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Stabilization of Structure of Monomeric and Multimeric Proteins – Role of Cosolvents

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A number of methods are used to stabilise macromolecules mainly either by altering the external conditions of the molecule or by genetically manipulating the amino acids to achieve the stability. In this article, the focus is on altering the external conditions in such a way as to induce stabilization of the macromolecules (a) monomeric and (b) multimeric proteins. In protein stabilization, either thermal or otherwise, one parameter of immediate concern is the bulk solvent, which is water, and its role in protein stabilization. Any additive to water in general which changes the structure of water not only in the bulk solvent but also near the protein has a profound effect on the thermal and even activity protection of proteins/enzymes. It is well known that certain reagents such as polyhydric alcohols and to a large extent some of the sugars, have a significant effect on the preferential hydration and also on the preferential interaction of these small molecules and their indirect effect on water structure. The results from our laboratory with reference to monomeric protein/enzyme such as wheat germ lipase(WGL) and rice bran lipase(RBL) and multimeric protein such as human hemoglobin(mHb) with reference to the mechanism of their stabilization in the presence of cosolvents are reviewed. The results suggest that the added cosolvents induce structural stability to wheat germ lipase and rice bran lipase. The activity measurements of heat treated lipase and thermal denaturation temperature evaluation suggest an increased thermal stability of the enzyme towards inactivation and unfolding of the molecule in presence of these cosolvents. The partial specific volume studies indicate preferential hydration overriding in presence of these cosolvents. The fluorescence and ultraviolet difference spectral measurements have shown alterations in the hydrophobic micro environment of chromophores in the proteins investigated. The results of thermal denaturation experiments of mHb in glycerol, sucrose, sorbitol and trehalose show that enhancement of thermal stability occurs in these cosolvents. The fluorescence emission spectral measurements of mHb with cosolvents has shown that there are structural alterations to mHb as evidenced by shift in its emission maxima and exposure of buried tryptophan residues to bulk solvent. This review tries to bring about the difference between (if any) the monomeric and multimeric proteins stabilization/destabilization in presence of the cosolvents.

Key Words: Wheat germ lipase, Rice bran lipase, Human hemoglobin, Cosolvents, Activity, Stability, Preferential interaction, Thermal denaturation

Introduction

Protein stability is normally defined as resistance to irreversible inactivation (Wetzel 1987). The native conformation of proteins is stable at physiological *p*H values, lower temperatures and mild salt concentrations and easily altered at extremely acidic and alkaline *p*H and high temperatures. The maintenance of protein/enzyme structure as well as function is of prime importance from both scientific and commercial point of view. Protein stability results from a balance between large and opposing entropic and enthalpic effects, both of which are highly temperature dependent (Janin 1984, Kuntz 1971).

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A variety of attempts have been made to elucidate the nature of stabilization of proteins (Amelunxen & Murdock 1978, Jaenicke 1981, 2000, Mozhaev & Martinek 1984, Brock 1985). Structural factors such as salt bridges, hydrogen bonding, binding of ligands, hydrophobic interactions, disulfide bonds and amino acid composition also play a major role in the stabilization of enzymes (Kristjansson & Kinsella 1991).

There are several methods of stabilization such as immobilization, chemical modification, additives, etc., which can be employed to make proteins/ enzymes more thermostable. Based on clear physicochemical models these methods lead to protein/enzyme stabilization (Klibanov 1983, Jaenicke 2000, O'Fagain 1995, Sangeeta Devi et al. 1998). In addition, site-directed mutagenesis has attracted attention in the recent years to obtain more stable enzymes/proteins, which have applications in therapeutic and industrial use. This can be achieved by substitution or deletion of certain amino acid/s from the primary structure of a protein or a triplet codon in the nucleotide sequence which mainly depends on the availability of the complete amino acid or nucleotide sequence, three-dimensional structure and genomic location of the protein. Later parts include the cloning and expression in a suitable host to produce the genetically engineered protein in large quantities.

The problem of stability is of great concern where these proteins/enzymes are used in biotechnological processes such as therapeutics, diagnostics, bioreactors, biosensors and fine chemicals. There are several reports available in the literature on the subject of stabilization of proteins/ enzymes (Schmid 1979, Torchilin & Martinek 1979, Klibanov 1983, Mozhaev et al. 1988a, O' Fagain et al. 1988, Querol et al. 1996) and reviews in which this has been discussed in greater detail in a number of references (Mozhaev et al. 1988b, Kristjansson & Kinsella 1991, O'Fagain & Kennedy 1991, O'Fagain 1995, Janecek 1993).

It is a general practice to stabilize proteins by the addition of cosolvents such as sugars and polyhydric alcohols. The cosolvent-protein interaction is a surface phenomenon where more water is structured around the protein molecule, a phenomenon known as preferential hydration, which is one of the major reasons to the stabilization of the protein. Here the cosolvent molecule act as thermodynamic boosters (Lee et al. 1979, Timasheff et al. 1976, Gekko & Timasheff 1981).

These thermodynamic booster solvents are known to increase the free energy of stabilization of native structure of proteins and stabilize the proteins by different mechanisms have led the conclusion that the macroeffects induced in proteins by these cosolvents are the direct consequence of their preferential interactions with the proteins. Preferential interactions- the redistribution of solvent composition in the presence of a protein- are commonly measured by equilibrium solution methods, such as dialysis equilibrium, vapor pressure equilibration, or light scattering. The observed interactions may be *preferential binding* (excess of cosolvent over the bulk solvent composition) or *preferential exclusion* (deficiency of cosolvent in the vicinity of the protein). The last corresponds to an excess of water, that is, to *preferential hydration*. These measured changes in solvent composition are the direct consequence of the mutual perturbation of the chemical potentials of the protein and cosolvent by each other.As such, they are true thermodynamic parameters and should be referred to as *thermodynamic binding* (Timasheff 1995). However the mechanism of such booster solvents are not yet clearly and completely known. Free amino acids stabilize the protein at low concentrations by increasing the hydrogen bonding (Arakawa & Timasheff 1983). Salts at low concentrations induce stability by preferential binding to hydrophobic sites (Arakawa & Timasheff 1982b,1985), sugars such as sucrose and trehalose and polyols by increasing the surface tension of the solvent (Lee & Timasheff 1981, Arakawa & Timasheff 1982a, 1982b, Lin & Timasheff 1996, Kaushik & Bhat 1998), glycerol by solvophobicity of non-polar residues on protein surface (Gekko & Morikawa 1981b, Na & Timasheff 1981), 2-methyl-2,4-pentanediol (MPD) by preferential exclusion due to steric repulsion from the protein charge (Pittz & Timasheff 1978) and polyethylene glycols, both by steric and charge repulsion mechanisms (Lee & Lee 1979,1981, Arakawa & Timasheff 1985). There is apparently negative binding of cosolvents where the protein is said to be preferentially hydrated

(Timasheff & Arakawa 1989). In the presence of cosolvents, the two state equilibrium between the native and denatured states of a protein is shifted towards the native or stable form of the protein (Timasheff & Arakawa 1989).

Using the above principles of preferential interaction, many industrial enzymes such as α-amylase (Brumm & Teague 1989, Rajendran et al. 1995), chymotrypsinogen (Gekko & Morikawa 1981b) and carbonic anhydrase (Cioco 1995) are stabilized against thermal inactivation.

Of particular interest in this article is to understand such stability factors in a monomeric protein such as lipase from wheat germ & rice bran and a multimer which has been well characterized such as Hemoglobin (Hb) and see what are the mechanistic differences between these classes of proteins. It is interesting that when we look at the stability factors the changes that is brought in the bulk solvent has a very profound effect on the stabilization/destabilization of macromolecules. In this process even though the surface phenomenon of multiplicity of charges plays a major role, the necessity of defining such a system is also equally important. The fundamental question of fine balance between native to denatured or stabilized to unstabilized state is still not clearly understood and this article documents some of the data that are important in this field. The article also tries to analyse the literature data with a thermodynamic backdrop both from the author laboratory and elsewhere.

Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) constitute one of the well characterized families of hydrolases. They play an important role in the turnover of lipids and, thus are very important in the area of lipid chemistry and biochemistry. The natural substrates of lipases are triacylglycerols having very low solubility in water. They hydrolyze the ester bonds in tri-, di- and monoacylglycerols, although some are known to degrade fairly a broad range of compounds containing an ester linkage.

Lipases are ubiquitous enzymes playing a pivotal role in all aspects of fat and lipid metabolism in variety of organisms. In humans and other vertebrates, a variety of lipases control the digestion, absorption and reconstitution of fat as well as lipoprotein metabolism (Desnuelle 1986). In plants, during post germination, the metabolism of oil reserves provide energy and carbon skeleton for embryonic growth and is controlled by the action of lipases (Huang 1987). Microorganisms such as bacteria and fungi are also known to produce a wide spectrum of extra-cellular lipid-degrading enzymes to breakdown the insoluble lipid into soluble polar components to facilitate absorption (Lie et al. 1991).

Lipolytic enzymes are defined as the long-chain fatty acid ester hydrolases (Brockerhoff & Jensen 1974). It was shown that a typical lipase also catalyses the hydrolysis of esters other than triacylglycerols (Borgstrom & Brockman 1984). A true lipase may be defined as an enzyme which attacks triacylglycerols and acts only at an oil-water interface (Galliard 1980). The enzymes from different sources have different specificities related to the chain length of the fatty acid and also the nature of the alcohol moiety. It is also true that lipases could hydrolyze water soluble substrates which is characteristic of esterases. Thus, lipases can be considered as special class of esterases, i.e., esterases with a high activity towards water-insoluble substrates (Sarda & Desnuelle 1958, Entressangles & Desnuelle 1968). It has been shown that in the case of lipases, the lipid-water interface is the primary site of lipolysis. The hydrophobicity index (Bigelow 1967) calculated based on the amino acid composition of lipases clearly shows that they are among the more hydrophilic proteins. They are small globular proteins of high solubility in water and some of them are glycoproteins which possess a hydrophobic patch on their surface and, thus, adsorb to hydrophobic surfaces (Elenskii et al. 1987). It is also important to note that true lipases are capable of catalyzing a variety of esterfication and transesterification reactions which are of great industrial importance.

Among lipases, the mammalian lipases are the ones which are studied extensively during the last two decades because of their medical importance. There are several of them starting from lingual lipase to hormone sensitive lipase, which act sequentially on neutral lipids following ingestion. In this sequence, we come across pancreatic lipase, pancreatic co lipase, pancreatic carboxyl ester lipase

(cholesterol esterase), milk lipase, lipoprotein lipase, hepatic lipase, lysosomal acid lipase and adipose tissue lipases (Borgstrom & Brockman 1984).

The lipases from other sources, viz., plant lipases, fungal lipases and bacterial lipases, have not received much attention as compared to mammalian lipases. Even then, the first lipase whose X-ray structure was determined is that from the fungus, *Geotrichum candidum*. As these lipases have not been studied indepth for their mechanism of action as many other mammalian lipases, the information on them are documented on the basis of their sources; bacteria, fungi and plants (Borgstrom & Brockman 1984). The advances in the purification, characterization and structure determination of lipases in plants have been well documented (Brockerhoff & Jensen 1974, Galliard 1980, 1983, Huang 1984, Borgstrom & Brockman 1984, Huang 1987, Antonian 1988, Huang et al. 1988).

Structure of Lipases in general – A Comparative Review

Clearly, high-resolution three-dimensional structures are needed to address the problem of interfacial activation of lipases. Since 1990, crystallographers have solved the high resolution crystal structures of lipases from fungi (Brady et al. 1990, Brzozowski et al. 1991, Schrag et al. 1991, Grochulski et al. 1993, Uppenberg et al. 1994, Schrag & Cygler 1993, Grochulski et al. 1994, Derewenda et al. 1994a), mammals (Winkler et al. 1990, van Tilbeurgh et al. 1993, Bourne et al. 1994) and bacteria (Noble et al. 1993, Lang et al. 1996, Kim et al. 1997). These structures showed that despite a striking diversity of sequences and chain lengths, all the enzymes share many common features such as : (i) have one or two domain α/β hydrolases. The catalytic domains contain a central β-pleated sheet between eight and eleven strands which are closely superimposable. The stoichiometry of the active atoms in this triad is closely conserved and the residues are always ordered Ser-Asp-His with respect to sequence and (ii) the catalytic serine is located on a turn between β-strand and an α-helix and is buried by one or more helices (Lawson et al. 1992).

Catalytic Site of Lipases

The arrangement of residues that form the triad in lipases are very similar to that of serine proteinases in terms of His-Ser-Asp/Glu. The critical role of these residues in catalysis has been confirmed for various enzymes of this family by site-directed mutagenesis (Cygler et al. 1993) and chemical modification using phenyl methyl sulfonyl fluoride.

The α/β-hydrolase fold contains a core of predominantly parallel β-sheets surrounded by α-helices. This fold also assembles the catalytic machinery, a catalytic triad of serine, histidine and either aspartic or glutamic acid and several oxyanion-stabilizing residues. The nucleophilic serine rests at a hairpin turn between a β-strand and an α-helix, the remaining two residues of the catalytic triad. In human pancreatic lipase, the topological origin of the catalytic Asp residue differs (Kazlauskas 1994).

The essential serine residue is conserved in all lipases, including lipoprotein lipase and hepatic lipase. The common sequence found in some of these lipases, Gly-X-Ser-X-Gly agrees with the active site sequence in serine proteinases. These are tabulated in table 1. As it can be seen the hydrophilic sequence surrounding the active site of serine proteinases is replaced by a highly hydrophobic sequence in general in lipases. The hydrophobic environment of the active site serine may be an important factor for lipase-catalyzed hydrolysis at the oil-water interface (Wong 1995).

Table 1 *Amino acid sequences of various hydrolytic enzymes that contain the G X S X G consensus motif (Derewenda & Sharp 1993).*

Enzyme	Sequence
Human lipase gene family	
Human pancreatic lipase	N V H V I G H **S** L G A H A A
Human lipoprotein lipase	N V H L L G Y **S** L G A H A A
Human hepatic lipase	H V H L I G Y **S** L G A H V S
Other mammalian lipases	
Human gastric lipase	Q L H Y V G H **S** A G T T I G
Bile-salt stimulated lipase	N I T L F G E **S** A G G A S V
Hormone sensitive lipase	R I C L A G D **S** A G G N L C
Filamentous fungi family lipases	
Mucor miehei (RmL)	K V A V T G H **S** L G G A T A
Humicola lanuginosa (HlL)	R V V F T G H **S** L G G A L A
Penicillium camembertii	E L V V V G H **S** L G A A V A
Rhizopus delemar	K V I V T G H **S** L G G A Q A
Bacterial lipases	
Moraxella TA144 (LipJ)	N T H V G G N **S** M G G A I S
Moraxella TA144	R L G A I G W **S** M G G C G A
Staphylococcus aureus	K V H L V G H **S** M G G Q T I
Pseudomonas fragi	R V N L I G H **S** Q G A L T A

All amino acids that are tightly packed in the hydrophobic core of the β-α-Se orwotif are in bold type, the active site Ser is bold and underlined.

Mechanism of Lipase Activity

The unique characteristic of lipases for many years was the phenomenon termed 'interfacial activation'. This term is used to describe the greatly increased enzymatic activity of lipases which is observed when the substrate concentration exceeds the critical micelle concentration. The molecular basis of this interfacial phenomenon in lipases has been in argument for decades. Crystallographic investigations on the structure of enzyme-inhibitor complexes offer an explanation on a molecular basis with regard to how the enzyme binds to the lipid substrate and initiates hydrolysis of the ester bond at the micelle surface.

