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IN MEMORIUM: DR. VINOD BHAKUNI – PROTEIN UNFOLDING EXPERT

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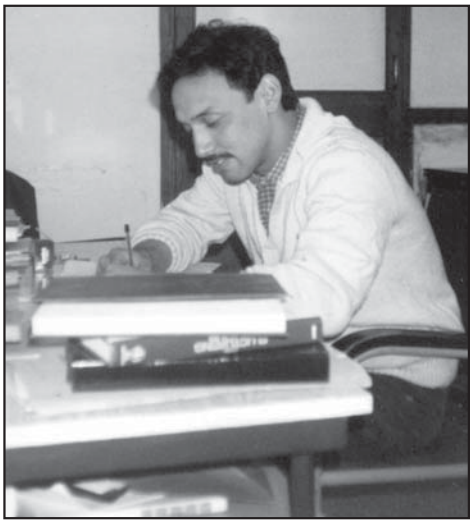
Vinod Bhakuni, the well known biophysicist passed away on July 15, 2011 of a sudden heart attack in Lucknow. With his death, the Indian scientific community lost an accomplished colleague who enriched equilibrium protein unfolding, stability and function by his contribution for about two decades. He was the Head of the department and In-charge of Molecular and Structural Biology division of the CSIR-Central Drug Research Institute, Lucknow.



Upon joining CDRI as a scientist in 1987

Vinod Bhakuni was born on May 24, 1962 at Pithoragarh, Uttar Pradesh (now in Uttaranchal). He obtained M.Sc (Spl) in Organic Chemistry from Lucknow University and Ph.D in Chemistry from CSIR-CDRI with Dr. C. M. Gupta, as advisor. Dr. Bhakuni had spent about two years working on thermodynamics of protein folding with Ernesto Freire at Johns Hopkins University, Baltimore, USA as a visiting postdoctoral fellow. His interest remained in the field and after returning back to India in 1992, he established his group at CSIR-CDRI working on the different aspects of protein folding and stability. His deep interest in the area originated from his acute sense of understanding of the significance of folding intermediates and their stability in protein unfolding pathway and its applications. The various projects he undertook are shown schematically in Fig. 1, and are briefly discussed below.

Folding Pathway of Proteins

The extent of unfolding of denatured states of protein under different conditions has long been of interest because of the possible relevance of their conformation to the protein folding pathway. The characterization of intermediates and the factors involved in their stability may provide important insight into the interactions responsible for their formation as well as their role

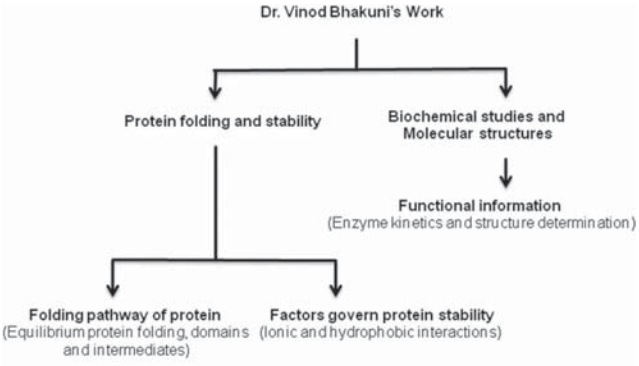


Figure 1: **Schematics of Dr. Vinod Bhakuni's Research Initiatives**

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in protein folding. Dr. Bhakuni studied several model proteins to understand the different aspects of protein unfolding, stability and function. The earliest investigation of his group was on tetrameric bovine liver catalase. His group reported the presence of unique oligomeric intermediate of catalase. The enzyme at low GdmCl concentration (>0.3M) dissociate from native tetrameric form to a functionally active dimeric form (Prakash *et al.*, 2002).

The studies on Glucose oxidase, an acidic dimeric enzyme from *Aspergillus niger*, revealed another interesting observation. In the presence of urea or GdmCl, GOD unfolded through a monomeric intermediate. However, a violation of 2-fold rule was observed (Akhtar *et al.*, 2002). Ideally, the amount of different denaturants viz. GdmCl and urea required to unfold a multimeric protein is different. In all the reports till the study was published, amount of urea required to denature oligomeric proteins was found to be twice or more than that of GdmCl. However in case of GOD, the amounts of the two denaturants required were more or less same (Table 1).

To understand the role of domains in the stability and folding of protein, a detailed study on Serine Hydroxymethyltransferases were carried out (Bhatt *et al.*, 2004). The SHMT from mesophilic *Bacillus subtilis* and thermophilic *Bacillus stearothermophylus* are highly homologous (homology-89%, identity-77%) and composed of two domains, a large N-terminus PLP binding domain and a C-terminal domain. As expected, the bstSHMT is more stable as compared to the bsSHMT. However, their folding patterns were different as bsSHMT showed a non-cooperative unfolding. Furthermore, the domain swapping studies (the carboxy terminal domain of bs- and bst-SHMT were swapped) shows that the folding and stability of SHMTs are governed by its C-terminal domain (Fig. 2). The chimera containing N-terminus of bsSHMT and C-terminus of bstSHMT showed the change in its non-cooperative unfolding to cooperative unfolding and vice versa.

