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Modulation of the thermodynamic stability of proteins by polyols: Significance of polyol hydrophobicity and impact on the chemical potential of water

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ABSTRACT

The exact mechanism of the modulation of chemical potential of proteins by polyols is not yet well understood. Present study investigates the role of hydrophobicity of polyols, and their impact on water activity and/or surface tension, in determining their stabilization/destabilization potential. Results with ribose and methyl-glucose show that the enhanced stability of proteins is not mediated via the effect on interfacial tension, a hypothesis that has so far been restricted to glycerol. An exemplary correlation between thermodynamic stabilization ($\Delta G_{f\text{-}uf}$), and polyol osmolality, confirms/generalizes the prominent role of water activity in the observed stabilization effects. Results show that even seemingly hydrophilic sugars such as deoxy-ribose can interact favorably with proteins, suggesting that properties other than the presence of hydroxyl groups also contribute to the net effect of polyols. We demonstrate that the hydrophobicity index of polyols and the net stabilization effect afforded to proteins have an excellent inverse correlation. These studies show that the weak hydrophobicity of polyols is critical for promoting their interactions with proteins, weakening of the hydrophobic forces within the protein interior and counteracting the polyol induced-solvent mediated stabilization effect.

1. Introduction

Sugars and polyols are widely used to impart thermodynamic stability to biological macromolecules in solution (Back et al., 1979; Lee and Timasheff, 1981; Uedaira and Udeaira, 1980). The general observations are that these additives prevent the loss of enzymatic activity (Bradbury and Jakoby, 1972), increase the thermal unfolding temperature and inhibit irreversible aggregation of proteins (Chi et al., 2003). To ensure the safety, efficacy and elegance of the aqueous formulations of proteins, it is important that the molecules be kept in a non aggregated state, and hence polyols are widely used as excipients in liquid formulations to enhance the shelf life of the active and non immunogenic species of a biological macromolecule.

The mechanism of stabilization of proteins by polyols has been primarily explained on the basis of the theory of preferential exclusion and is discussed in detail elsewhere (Gekko and Timasheff, 1981a,b; Lee and Timasheff, 1981; Xie and Timasheff, 1997a,b). However, the reason for the molecular origin of such preferential exclusion is still under debate. Sugars such as sucrose and trehalose increase the surface tension of water and are believed to be excluded from the protein domain because they increase the free energy at the protein water interface (Kaushik and Bhat, 1998; Lin and Timasheff, 1996). Glycerol on the other hand lowers the surface tension of water and has been hypothesized to preferentially hydrate proteins by enhancing the solvent ordering around the hydrophobic groups of the protein molecules (Gekko and Timasheff, 1981a; Kaushik and Bhat, 1998; Tiwari and Bhat, 2006). Any increase in the hydrophobic surface area of proteins on unfolding would thus be rendered even more unfavorable in the presence of glycerol. Liu et al. used molecular dynamic simulations and observed good correlation between protein stabilization effect at equivalent molarity of polyols and their molecular volumes (Liu et al., 2010). Additional work by these authors points to the role of indirect interactions such as ordering of water structure by polyols. Such ordering results in a decrease in the entropy of water present in the first hydration shell around the protein molecules. Greater entropic decrease on unfolding thus shifts the equilibrium towards the folded state. It is however unclear from the study as to why different polyols behave differently (in terms of stabilization effect) when used at equivalent weight percentages of the solution. For example, why is 20% (w/v) sorbitol a better stabilizer than 20% (w/v) glycerol.

The role of polyols on the surface free energy of water (surface tension/interfacial tension) in governing the extent of polyol exclusion, and hence determining protein stability, remains unclear.

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Recently, several authors have stressed on the importance of the effect of polyols on the chemical potential of water in determining their stabilization potential (Courtenay et al., 2000; Hammou et al., 1998; McClements, 2002; Miyawaki, 2007; Parsegian et al., 2000). Miyawaki (2007) investigated the difference in the hydration of the folded and unfolded states of the protein molecules and the relationship between this differential hydration and the observed effects of sugars on the thermal stability of proteins. Since, the number of bound water molecules was much higher for the unfolded state of the protein; it was hypothesized that sugars could produce the observed stabilization effect by merely affecting the chemical potential of water. However, the work was limited to a few cyclic sugars (no linear polyols were used) and one protein, and no explanation was given as to why different sugars provide differential stability at equivalent water chemical potential. Support for water activity hypothesis also comes from the work published by Parsegian et al. (2000) wherein, it was demonstrated that both osmotic stress (due to the affect of polyols on the chemical potential of water) and preferential hydration have the same thermodynamic origin (Preisler et al., 1995). It should thus be realized that an essential consequence of the effect of decrease in the chemical potential of water by polyols, and hence an increase in the chemical potential of proteins, is the exclusion of polyols from protein domain. The exact relationship between hydration water and preferential hydration hitherto remains under debate.