The structures of different lipases differ from one another predominantly in the number and position of the surface loops. In smaller lipases such as *Rhizomucor miehei* lipase, a single α-helix forms the lid while larger lipases such as *Candida rugosa* lipase have two α-helices to form the lid.

Applications of Lipases

Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheese, the acceleration of cheese ripening, the manufacture of cheese-like products, enzyme-modified cheese (EMC) and the lipolysis of butterfat and cream. EMC can be used as an ingredient in other products such as sauces, soups and snacks. Lipases have also been found useful for the resolution of racemic alcohols in the preparation of prostaglandins, steroids and carboxylic nucleoside analogues (Wooley & Petersen 1994).

Lipases have also tremendous potential in small-scale enzymic fat-splitting processes for the production of high value polyunsaturated fatty acids and the manufacture of soap. The three major reactions – hydrolysis, glycerolysis and alcoholysis have been performed directly on mixed substrates using immobilized lipases (Wooley & Petersen 1994). Lipases are widely used in the production of personal care products such as skin and sun-tan creams, bath oils etc. Wax esters (esters of fatty acids and fatty alcohols) have similar application in personal care products which are being manufactured enzymatically (Wooley & Petersen 1994).

In addition to above, lipases have successfully used the synthesis of optically active polymers which are used as adsorbants, in paper manufacturing – apparently, the treatment of pulp with lipase leads to a higher quality product and reduced cleaning requirements. Similarly, the enzyme has also been used in association with a microbial cocktail for the treatment of fat-rich effluents from ice-cream plant. It can also be used effectively in waste processing of many food industries (Wooley & Petersen 1994).

Plant Lipases

Today many plant lipases are the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemists, biotechnologists, microbiologists, food technologists and biochemical & process engineers due to its versatility. Information on lipolytic enzymes in higher plants is important in understanding their physiological roles as well as their action in agricultural products during storage. In post-germination of oil seeds, the mobilization of oil reserves is essential in providing energy and carbon skeletons for embryonic growth. The turnover of membrane lipids in various tissues is dependent upon lipolytic enzymes. Lipolytic enzymes catalyze the initial steps during lipid mobilization and thus may be rate-controlling in germination and post-germination growth.

In agricultural products, the crushing or improper storage of seeds or other agricultural products may lead to an increase in lipolytic activity. This increase results in an accumulation of free fatty acids in oil seeds and the removal of fatty acids from the food grade oil products may prove quite costly.Also, lipolytic activities during seed storage may cause a loss of seed vigor or ability of the seeds to germinate rapidly. Further, lipolytic activities cause rancidity due to the accumulation of free fatty acids which are prone to autooxidative processes which lead to severe impairment of the quality of the final products.

Stability of Monomeric Proteins in Cosolvents

The effect of various cosolvents such as glucose, sucrose, glycerol, DMSO and sorbitol on wheat germ lipase and rice bran lipase is studied by a number of groups to understand the effect of these cosolvents on the structure, activity and stability of the enzyme.The effect of concentration of individual cosolvent as a function of protein concentration is determined principally by partial specific volume measurements to obtain preferential hydration and interaction

parameters. This is supported by activity measurements, fluorescence spectra and thermal stability measurements of wheat germ lipase and rice bran lipase. These data would ultimately provide the magnitude of energetics of the cosolvent – wheat germ and rice bran lipase interaction to understand the various molecular level forces that are responsible for the stability of the native molecule in these cosolvents.

Wheat Germ Lipase (WGL)

Studies on wheat germ lipase were initiated as early as 1948 by Singer who isolated and studied the properties of the enzyme to some extent (Singer & Hofstee 1948a, b). Wheat germ lipase is an esterase and was found to be highly active on triacetin and tributyrin and inactive on long-chain triacylglycerols. Initial studies of wheat germ lipase were focused on activity measurements of wheat germ proteins at different steps of purification and relative rates of hydrolysis with various esters (Singer & Hofstee 1948a). They also carried out the kinetic analysis of hydrolysis of various esters by wheat germ lipase (Singer & Hofstee 1948b). The effect of hydrogen ion concentration and the effect of heat on the activity of the enzyme have also been documented (Singer & Hofstee 1948a, b).

Three distinct esterolytic activities have been reported in the aqueous extracts of wheat germ lipase (Stauffer & Glass 1966), namely, (i) an esterase (ii) a tributyrinase and (iii) a lipase which catalyses the hydrolysis of emulsified mono-olein.

The stabilization of wheat germ lipase in the presence of cosolvents such as glucose, sucrose, glycerol, Dimethyl sulphoxide (DMSO) and sorbitol is worked out by Prakash and Coworkers (Rajeshwara 1994, Rajeshwara & Prakash 1996) through the measurement of activity of the enzyme as a function of temperature. The specific activity of the enzyme has been compared with the specific activity value of the enzyme exposed to 70°C for 10 min in the presence of different cosolvents (at both 25% and 40% concentration). In table 2 is shown the percent retention of activity values of wheat germ lipase in the presence of various cosolvents and the fold increase in thermal stability of the enzyme as reported by Rajeshwara (1994) and Rajeshwara and Prakash (1996).

From table 2 it can be seen that the heat-treated enzyme has only 2% residual activity in phosphate buffer. The maximum retention of activity is 24% or 42% in the presence of glucose or sorbitol at 25% or 40% concentration, respectively. The retention of activity is 19% or 32% in the presence of 25% or 40% sucrose, respectively. Similarly, in the presence of 25% or 40% glycerol, the residual activity value is 8.8 % or 16.1% respectively. In contrast, DMSO is found to stabilize the enzyme only at lower concentrations. From these results, the hierarchy of

Table 2 *Specific Activity of Wheat Germ Lipase upon Heating at 70°C for 10 min in the Presence of Phosphate Buffer containing Cosolvents*

Cosolvent	Concn. (%)	Specific activity (equiv mg^{-1} h^{-1})	% Retention	Fold increase in stability[b]
Buffer[a]				
(a) Native (unheated)		12.00 ± 1.00	100.0	—
(b) Heated		0.30 ± 0.05	2.0*	—
Glucose	25	3.64 ± 0.30	24.0	12.0 ± 1.4
	40	5.72 + 0.48	41.9	20.9 ± 2.5
Sucrose	25	2.88 ± 0.24	19.0	9.5 ± 1.1
	40	4.40 ± 0.36	32.2	16.1 ± 1.9
Glycerol	25	1.34 ± 0.11	8.8	4.4 ± 0.5
	40	2.20 ± 0.18	16.1	8.1 ± 0.9
DMSO	25	1.17 ± 0.10	9.6	4.8 ± 0.6
	40	0.22 ± 0.02	1.8	0.9 ± 0.1
Sorbitol	25	3.64 ± 0.30	24.0	12.0 ± 1.4
	40	5.72 ± 0.48	41.9	20.9 ± 2.5

[a]*pH 7.0, 0.02 M phosphate buffer.* [b]*Calculated on the basis of ratio of percent retention of activity of the enzyme heated in buffer alone as compared to that in different cocolvents. (Ref : Rajeshwara & Prakash 1996)*

effectivness of these cosolvents toward thermal stabilization of the enzyme is shown by them as:

Glucose ≥ Sorbitol > Sucrose > Glycerol > DMSO > Only buffer

The effect of concentration of lipase on the partial specific volume were measured in 0.02 M phosphate buffer, *p*H 7.0. The isomolal and isopotential values at zero concentration were 0.730 ± 0.001 and 0.731 ± 0.002 ml/g respectively. The partial specific volume in the presence of different cosolvents like glucose, sucrose, glycerol and DMSO indicated that lipase is preferentially hydrated to different extents in all the cosolvents used.