He chose various other model proteins, of which some are listed in Table 2, to investigate the folding intermediates and pathways. One of the model proteins, Serine Hydroxymethyltransferase from *Mycobacterium tuberculosis* also showed a unique stoichiometry of PLP binding. All the known SHMT have one PLP per enzyme monomer where as in case of SHM1 it is one PLP per enzyme dimer. The difference might be due to two double mutations (VS*TTV*HKT) in the PLP binding pocket of SHMT as compared to all the other known SHMTs where this sequence is VTTTTHKT (Chaturvedi and Bhakuni, 2003).

Factors Governing Protein Stability

At the time when Bhakuni began his scientific career, understanding the role of different factors

Table 1
Comparison of Urea and GdmCl C_m Values for Proteins

Protein	*oligomeric state*	C_m *(urea, M)*	C_m *(GdmCl, M)*	C_m *(urea)/* C_m*(GdMCl)*
vitronectin	tetramer	6	2.8	2.1
phosphofructokinase	tetramer	2.8	0.6	4.6
triosephosphate isomerase	dimer	>6.0	1.4	>4.3
ceratinase	dimer	5.2	2.0	2.6
glucose oxidase	dimer	3.6	3	1.2
bovine growth hormone	dimer	8.3	3.8	2.1
invertase	dimer	5.25	1.6	3.28
glutathoine transferase	dimer	5.5	1.5	3.66
RNase T1	monomer	4.6	2.9	1.59
RNase A	monomer	6.6	3.0	2.2
maltose binding protein	monomer	3.5	1.05	3.33
ubiquitin	monomer	3	3.9	0.77

Table 2
List of Few Proteins for which the Protein Unfolding was Studied (Kumar and Bhakuni, 2010; Mishra *et al.*, 2010; Tripathi *et al.*, 2009; Mishra and Bhakuni, 2003; Chaturvedi and Bhakuni, 2003)

Protein	*Pathway/Intermediates*
Mycobacterium tuberculosis malate synthase G	Non cooperative / monomeric intermediate
Entamoeba hystolytica Phosphoserine aminotransferase	Non cooperative / monomeric intermediate
Plasmodium vivax Glutathione S transferase	Non Cooperative /functionally active dimeric intermediate
E. Coli Methylenetetrahydrofolate reductase	Cooperative / no intermediate