Timasheff and coworkers have argued that preferential exclusion effect can get compensated by preferential binding effect leading to a decrease in the stability parameters. Although, the origin of weak attractive interactions between molecules such as salts and proteins is not difficult to understand, the origin of the weak attractive interactions between highly polar trehalose, which has been observed to decrease the chemical potential of the protein under certain solution conditions (Xie and Timasheff, 1997a), and proteins remains unclear. Bolen et al. found that the transfer free energies of the side chains of some of the amino acids from water to sucrose solution are negative indicating favorable interactions between these amino acids and sucrose (Auton and Bolen, 2007; Bolen, 2004; Liu and Bolen, 1995; Qu et al., 1998).

Several polyols including sucrose have been observed to increase the solubility of proteins in solution (Antipova and Semenova, 1996; Conti et al., 1997; Farnum and Zukoski, 1999), an observation which theoretically counteracts exclusion. Since polyols increase the chemical potential of the protein molecules, it is anticipated that preferentially excluded co-solvents will favor the solid state over the dissolved state as this would minimize the area of the protein molecules that is exposed to the co-solvent environment. Further support to the observed effects of polyols on protein solubility (i.e. the increase in solubility by polyols) comes from the determinations of the second virial coefficients (B_{22} or A_2) of proteins in solution (Bajaj et al., 2004; Bonnette et al., 1999; Tessier et al., 2002). Experimental determination of B_{22} by different techniques such as light scattering and ultracentrifugation has shown that polyols in general decrease protein–protein attractive interactions in solution (Valente et al., 2005; Weatherly and Pielak, 2001).

A positive impact of a polyol on the stability of the protein may not necessarily translate into a positive impact on the solubility of the protein. In order to obtain maximum benefit out of the added excipient, careful optimization of the stability and solubility characteristics of the protein in the presence of the polyol is important. In the present market scenario, wherein monoclonal antibodies (mAbs) and mAb-like proteins constitute a major portion (>60%) of the total protein drugs currently in clinical and preclinical testing, the utility of sugars becomes even more important. Since, many diseases that are being targeted by these relatively low potency proteins are chronic and require frequent dosing, providing at home-outpatient administration option to patients by the subcutaneous or intra-muscular route is the desired way of delivery to increase patient compliance. The volume limitation (<1.5 ml) presented by these delivery routes, however, necessitates that the antibody and Ig-like therapeutics be formulated at high concentrations (>100 mg/ml). Increased aggregation propensity at high concentrations thus necessitate that excipients be used to minimize instabilities and hence increase the shelf life of the concerned biological macromolecule. Concerns however arise as development of high concentration aqueous formulations pose solubility issues for some of these molecules.

Despite the presence of a vast amount of literature, the mechanism of protein stabilization/destabilization by polyols and the impact of polyols on protein solubility remain unclear. This lack of understanding prevents the best and the most productive utilization of an optimum polyol and/or sugar in the liquid formulation of a biological macromolecule. The present study was aimed towards answering the following fundamental questions: (1) what is the relationship between the effect of polyols on the surface tension of water (or on the activity of water) and their stabilization potential? (2) What is the nature and extent of the weak attractive interactions, if any, between polyols and proteins, and how may these interactions impact the stabilization effect and the solubility of the protein?

2. Materials and methods

2.1. Materials

All buffer reagents were of the highest purity grade available from commercial sources and were used without further purification. Ethylene glycol, glycerol, L-tyrosine, L-phenylalanine, L-tryptophan and biphenyl were obtained from Acros (Geel, Belgium). 2-Deoxy-D-ribose, D-ribose, alpha-methyl D-glucoside and sucrose were obtained from Fischer Scientific (Fair Lawn, NJ). Trehalose dihydrate, maltose monohydrate, lysozyme and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St Louis, MO). IFNα2a was donated generously by Hoffmann-La Roche and was supplied as 1.6 mg/ml solution in 25 mM acetate buffer, containing 120 mM NaCl (total ionic strength = 142 mM). The protein was stored at −80 °C in vials and each vial was thawed before use.

2.2. DSC studies

A VP-DSC micro-calorimeter from Microcal Inc. (Northampton, MA) was used. Studies with lysozyme were conducted at pH 6.5 and 0.006 M ionic strength. The concentration of lysozyme was kept constant at 0.38 mM. Studies with BSA were conducted at pH 6.5 and 0.001 M ionic strength. Use of such low ionic strength was essential in order to ensure minimal interference from salt effects. Scans were taken from 10 °C to 90 °C at a scan rate of 1 °C/min with a pre-scan thermostat of 10 min. The instrument was allowed to run through the night with multiple scans of the buffer in both the sample and the reference compartments before the measurement of the actual sample. Buffer scan was subtracted from the sample scan and corrected for baseline. Buffers and samples were filtered through a 0.2 μm Millipore (Bradford, MA) syringe filters just before degassing. All samples and buffers were degassed for 5 min in Thermovac, the degassing accessory from Microcal Inc. (Northampton, MA), before their introduction into the sample holders of the DSC instrument. Thermodynamic parameters were obtained by fitting to a two state model. Good post transition baselines were obtained for both lysozyme and BSA. Lysozyme transitions showed good reversibility in the absence or presence of polyols (Fig. 1). The transition in the presence of deoxy-ribose was only partially reversible (40% enthalpy recovery in the rescan). The transitions under all