The value of the preferential interaction parameter, $(\delta g_3/\delta g_2)_{T,\mu 1,\mu 3}$ calculated for these cosolvents from the partial specific volume are shown in table 3. These values were negative, suggesting preferential exclusion of the solvent components from the domain of the protein molecule. The same interaction parameter on a mol/mol basis was also calculated and is shown in the last column of table 3. From the table it is clear that the value of the preferential interaction parameter on a mol/mol basis is highest in the case of 25% DMSO (Rajeshwara 1994, Rajeshwara & Prakash 1994). Since the cosolvent can vary enormously and independently in their interaction with lipase, a better way to compare the different solvents would be through the actual amount of water molecules present in the domain of the protein.

The results of fluorescence emission spectral changes of WGL as a function of cosolvent concen-tration in glucose, sucrose and DMSO indicates the fluorescence emission intensity changes gradually with increase in cosolvent concentration. The results also reflects that in the presence of glucose, sucrose and DMSO the quenching of emission intensity occurs as a function of cosolvent concentration (Rajeshwara 1994). The preferential hydration and conformational change that might have taken place in lipase molecule must be in the region where aromatic chromophores are absent (Rajeshwara & Prakash 1994).

The stability of the enzyme as evidenced by activity measurements, partial specific volume measurements and fluorescence emission spectra is also be supported in terms of apparent thermal denaturation temperature ($T_{m,\,app}$). The apparent thermal denaturation temperatures of the enzyme in presence of cosolvents as a function of cosolvent composition is shown in table 4. The data by the same authors indicates that the ($T_{m,\,app}$) increases to different extent in presence of these cosolvents, the highest being 66 ± 2°C observed in presence of 40% glucose (Rajeshwara 1994, Rajeshwara & Prakash 1994).

This increase in ($T_{m,\,app}$) of WGL in presence of an added cosolvent is a result of protection of the

Table 3 *Preferential interaction parameters of Wheat Germ Lipase in cosolvents*

Cosolvent	Conc. % (w/v)	ϕ_2^0 (ml/g)	$\phi_2'^0$ (ml/g)	$(\delta g_3/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\delta g_1/\delta g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\delta m_3/\delta m_2)_{T,\mu_1,\mu_3}$ (mol/mol)
Glucose	25	0.721 ± 0.001	0.742 ± 0.002	-0.073 ± 0.009	0.248 ± 0.032	-17.12 ± 2.17
Sucrose	25	0.720 ± 0.002	0.755 ± 0.002	- 0.119 ± 0.012	0.402 ± 0.141	-14.57 ± 1.41
Glycerol	10	0.721 ± 0.002	0.732 ± 0.001	-0.054 ±0.013	0.500 ±0.116	-24.69 +5.75
	20	0.720 ± 0.002	0.734 ± 0.002	- 0.073 ± 0.016	0.309 ± 0.015	- 33.31 ± 6.92
DMSO	25	0.715 ± 0.001	0.734 ± 0.002	- 0.141 ± 0.020	0.442 ± 0.086	-75.67 ± 14.81
Sorbitol	25	0.727 ± 0.001	0.760 ± 0.001	- 0.128 ± 0.008	0.426 ± 0.027	-29.43 ± 1.79

(Ref: Rajeshwara and Prakash 1994)

Table 4 *Apparent T_m of Wheat Germ Lipase in presence of of cosolvents*

Cosolvent concentration (%)	Apparent $T_m \pm 1°C$			
	Glucose	Sucrose	Glycerol	DMSO
10	58	58	58	58
20	61	60	60	61
30	64	62	63	63
40	66	64	64	64

• *Wheat Germ Lipase has an apparent T_m of 56 ± 1°C. (Ref : Rajeshwara and Prakash, 1994)*

enzyme by the cosolvent against thermal denaturation. This increase in temperature of denaturation is more as the concentration of the cosolvent is increased. The results suggest that the enzyme is stable to higher temperatures in the presence of these cosolvents. In other words, the process of denaturation is delayed depending upon the nature and concentration of cosolvent used. Based on the results obtained, the effectiveness in stabilizing the enzyme at elevated temperature follows the order as suggested by the above authors :

Glucose > Sorbitol > Sucrose > Glycerol > DMSO > Only in buffer

Thus, the above results suggest that the added cosolvents induce structural stability to wheat germ lipase. The partial specific volume studies indicated extent of preferential hydration of the enzyme. The fluorescence and spectral measurements showed alterations in the hydrophobic environment of chromophores. The activity measurements of heat treated lipase and thermal denaturation temperature evaluation suggest an increased thermal stability of the enzyme towards inactivation and unfolding of the molecule in presence of these cosolvents. In a three component system, preferential hydration is a known phenomenon where the third component is a stabilizer of the structure of the macromolecule as shown for various proteins in presence of sucrose (Lee & Timasheff 1981) glycerol (Gekko & Timasheff 1981), glucose (Arakawa & Timasheff 1982a) and some salts (Arakawa & Timasheff 1982b).

The data obtained by fluorescence emission measurements and ultraviolet difference spectra by Rajeshwara and Prakash (1994) supports the hypothesis that the change in microenvironment of aromatic chromophores of wheat germ lipase is occurring due to the addition of a cosolvent. Quenching of fluorescence emission intensity would imply alteration of microenvironment of the protein surface, especially the tryptophan residues.

It is also very well documented that the cosolvent stabilization results in enhanced stability in thermal denaturation of enzymes (Gerlsma & Stuur 1972). This phenomenon of thermal stabilization of proteins in general has been observed for ovalbumin in presence of sucrose (Simpson & Kauzmann 1953), lysozyme and ribonuclease in presence of poly-hydric alcohols (Gerlsma & Stuur 1972), egg white proteins in presence of sucrose (Donovan 1977) and for various proteins in presence of sucrose, glucose, sorbitol and glycerol (Back et al.1979). Wheat germ lipase also shows similar behaviour and there is a considerable increase in the thermal denaturation temperature of the enzyme as a function of cosolvent concentration. These results partly explain the observed retention of activity of the heat treated monomeric enzyme in presence of these cosolvents.

Rice Bran Lipase (RBL)

In general, lipase activities are present in different parts of cereal grains. In the case of rice, most of the lipase activities are present in the outer layers (bran) of the grains. The first report on the isolation and purification of rice bran lipase was given by Funatsu et al. (1971) and Shastry and Rao (1971). Funatsu et al. (1971) purified the enzyme by selective ammonium sulfate precipitation, series of chromatographic techniques in the presence of calcium ions. The purified enzyme showed a specific activity of 4-7 units/mg protein and was found to have molecular weight of 40 kDa with two subunits. The chemical properties of rice bran lipase were reported by Aizono et al. (1971). They also have determined some of the enzymatic properties with reference to *p*H, temperature stability, effect of calcium ions, Ethylene Diamine Tetra Acetic acid (EDTA) and the substrate specificity (Aizono et al. 1973).

The retention of activity of an enzyme is a measure of its stability in solution. The heat treatment will lead to inactivation of the enzyme to different extents depending upon the temperature and period of heating. The extent of stability offered by cosolvents such as glucose, sucrose, glycerol, DMSO and sorbitol towards rice bran lipase has been determined by the measurement of activity of the enzyme (Rajeshwara 1994). The activity of rice bran lipase in its native state has been compared with the activity of the enzyme obtained when heated in presence of these cosolvents at 70^0C for 5 min. both

at 25 and 40% concentrations (Rajeshwara 1994).

