations of all the three cosolvents were found to inhibit the protein aggregation which was evident by the decrease in absorbance at 292 nm in the post denaturation region (Fig. 5A, B and C). Glycine and its methyl derivatives are known to stabilize many proteins during thermal denaturation and their protein structure stabilizing property

has also been well explained (**Arakawa and Timasheff, 1983, 1985**). Methylated amines are also known to affect the thermal stability of DNA (**Nordstrom *et al.*, 2006**). Stability effect of certain additives like sugars (**Lee et al., 1975; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982 a, b**), and salts (**Arakawa and Timasheff, 1982 c**) etc on the thermal stability of proteins has been related to the preferential hydration of the protein molecules in their presence. It was proposed that the preferential hydration property of these additives was due to their capability to raise the surface tension of water (**International critical table, 1928**). However, it was found that many additives like urea increase the surface tension of water but preferentially bind to the protein molecules and therefore lead to its destabilization (**Prakash *et al.*, 1981**). At the same time additives like glycerol and betaine which lower the surface tension of water lead to preferential hydration and help in the stability of proteins (**Gekko and Timasheff, 1981 b; Arakawa and Timasheff, 1985**). Thus, it has been proposed that increase in the surface tension of water is not the sole factor for the preferential hydration mechanism and apart from surface tension other factors responsible for the stability of protein could also exist (**Arakawa and Timasheff, 1983 and 1985; Tiwari and Bhat, 2006**).

It has been proposed that the osmolyte effect on the protein stability is based upon a fine balance between two forces (**Bolen and Baskakov, 2001**): (1) The thermodynamically unfavorable interaction of osmolyte with the protein backbone (known as osmophobic effect) is responsible for the protein stabilization (**Bolen and Baskakov, 2001**). (2) The thermodynamically favourable interaction of the osmolyte with protein side chain destabilizes the protein (**Bolen and Baskakov, 2001; Bolen, 2004; Das *et al.*, 2007; Ignatova and Gierasch, 2007**).

As discussed above, betaine and sarcosine are well known protein stabilizers but during thermal denaturation of BCA II, betaine and sarcosine had no affect on $T_{agg}$. Thus, for sarcosine and betaine it appears that the two effects, one involving stabilization of native proteins as a result of preferential hydration and the other causing destabilization due to favourable interaction of amino acid side chain of the unfolded polypeptide chain with the two osmolytes, could be nearly equal in magnitude resulting in no change in the $T_{agg}$. The stabilization effect of glycine is attributed to its ability of increasing the surface tension of water (**Arakawa and Timasheff, 1983; 1985**). Contrary to its stabilization

property, glycine was found to decrease the $T_{agg}$ and temperature onset of denaturation of native BCA II during thermal denaturation experiment. It is possible that glycine may have some favorable interactions with some of the side chains of BCA II polypeptide which could dominate over the general preferential hydration effect of the native protein and result in decreased $T_{agg}$ and therefore destabilization of native BCA II.

Taken together, the above data indicate that amino acids like glycine and its N-methyl derivatives namely sarcosine and betaine, at high concentration act as an efficient thermo protectant against temperature induced aggregation, and among the three, the most effective was betaine. Though all the three cosolvents were able to prevent aggregation of native BCA II at high temperature, they behaved differently during thermal denaturation. While glycine decreased the $T_{agg}$ of native BCA II showing its destabilization effect, sarcosine and betaine had negligible effect on the $T_{agg}$. The above cosolvents also lead to different results during refolding studies. While glycine at all the concentrations and betaine at higher concentration reduced the refolding yield significantly, sarcosine had no effect on the folding of denatured BCA II. Thus, the effect of these cosolvents during refolding of denatured BCA II, prevention of aggregation of native protein during refolding and after high temperature incubation, and thermal denaturation is based upon a fine balance between two forces. One is the unfavorable interactions between polypeptide backbone and the osmolyte and the other one is favorable interaction of osmolyte with the side chain of the protein. It therefore appear that the effect of amino acids osmolytes would depend much on both the nature of the protein and, hence, protein-osmolyte interactions, as well as the nature of osmolyte itself in terms of its polar and non polar nature.

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