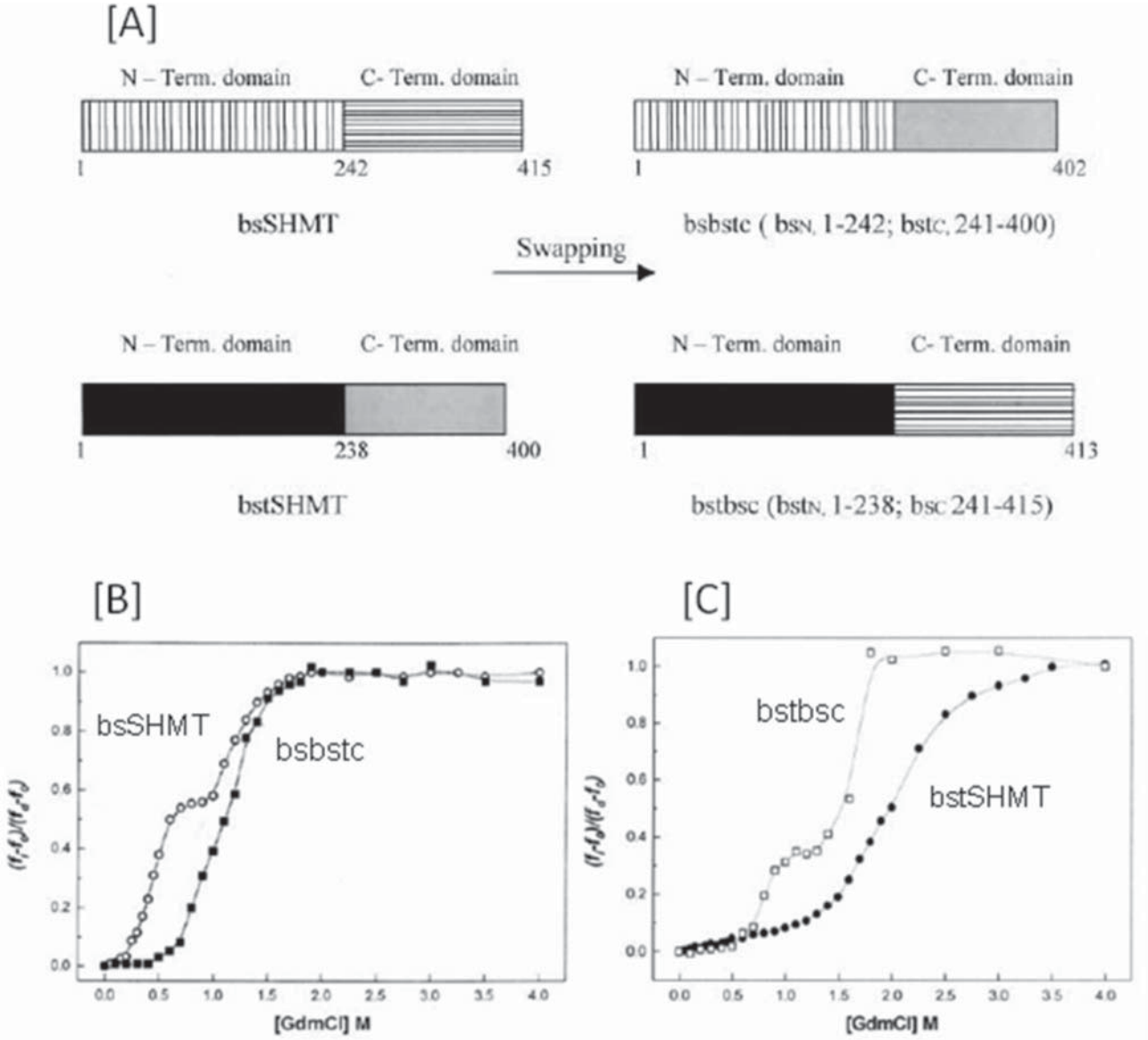


Figure 2: **(A) Schematic diagram showing the swapping of the C-terminal domains between bst- and bs-SHMT. The numbers denote the place of amino acid in the primary sequence of the protein. (B) Equilibrium GdmCl-induced unfolding of bsSHMT and its chimera. Plot of fractional change in the wavelength of maximum fluorescence emission of PyP for bsSHMT and its chimera. (C) Plot of fractional change in the wavelength of maximum fluorescence emission of PyP for bstSHMT and its chimera**

on protein stability was rather a hot area of protein folding. pH is known to influence the stability of a protein by altering the net charge on the protein. Many proteins denature at extreme pH because of the presence of destabilizing repulsive interaction between like charges in the native protein. The exact behaviour of a given protein at low or high pH is a complex interplay between varieties of stabilizing and destabilizing forces, some of which are sensitive to the environment. Salts have been known to affects the physio-chemical properties of the proteins like their solubility, stability and pKa. Dr. Bhakuni chose to understand the interplay of these forces in the subunit assembly of multimeric enzymes.

In mid nineties, his group worked on hen egg white lysozyme and bovine liver catalase. Lysozyme shows denaturation at low acidic pH where as the catalase at basic pH (Babu and Bhakuni, 1997; Prajapati *et al.*, 1998). The partially denatured proteins showed the loss in secondary and tertiary structure; however the increase in ionic strength confers a gain in its secondary and tertiary structures. During the late nineties a much cited work from his laboratory was published showing the first proof of the hydrophobic dye ANS in the refolding of proteins (Ali *et al.*, 1999).

The dual roles of ionic interactions (mono- and di-valent cations) on the stability of the different enzymes were also studied in details. The increase in ionic strength (di-valent cations) leads to the dissociation of the dimeric GOD (12). The binding of different activator cations (K^+, Mg^+) and the inhibitor cations (Cd^+, Zn^{2+}, Mg^{2+}) on the same TIM barrel domain of *Mycobacterium tuberculosis* isopropylmalate synthase and its significance in the function of the enzyme was also reported by the group recently (Singh and Bhakuni, 2008). Table 3 summarizes some of the work related to ionic perturbation of the proteins carried out using different model proteins.