In table 5 is shown percent retention of activity of rice bran lipase in presence of various cosolvents and number of folds increase in the thermal stability of the enzyme. From the table, it is seen that heat treated rice bran lipase has 22.2% of activity in phosphate buffer. The value of retention of activity calculated for rice bran lipase in presence of cosolvents indicated a highest value of 80.2% in presence of 40% sorbitol. Among all the cosolvents, glycerol was least effective. Thus, all the cosolvents used in the study tend to stabilize the activity of the enzyme against thermal inactivation. The increase in concentration of the cosolvents increased the stability of the enzyme except in the case of DMSO wherein the retention of activity is less at 40% concentration as compared to 25% concentration of the cosolvent. From the specific activity values of heat treated rice bran lipase in presence of phosphate buffer and cosolvents, the number of folds increase in the value of the activity has been calculated and is shown in table 5. From these results, the hierarchy of effectiveness of different cosolvents in preventing the thermal inactivation of rice bran lipase as indicated by the above authors :

Sorbitol > Glucose > Sucrose > Glycerol ≥ DMSO > Only in buffer

The above results suggest that the enzyme, rice bran lipase is inactivated to an extent of 78% when heated to 70°C for 5 min in presence of 0.05 M phosphate buffer at *p*H 7.4 alone. The addition of cosolvents such as glucose, sucrose, glycerol, DMSO and sorbitol offered protection to the enzyme against thermal inactivation. The highest protection observed is in presence of 40% sorbitol. In this series, glycerol and DMSO are least effective as protectants of the activity of the enzyme. This is not so of wheat germ lipase as has been described earlier. The cause for such stability of the enzyme as a result of addition of these cosolvents has been studied in detail by the measurement of partial specific volume of the enzyme.

Thus, the apparent partial specific volumes of rice bran lipase in presence of different cosolvents such as glucose, sucrose, glycerol, DMSO and sorbitol determined at 25% concentration of the cosolvents indicated significant difference between isomolal and isopotential measurements. In all the cases, isopotential values are higher as compared to isomolal values. The difference in the two values being highest in presence of sucrose and sorbitol and the least being in presence of glycerol and DMSO. The preferential interaction parameter calculated for these cosolvents indicated the exclusion of

Table 5 *Specific Activity of Rice Bran Lipase upon Heating at 70°C for 5 min in the Presence of Phosphate Buffer containing Cosolvents*

Cosolvent	Concn. (%)	Specific activity (equiv mg^{-1} h^{-1})	% Retention	Fold increase in stability[b]
Buffer[a]				
(a) Native (unheated)		18	100	-
(b) Heated		4	22	-
Glucose	25	10.8	60.0	2.70
	40	13.2	73.3	3.30
Sucrose	25	9.7	53.9	2.43
	40	12.6	70.1	3.16
Glycerol	25	8.7	48.5	2.18
	40	9.7	53.9	2.43
DMSO	25	10.8	60.0	2.70
	40	9.6	53.3	2.40
Sorbitol	25	9.6	53.3	2.40
	40	14.4	80.2	3.61

[a]*pH 7.4, 0.05 M phosphate buffer.* [b]*Calculated on the basis of ratio of percent retention of activity of the enzyme heated in buffer alone as compared to that in different cosolvents. (Ref: Rajeshwara 1994)*

cosolvents and the highest exclusion being in the case of DMSO. Among other cosolvents, the exclusion is least in the case of glucose.

These results of partial specific volume of rice bran lipase in presence of different cosolvents has indicated that the enzyme is stabilized by preferential exclusion (Rajeshwara 1994). From the preferential interaction parameter values of the enzyme on a g/g basis (ξ_3), the mol/mol preferential interaction parameters $(\delta m_3/\delta m_2)_{T, \mu 1, \mu 3}$, are calculated based on the preferential interaction parameter and solvent composition (g_3) values. These values of preferential hydration are presented in table 6. The value of preferential hydration is highest in the case of 25% DMSO, which is equal to 0.951 g/g and the lowest value is 0.447 g/g obtained in the presence of 25% glucose. The preferential hydration values in the presence of other cosolvents lies in between the values of DMSO and glucose, the magnitude of which decreases in the order as indicated by the above authors :

Sorbitol > Sucrose > Glycerol

As discussed earlier, the thermal stability of rice bran lipase is found to be higher in presence of sorbitol and glucose as seen from the activity measurements of the enzyme after heat treatment. But the preferential hydration values obtained are not higher in these two cases as compared to DMSO and glycerol. This gives an indication that several mechanisms may be operating simultaneously in bringing about the stability for the molecule.

The preferential interaction parameter value calculated on a mol/mol basis is a direct measure of number of moles of cosolvent molecules excluded from the surface of the enzyme. As shown in table 6 the maximum value has been observed in the presence of DMSO (-116.15 mol/mol) and the lowest value is –18.96 mol/mol in the case of sucrose. These values are derived from the preferential interaction parameter value on a g/g basis (ξ_3) and taking into the consideration of the molecular weight of the enzyme and the cosolvent. Though the highest exclusion observed is in the case of DMSO, this exclusion is not solely responsible for the enhanced thermal stability of the molecule. The measurement of specific activity of heat treated enzyme in presence of these cosolvents showed a maximum stability of the enzyme in presence of sorbitol and glucose which are having lesser values of mol/mol interaction parameter (table 6). Similar to the stabilization of rice bran lipase in presence of these cosolvents, wheat germ lipase has also shown to have an increased thermal stability. In the case of wheat germ lipase also, the stabilization is partly due to the preferential exclusion of cosolvent molecules from the domain of the enzyme molecule. However, one can see the effect of such cosolvents on the non polar microenvironment of groups such as tryptophan as evidenced by fluorescence emission spectra of RBL in the presence and in the absence of 10% and 30% cosolvent concentrations measured in the wavelength range of 300-400 nm in order to obtain information on the cumulative effect of cosolvents on the tryptophan environment (Rajeshwara 1994). The data suggests that the tryptophan residues of RBL experienced lesser

Table 6 *Preferential interaction parameters of Rice Bran Lipase with cosolvents at 25% (w/v) concentration*

Solvent	ϕ_2^0 (ml/g)	$\phi_2^{'0}$ (ml/g)	$(\delta g_3/\delta g_2)_{T, \mu_1, \mu_3}$ (g/g)	$(\delta g_1/\delta g_2)_{T, \mu_1, \mu_3}$ (g/g)	$(\delta m_3/\delta m_2)_{T, \mu_1, \mu_3}$ (mol/mol)
Glucose	0.693 ± 0.001	0.731 ± 0.001	-0.132 ± 0.007	0.447 ± 0.024	-21.93 ± 1.17
Sucrose	0.693 ± 0.001	0.750 ± 0.002	- 0.193 ± 0.010	0.658 ± 0.034	-18.96 ± 1.13
Glycerol	0.693 ± 0.002	0.722 ± 0.002	-0.164 ±0.023	0.533 ±0.073	-53.37 ±7.36
DMSO	0.693 ± 0.001	0.722 ± 0.002	- 0.302 ± 0.031	0.951 ± 0.097	-116.15 ± 11.90
Sorbitol	0.694 ± 0.001	0.748 ± 0.002	- 0.208 ± 0.012	0.699 ± 0.040	-34.30 ± 1.98

(Ref: Rajeshwara, 1994)

quantum efficiency of fluorescence emission as the cosolvent concentration increases. This also implies that the tryptophan residues of rice bran lipase are not experiencing a large change in the polarity of the bulk solvent in presence of cosolvent concentrations.

The stability of RBL as evidenced by activity measurements, partial specific volume measurements and fluorescence emission spectra can also be supported in terms of apparent thermal denaturation temperature ($T_{m, app}$) of the enzyme (Rajeshwara 1994). The apparent thermal denaturation temperature ($T_{m, app}$)of the enzyme determined in presence of different concentrations of these cosolvents are shown in table 7. As shown in the table 7 increase in concentration of the cosolvent increased the $T_{m, app}$ value in all the cases except in the case of higher concentrations of DMSO. The increase in $T_{m, app}$ of the enzyme in presence of these cosolvents is as a result of stabilization of the enzyme by cosolvent molecules. The addition of a cosolvent results in an increase in hydration of the enzyme which delays the process of denaturation of the enzyme. Based on the results obtained above, order of effectivenss of protecting the enzyme against thermal denaturation is as indicated by the above authors :

Sorbitol > Glucose $\geq$ Sucrose $\geq$ Glycerol > DMSO > Only in Buffer

The data suggests that sorbitol and glucose molecules are able to stabilize the enzyme to a maximum extent and DMSO being least effective in protecting the enzyme. This data correlates well with the results obtained by activity measurements of the heat treated enzyme in presence of these cosolvents.

Table 7 *Apparent T_m of Rice Bran Lipase in presence of of cosolvents*

Cosolvent concentration	Apparent $T_m \pm 1°C$				
(%)	Glucose	Sucrose	Glycerol	DMSO	Sorbitol
10	70	69	68	68	68
20	72	71	70	69	70
30	73	74	74	72	71
40	75	75	75	72	76

** Rice Bran Lipase has an apparent T_m of 67 ± 1°C.*
(Ref: Rajeshwara 1994)

All these data of the effect of different cosolvents indicated the stabilization of rice bran lipase molecule. The partial specific volume experiments indicated the magnitude of preferential interaction parameter both on a g/g basis as well as mol/mol basis. The preferential hydration values of the enzyme have been obtained which is a measure of stability of the molecule. The fluorescence emission spectral measurements showed alterations in the hydrophobic environment of tryptophan residues as a result of cosolvent addition. The activity measurements of heat treated enzyme and the apparent thermal denaturation temperature of rice bran lipase in presence of these cosolvents suggested the enhanced thermal stability of the enzyme against unfolding and inactivation of the molecule.

Multimeric Protein

Hemoglobin

Hemoglobin is one of the transport proteins characterized by its specific physiological function (that of oxygen transport) and, in sickle-cell anemia where a point mutation was demonstrated with the replacement of a single amino acid change. Hemoglobin is an allosterically regulated tetrameric metalloprotein. Hb has been extensively studied as a multisubunit protein. It offers an excellent model system for the analysis of protein-protein interactions, ligand binding, cooperativity and the thermodynamics of macromolecular assembly (Antonini & Brunori 1970, 1971).

Structure of Hemoglobin (Hb)

The hemoglobin tetramer is a spheroidal molecule of dimensions 64 x 55 x 50 Å. The molecule consists of 574 amino acids with a molecular weight of 64,500 (Braunitzer et al. 1961, Konigsberg et al. 1963, Goldstein et al. 1963). The tetrameric structure comprise of four subunits (two α and two β chains), each of which contains a heme group where oxygen is bound. The α-chain consists of 141 amino acids and β-chain 146 amino acids. The α and β- chains are homologous and the primary structures of these two chains are related to the primary structure of myoglobin (Watson & Kendrew 1961). Each of the polypeptide chain consists of geometrically ordered 8 helical regions and also non-helical regions. Approximately 70% of the residues in the protein participate in the α-helical secondary structure.

Binding of Ligands to Human Hb

The heme groups of Hb reversibly combine with gaseous ligands (O_2, CO and NO), lower alkyl isocyanides, and nitroso aromatic compounds with different affinities and cooperativities. These ligands are termed heme ligands. The binding of the heme ligands is influenced by reversible binding of other types of ligands to specific site on the globin moiety. These ligands which are called nonheme ligands, are H^+, CO_2, and various anions, such as 2,3- diphosphoglycerate(DPG), adenosine triphosphate (ATP), inositol hexaphosphate (IHP) and chloride ion (Rossi Fanelli et al. 1964, Antonini & Brunori 1970, 1971).

Effect of Cosolvents on Human Hb

The role of Hb solutions as oxygen carriers in biotechnology are numerous, such as in the oxygen supply to the biocatalysts or in the preparation of blood substitutes. Human oxyHb undergoes structural change readily into mHb which is devoid of its function as an O_2 carrier. The cosolvents stabilize oxyHb by preventing autoxida-tion, which is a major barrier in preparing protein solution, storage and utilization.

Several workers have described immobiliza-tion of Hb by formation of a covalent bond (Brunori et al. 1977, Rossi- Fanelli et al. 1978), cross-linking (Guillochon et al. 1981) and entrapment (Gaber et al. 1983, Bonaventura et al. 1984). In some cases Hb was stabilized by using cross linker against denaturation (White & Olsen 1987). Asakura et al. (1978) showed that solvents such as alcohols and ketones stabilize Hb at low concentrations against denaturation by mechanical shaking. Cupane et al. (1982) reported that the heat denaturation of mHb was slowed by the presence of lower concentrations of methanol and ethanol.

Haire and Hedlund (1983) studied Hb function in water- ethylene glycol cosolvent system in relation to oxygen binding and hydration. Their results showed that at higher concentrations of ethylene glycol perturbations of protein hydration takes place leading to stabilization of the high-affinity form of Hb. Various workers studied the hydration and allosteric transitions in Hb in sucrose and glucose (Colombo et al. 1992, Bellelli et al. 1993, Colombo & Bonilla-Rodriguez 1996). Glycerol stabilizes strongly human oxyHb, by means of enhancing the structure of water and forming few hydrophobic interactions with globin and thus preventing autoxidation (Nedjar-Arroume et al. 1991, 1993).

The apparent T_m of human methemoglobin (mHb) in colsolvents namely glycerol, sucrose, sorbitol and trehalose are tabulated in table 8 as reported by Ramesh Kumar (1997) and Prakash et al. (2000). From the table, it can be seen that at lower concentration (5%) of cosolvents the apparent T_m of mHb is found to be same with a value of 61°C. But as the concentration of cosolvent is increased, thermal transition temperature of mHb differs to different extents and depends upon the nature of cosolvent. At 40% concentration of glycerol, sucrose, sorbitol and trehalose apparent T_m of mHb is found to be 67, 71, 70 and 69°C respectively. The above data show that the order of magnitude of thermal stability of mHb in presence of 40% cosolvent concentration is as reported by the above authors :

Sucrose > Sorbitol > Trehalose > Glycerol

The thermal denaturation experiments were also performed for mHb at 406 nm in presence of cosolvents. In this case, 0.9 M GuHCl was used in the buffer to prevent precipitation. At 406 nm the absorbance decreases from 25°C to 95°C and the protein is in clear solution up to 95°C. White and Olsen (1987) and Olsen (1994) used 0.9 M GuHCl in the buffer to study thermal denaturation of mHb and some cross linked mHb. But in case of thermal denaturation profiles conducted at 287 nm the protein is precipitated upon heating and the curve acquires sigmoidal shape where coagulation temperature (apparent T_m) can be observed. This is mainly because there is no denaturant (0.9 M GuHCl) present in the solution.

Table 8 *Apparent T_m of mHb* in presence of varying concentrations of cosolvents*

Cosolvent concentration (%)	Apparent $T_m \pm 1$°C			
	Glycerol	Sucrose	Sorbitol	Trehalose
5	61	62	61	61
10	61	64	62	62
20	62	66	64	65
30	63	68	67	66
40	67	71	70	69

** mHb has an apparent T_m of 60 ± 1°C. Thermal denaturation experiments were performed at 287 nm in 0.02 M Tris-HCl, pH 7.4. (Ref: Ramesh Kumar 1997, Prakash et al. 2000)*

The transition temperature (T_m) of mHb in presence of different concentrations of glycerol, sucrose, sorbitol and trehalose as reported by Ramesh Kumar (1997) are shown in table 9. From the table it is observed that at 5% concentration of glycerol T_m of mHb increased to 51°C from 49°C and at 10% and 20% T_m increased marginally to a value of 52°C while at 40% glycerol the T_m of mHb was found to be 54°C. These results suggest that there is a 5°C increment in T_m at 40% glycerol concentration as compared to the control mHb. This data show that thermal stability of mHb has increased due to interaction of glycerol with the protein.