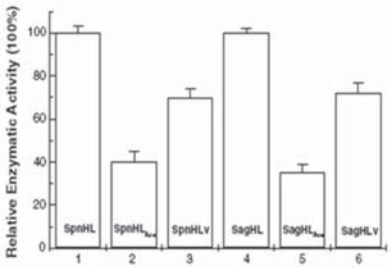
Biomolecular Studies and Molecular Structures

More recently, his interests revolved around translating his expertise in protein unfolding and stability in understanding the functional aspect of proteins especially of Hyaluronate lyases from *Streptococcus pneumonia* and *Streptococcus agalactiae*. HL is composed of three domain, βI domain, α-domain and C-terminal domain connected through linkers. The group studied these enzymes and showed the importance of carboxy terminal domain in increasing the surface potential of the catalytic interface in recruiting the polymeric hyaluronan. The role of linker was established in regulating the C-terminal domain in a conformation suitable for maximum activity (Fig. 3). The group also observed that there is no interaction of Ca^{2+} with its C-terminal domain; instead these ions interact with the substrate hyaluronan to change its conformation. The possible inhibitor (GTC, L-NAME) of the HLs were also reported (Akhtar and Bhakuni, 2003; Akhtar *et al.*, 2006; Akhtar and Bhakuni, 2007). For the last two or three years, Bhakuni was also working on different aspects of amyloidogenesis to understand its mechanism of formation and remedy. The trimeric HL from bacteriophage at acidic pH starts aggregating as observed by thioflavin and other binding assays. HylP2 was observed to form an unusual functionally active fibril through unfolding of its N-terminal domain (Mishra *et al.*, 2006; Mishra and Bhakuni, 2009).

Table 3
List of Few Proteins for which the Protein Stability was Studied (Ref. Tripathi *et al.*, 2009; Bhatt *et al.*, 2005; Kumar and Bhakuni, 2008; Tripathi *et al.*, 2008)

Protein	*Salt or pH*	*Enzymatic activity*	*Effect on the structure*
FprA	Upto 1M NaCl or KCl	No change	Stabilize the protein
	>10mM MgCl2 /CaCl2	Loss	2° structure break, cofactor release
Isocitrate Lyase	Mg++, Mn++	Increase	No change
	Zn++, Cd++	Loss	Loss in 2° structure
PvGST	Mono and Divalent salts	Loss	Loss in 2° structure
Monothiol glutaredoxin	Acidic pH		No change
	Alkaline pH		Loss in 2° and 3° structure

[A]



[B]

Protein	Substrate	K_m (mM)	V_{max} (μmol/min)
SpnHL	HA/HA$_6$	0.13 ± 0.03	114 ± 4
Chimera-I	HA/HA$_6$	0.14 ± 0.05	112 ± 8
SpnN:SpnC complex	HA	0.15 ± 0.04	37 ± 2
SpnN:SpnC complex	HA$_6$	0.15 ± 0.02	67 ± 3
SpnN or α-domain	HA/HA$_6$	0.29 ± 0.05	27 ± 3
SagHL	HA/HA$_6$	0.20 ± 0.05	71 ± 5
Chimera-II	HA/HA$_6$	0.19 ± 0.07	73 ± 9
Sag Domain-I:SagC complex	HA	0.20 ± 0.05	24 ± 4
Sag Domain-I:SagC complex	HA$_6$	0.19 ± 0.05	44 ± 5
Sag DomainI/α-domain	HA/HA$_6$	0.32 ± 0.04	22 ± 1

Figure 3: **The C-terminal domain modulates the enzymatic activity of α-domain. (A) Enzymatic activity profile of different proteins at 37°C with polymeric HA and its smaller fragment (HA6/HA8/HA10). SagHL (S. agalactiae HL), Sag Domain-I (SagHL without C-terminal domain), SagC (C-terminal domain of SagHL; SpnHL (S. pneumoniae HL), SpnN (N-terminal domain of SpnHL, SpnC (C-terminal domain of SpnHL). (B) Kinetic properties of recombinant enzyme and its different domain**

Dr. Vinod Bhakuni will be Remembered

In the course of his about 20 years of stay at CSIR-CDRI, Bhakuni had more than 50 publications in SCI journals and established a sound reputation of himself and the department (2003-2011). He was the recipient of many awards including the prestigious Shanti Swaroop Bhatnagar Prize in 2006 and was conferred the fellowship of all the three national academies.

Receiving the Bhatnagar Prize in 2006

Vinod Bhakuni's demise is a deep personal loss for many others and us. He is survived by his parents, wife, son and a daughter. He will be remembered by a large body of friends and well-wishers and in some sense he would always remain with us.

Acknowledgement

We thank our laboratory members for many contributions and comments. We apologize to colleagues whose work was not cited. This is communication number 8140 from CSIR-CDRI, Lucknow.

Abbreviations

GdmCl, guanidinium hydrochloride; GOD, glucose oxidase; SHMTs, serine hydroxymethyltransferases; bsSHMT, SHMT from *Bacillus subtilis*; bstSHMT, SHMT from *Bacillus stearothermophylus*; SHM1, serine hydroxymethyltransferase from *Mycobacterium tuberculosis*; HL, hyaluronate lyases; SpnHL, HL from *Streptococcus pneumonia*; SagHL, HL from *Streptococcus agalactiae*; HylP2, HL from bacteriophage; PLP, pyridoxal- 5'-phosphate; PyP, pyridoxamine-P.

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