In case of sucrose, at 5% concentration there is a rapid increase in T_m to a value of 54°C. At 40% sucrose concentration there is 10°C increment of T_m is as observed compared to control mHb. Here also upon addition of sucrose thermal stability of mHb increased. At 5% sorbitol concentration, there is a marginal increment of T_m to 50°C. At higher concentrations of sorbitol, i.e. at 40%, T_m of mHb is 60°C which suggests that there is an increment of 11°C as compared to control. These results suggest that mHb gets stabilized due to interaction with sorbitol. The above data show that the order of thermal stability of mHb in presence of cosolvents at higher concentration as reported by the above author is :

Sorbitol > Sucrose $\geq$ Trehalose > Glycerol

Fluorescence emission spectral measurements of mHb were carried out in presence of glycerol, sucrose, sorbitol and trehalose as a function of cosolvent concentration. Any change that is occurring in mHb due to stabilization upon addition of cosolvents can be monitored through fluorescence emission spectral measurements. These studies are also monitored as a function of temperature. The different temperatures that are employed for intrinsic fluorescence were 10, 25, 37 and 45°C. The control mHb is having the emission maximum at 335 nm at 25°C. In presence of different concentrations of glycerol, there is no shift in emission maxima of mHb, but at higher concentration of sucrose (40%) the flurescence emission maximum of mHb shifts to 344 nm from 335 nm. These results suggest that tryptophan residues are exposed to bulk solvent and bring about the change in the microenvironment at $\alpha_1\beta_2$ contact surface where β37 tryptophan is present. In presence of 40% sorbitol concentration the emission maximum shifts to 340 nm. In case of 40% trehalose concentration the fluorescence emission maximum is found to be 320 nm. So there is a blue shift of emission maximum of 15nm was observed compared to control (Prakash et al. 2000). These data suggests that upon binding of sorbitol or trehalose subunit structural changes are occurring in mHb molecule.

The fluorescence data suggest that 6 tryptophan residues present in mHb molecule are not experiencing a larger change in the polarity of the bulk solvent in presence of glycerol. At 10% sucrose concentration 73% relative fluorescence intensity is observed and it decreases gradually with increase in concentration of sucrose. At 40% sucrose concentration there is a large decrease in fluorescence intensity (Ramesh Kumar 1997, Prakash et al. 2000). These results suggest that in mHb changes are happening upon binding with sucrose. There are reports that upon binding with sucrose Hb molecule dissociates into α and β dimers and large buried surface is exposed to solvent (Bellelli et al. 1993). So these results suggest that probably tryptophan at 37th position in β chain is exposed to bulk solvent as it partially dissociates into α and β dimers.

At 10% sorbitol concentration the percentage relative fluorescence intensity is found to be 76 and it gradually decreases with increasing concentration of sorbitol. There is a decrement of 42% relative fluorescence at 40% sorbitol concentration. The data suggest that changes are occurring in micro-environment of tryptophan residues which are exposed to bulk solvent. At 10% concentration of

Table 9 *Thermal transition temperature (T_m) of mHb* in presence of varying concentrations of cosolvents*

Cosolvent concentration (%)	Transition Temperature, (T_m) ± 1°C			
	Glycerol	Sucrose	Sorbitol	Trehalose
5	51	54	50	50
10	52	55	51	51
20	52	55	54	52
30	53	56	56	55
40	54	59	60	59

** mHb has transition temperature (Tm) of 49 ± 1°C. Thermal denaturation experiments were performed at 406 nm in 0.02 M Tris-HCl, pH 7.4 containing 0.9 M GuHCl.*

(Ref: Ramesh Kumar 1997; Prakash et al. 2000)

trehalose the percentage relative fluorescence intensity is found to be 72 and it decreases gradually with increase in concentration of trehalose as at 40% trehalose concentration the percentage relative fluorescence is found to be 42. These results suggest that there is a decrement of fluorescence intensity and change in emission maxima upon binding of trehalose to mHb molecule.

Nedjar-Arroume et al. (1991, 1993) have studied the stabilization of oxyHb in presence of various solvents. They reported strong stability of oxyHb in presence of glycerol and it is due to enhancing the structure of water and forming few hydrophobic interactions with globin. Kwiatkowski and Noble (1993) reported that glycerol lowers oxygen affinity of Hb possibly by shifting the equilibrium between the T state and the high affinity R state in the direction of the T state. Sucrose reduces the oxygen affinity of Hb and this effect is due to stabilization of deoxy-T quaternary state of Hb, via reduction of the activity coefficient of water (Colombo et al. 1992, Bellelli et al. 1993).

It has been shown that trehalose has a significantly higher glass transition temperature than glucose and other sugars. Recently Sastry and Agmon (1997) have reported that trehalose prevents the escape of the few vital internal water molecules in Mb and preserves the internal mobility of the protein. In this study also trehalose may be preventing the escape of internal water molecules. The structural change induced by glycerol could involve dehydration of the heme pocket of mHb.

Solvents may affect proteins through viscosity (Ansari et al. 1992) and osmotic pressure (Rand 1992). The latter determines the hydration state of the protein. It appears that surface hydration layer and the few internal water molecules are vital to normal protein function, conferring to it its internal stability and flexibility (Doster et al. 1993).

The results of this thermal denaturation experiments on mHb in presence of glycerol, sucrose, sorbitol and trehalose show that enhancement of thermal stability occurred with different extents in all cosolvents. The fluorescence emission spectral measurements of mHb in presence of cosolvents showed that there are structural alterations to mHb due to shift in emission maxima and exposure of buried tryptophan residues to bulk solvent (Ramesh Kumar 1997). This study throws more light on the mechanism of stabilization of mHb induced by structural stabilizing cosolvents employed.

It is not clear as why there is a decrease in fluorescence intensity of mHb in presence of different cosolvents. Ananthanarayana and Bigelow (1969) have concluded unequivocally that perturbation of aromatic chromophores in solvents such as sucrose would alter the absorbance of the aromatic chromophores and have calculated the extent of such a perturbation on difference ultraviolet spectra. Studies using L-tryptophan ethyl ester at different concentrations of cosolvents show an increase in fluorescence intensity at higher concentrations of cosolvents such as glycerol and sucrose (Ramesh Kumar 1997, Prakash et al. 2000). In contrast, at lower concentrations there is a decrease in the fluorescence intensity.

Similar data have been observed in case of L-tryptophan ethyl ester in presence of glycerol (Ramesh Kumar & Prakash, unpublished results) where there was an initial decrease which significantly increased progressively later. All these data point to the fact that fluorescence of model compounds such as L-tryptophan ethyl ester at 335 nm is altered in presence of these cosolvents. This may be one of the reason why the fluorescence intensity of mHb has decreased in presence of these cosolvents. However, there are other effects such as preferential hydration, association-dissociation of subunits which also play additional complementary role. There could also be other factors in protein which contribute to the alteration of protein fluorescence in these cosolvents. To explain the entire phenomena by model compound data through tryptophan ethyl ester alone is rather difficult. However, the model compound data suggest the perturbation of the fluorescence data which account for the decrease in the relative fluorescence intensity. Further, the exposure of these inter subunit fluorophores may also contribute partly to such a decrease in the fluorescence intensity as a result of shifting of equilibrium from tetramer to monomer. The above reasoning can be substantiated by differential scanning calorimetric data of monomer to tetramer model of mHb at different *p*H values of 6.0 and 5.5 respectively.

The effect of certain cosolvents such as glycerol which is used as a model solvent in both the

differential scanning calorimetric studies as well as a function of *p*H studies, suggests that the transition of the associated to the dissociated state is favoured towards the dissociated state as a function of lowering of *p*H (in the present experi-ment from *p*H 6.0 to *p*H 5.5). From the apparent T_m measurements at higher concentration of glycerol and similarly in other solvents for example at 5% glycerol apparent T_m is 62°C whereas at the 40% glycerol it is 65°C. Similarly at 5% sorbitol it is 61°C and at 40% it is 70°C (Prakash et al. 2000). The coagulation phenomenon as a function of temperature is delayed in presence of these cosolvents thereby suggesting that the state favoured by cosolvents such as glycerol is the dissociated state and it takes a larger thermal energy to aggregate the molecule before it precipitates at higher temperatures. It is stressed that the domination of water as component 2 cannot be igno red in terms of structured water and clathrate structure around charged groups, hydrophobic moiety and hydrophobic pockets which contributes indirectly to stability of the protein molecule (Timasheff 1998, Prakash et al. 2000).

Conclusions

In conclusion, the role played by certain cosolvents brings in rather explicitly a clear cut separation of changes which involves direct interaction of a particular solvent effect for bringing in both the changes in the bulk solvent properties and the macromolecular properties. What is the driving force in such change of properties as being characteristic between a monomer and a multimer ? If one looks at the enthalpy - entropy compensation in most of the chemical/physical process especially with cosolvent – macro molecular interaction, it is interesting to note that the "first effects" is derived from the changes in micro environments around the macromolecule either by preferential interaction or by preferential hydration depending upon the surface characteristics of the macromolecule the number of crevices it has either through for example subunit – subunit interactions or due to conformational dent as well as chemical and physical nature of the cosolvents and of course the role of temperature, protein concentration and the presence of other salts also cannot be neglected. Further, when we try to look and also analyse the data of the macromolecular – cosolvent interactions either with a monomer or with a multimer what attracts one's attention is the inter subunit interfaces which newly gets exposed to the cosolvent especially from the subunits which are adhering to each other through hydrophobic interactions which might get disrupted as a result of cosolvent interaction or "addition of these, the subunits" being driven by the cosolvent, thus finally create a macromolecule which has an aggregate property of several of these subunits as a result of solvent dominated interaction in these systems. However, when one looks at either binding or exclusion, a critical step exists between the two. It has been shown that denaturants can even act as stabilizers at low concentration say upto 1 M concentration of urea or even upto 0.5 M concentration of GuHCl where the preferential hydration dominates, preferential interaction $\delta m_3/\delta m_2$ is negative and is a classical example of how a molecule like urea can reverse the phenomenon of destabilization of macromolecules in solution at lower concentration (Timasheff 1995, 1998).

The total thermodynamic interaction between macromolecule in this particular review being a protein and a cosolvent is that some or many such interactions will compensate the negative free energy due to specific ligand with firm occupancy of the free site. These are initially available and as a result of continuous displacements and interactions of the solvents when increased in its own concentration such sites ultimately can get saturated. But in the case of denaturants it can be seen that the sites that are exposed as a result of binding of the molecule as compared to in the case of the cosolvents and stabilizers, the reverse may be true where the macromolecule may get into compact form than it had ever been thus raising even the fundamental question whether there exists a supernative structure which is of course generated in the presence of these cosolvents and also have a thermodynamic stability.

It is this concept and the observation that is made in a monomer model that needs to be validated in a multimeric model, also with experimentation and thermodynamic calculations. Therefore, the present example of either wheat germ lipase or rice bran lipase (monomer) vis-à-vis Human methemoglobin, mHb (multimer) could be a very important area for analysis in terms of the stability of the molecule which need to be addressed both from the nature of the cosolvent as well as its concentration. Certain physical

parameters such as surface tension, viscosity, dielectric constant, polarity of the molecule, the concentration of the cosolvent and also the properties of the cosolvent with certain hydrophobic groups (which are bulkier) such as tryptophan, phenylalanine can make a lot of difference in terms of indirect effect of each individual contributions which can be an additive factor and can stabilize the molecule as mentioned above more than that of the native molecule towards thermal denaturation, precipitation, dissociation of subunits and more importantly in the case of enzyme retention of its activity towards a specific substrate.

However, a word of caution is very critical in interpretation of such monomer and multimer data and this has to be also addressed from the point of view of the activity coefficients of certain aromatic amino acids such as N-acetyl ethyl esters in certain polyhydric alcohols and sugars (Lakshmi & Nandi 1976) which clearly demonstrates that these components show an increased activity coefficients (decrease in solubility or "sugaring out") in these solutions studied such as sucrose and glucose. The molar free energy of transfer value was found in such studies to remain unaltered over a range of temperature. This certainly would give a clear explanation of why the thermal stability or the stabilization of a macromolecule occurs in aqueous solutions in certain cases only. Primarily it is the surface property of the macromolecule that determines the stabilization forces and may be the cosolvent can act as booster to such properties.

The role of solvent for a particular protein structure stabilization and similarly even in unfolding of the protein in presence of denaturant is equally vital. If one looks at the preferential interaction parameters in the presence of urea, GuHCl, 2-chloro ethanol, methoxy ethanol in a classical review by Timasheff et al. (1982) in Biophysics of water, one can clearly see that the bulk solvent effect as compared to the nature of cosolvent vis-à-vis the interaction with the peptide group and the side chain especially the non-polar residues which ultimately would relieve the hydrophobic pressure of water probably in the case of denaturants permitting this structure to loosen out (Prakash et al. 1981). This may be conversely true in the case of cosolvents wherein such exclusion of the cosolvent from the protein domain may be the primary driving force for the stabilization of the molecule. When one compares the data for a large number of proteins especially whose three dimensional structures is well known, it is vital to understand that the nature of interaction has to be addressed from an enthalpy-entropy compensation effects and unless and until the nature and the extent of hydrogen bonding is addressed, the entire framework will not become very clear. Therefore, in comparing many data in the literature, temperature is very critical and need to be kept in mind as at lower temperatures, the hydrogen bonds will be much stronger as compared to at higher temperature. Recently as has been indicated by Kita et al. (1994) and Lin and Timasheff (1996), the surface tension of water and its change by the addition of cosolvent has been thought of as playing a very significant and major role in the stabilization of macromolecules especially at elevated temperature in the presence of cosolvents.

However, when one compares some cosolvents with that of a number of other proteins whose surface parameters and surface characteristics are quite different and also when one looks at the compact protein native state that hitherto what one thought probably was the most stable state one sees there can be many more variants of such stable structures also. On the other hand, all ensembles of the compact native state may not be always biologically active. It is quite possible that a slight amount of 'denaturation' may be necessary in some of the molecules to bring in the enzymatic activity that may be due to creation of special crevices and structural features. This needs to be understood in relation to the clathrate structure of water and also the mobile water that moves as a monolayer with the protein in many of its conformational states in presence of these cosolvents.

However, one has to also take into account the role of the conformation in other parts of the macromolecule and how they can effect in terms of local stabilization of the activity. It is very vital to also give enough emphasis towards the interfacial steps between subunits which comes into play after dissociation of the molecule or in many cases, the macromolecule gets stabilized by an indirect factor at sometimes higher concentration of the same.

When one looks at the thermal denaturation

properties of some of the proteins and macromolecules, it is fascinating that the ΔC_p values from the differential scanning calorimetry data as compared to the transition temperatures (T_m) may not match with each other in terms of either the quantity of thermodynamic parameters that one looks upon with addition of sugar or the extent of stabilization that the cosolvents brings about in the case of certain cosolvents such as sorbitol, trehalose, fructose and xylitol (Gekko & Morikawa 1981b).

Hence, ultimately when one looks at the role of surface tension, the number of hydrophobic groups, the number of aromatic groups (hydrophilic) as well as the number of disulfide bonds, that the protein is made up of, each one of them becomes an additional factor to determine the stability of a monomeric protein molecule in presence of these cosolvents. This has been very ably demonstrated with the data on the stabilization of proteins (Lin & Timasheff 1996) in a number of cosolvents and its precise balance between preferential hydration and preferential interaction that ultimately would determine to a large extent the clear process of minimizing denaturation of a protein or in other words stabilization of the protein thus imparting an indirect way to successful structural stability from a solvent mediated and dominated mechanism. This delicate balance that exists between surface hydrophobicity of a multimeric protein as compared to a similar surface hydrophilicity of monomeric protein, the nature of the cosolvent, the temperature of the interaction, the hydrophobicity/hydrophilicity index of the protein, all of them would determine directly or indirectly and contributes to the thermodynamic stability of the said monomeric or multimeric protein from an energetics point of view.

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