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Comparison of the thermal stabilization of proteins by oligosaccharides and monosaccharide mixtures: Measurement and analysis in the context of excluded volume theory

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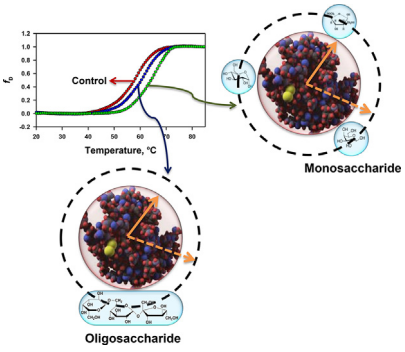
HIGHLIGHTS

- Denaturation of proteins was studied in the presence of monosaccharides mixtures.
- Monosaccharides have more stabilizing effect than their respective oligosaccharides.
- Stabilizing effect of sugar mixtures is accounted by excluded volume model.

GRAPHICAL ABSTRACT



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ABSTRACT

The thermal stability of apo α–lactalbumin (α–LA) and lysozyme was measured in the presence of mixtures of glucose, fructose, and galactose. Mixtures of these monosaccharides in the appropriate stoichiometric ratio were found to have a greater stabilizing effect on each of the two proteins than equal weight/volume concentrations of di- tri- and tetrasaccharides with identical subunit composition (sucrose, trehalose, raffinose, and stachyose). The excluded volume model for the effect of a single saccharide on the stability of a protein previously proposed by Beg et al. [Biochemistry 54 (2015) 3594] was extended to treat the case of saccharide mixtures. The extended model predicts quantitatively the stabilizing effect of all monosaccharide mixtures on α–LA and lysozyme reported here, as well as previously published results obtained for ribonuclease A [Biophys. Chem. 138 (2008) 120] to within experimental uncertainty.

1. Introduction

With the exception of urea, all naturally occurring osmolytes stabilize proteins with respect to unfolding under denaturing stress conditions [1]. Stabilizing osmolytes interact unfavorably with both native (N) and unfolded (U) states of the thermodynamic equilibrium (N ↔ U), but more unfavorably with the U state, thus shifting the equilibrium between the two states toward the N state [2,3]. The effect

Abbreviations: Apo, α–lactalbumin; α–LA, Ribonuclease A; RNase, Guanidinium chloride; GdmCl, Glucose; Glc, Galactose; Gal, Fructose, Fru

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of stabilizing osmolytes on protein stability was studied extensively by Timasheff, Bolen and their coworkers, who attributed the stabilizing effect to *preferential hydration* [2] and an *osmophobic effect* [3], respectively.

For some time we have been interested in the thermal stabilization of proteins by naturally occurring polyols [4–7], and recently reported the results of extensive measurements of reversible thermal denaturation of apo α–lactalbumin (α–LA) and lysozyme in the absence and presence of varying concentrations of seven mono– and oligosaccharides: glucose (Glc), galactose (Gal), fructose (Fru), sucrose, trehalose, raffinose, and stachyose at several pH values [8,9]. Quantitative analysis of these results led us to the following major conclusions. (1) The increase in unfolding free energy of both proteins in the presence of a saccharide depends linearly upon the concentration of added saccharide, and the incremental increase in unfolding free energy per mole of added saccharide was found to depend approximately linearly upon the extent of oligomerization of the saccharide. Both observations could be accounted for quantitatively by a simple statistical – thermodynamic model attributing the stabilization effect to volume exclusion deriving from steric repulsion between protein and saccharide molecules [8]. (2) The incremental increase in unfolding free energy of these proteins per mole of all saccharides increases as the pH of the solution diverges from the isoelectric point of the protein. This observation could be accounted for quantitatively by a straightforward electrostatic generalization of the excluded volume model for stabilization of proteins by sugars [9].

We previously published a study [6] in which the stabilizing effect of an oligosaccharide on ribonuclease A (RNase) was measured and compared with that of an equal weight/volume concentration of monosaccharides of the same stoichiometry and composition as the subunits in the oligosaccharide (for example, comparing the stabilizing effect of a 100 g/l solution of sucrose with that of a solution containing 50 g/l glucose and 50 g/l fructose). In the present work we add to the earlier data new measurements of the stabilization α–LA and lysozyme by monosaccharide mixtures. In addition, we present here a straightforward extension of the previously proposed excluded volume model to treat explicitly the effect of osmolyte mixtures on protein stability. It is shown that the extended excluded volume model can predict to within experimental uncertainty the stabilizing effect of all monosaccharide mixtures upon all three proteins studied.

2. Experimental

2.1. Materials

Proteins (bovine α–lactalbumin and hen egg white lysozyme) in lyophilized form, sodium cacodylate trihydrate and all sugars (glucose, fructose, galactose, sucrose, trehalose, raffinose and stachyose) used in this study were purchased from Sigma Chemical Co., and used without further purification. Glycine and ethylene glycol–bis (β–aminoethyl ether)–*N*,*N*,*N′*,*N′*–tetraacetic acid (EGTA) were purchased from Merck India. Guanidinium chloride (GdmCl) was the ultrapure sample from MP Biomedicals. All chemicals and reagents used were of analytical grade.

Concentrated solutions of both the proteins, holo *α*–lactalbumin and lysozyme, were prepared by dissolving lyophilized powder of each protein in 0.1 M KCl at pH 7.0. In this study, we have used apo form of α–lactalbumin (α–LA) which was prepared by adding 5 mM EGTA to the solution of holo form of the protein. After adding EGTA to the solution of holo protein, protein solution was extensively dialyzed against 0.1 M KCl at pH 7.0 and 4 °C. Similarly, solution of lysozyme was also extensively dialyzed against 0.1 M KCl at pH 7.0 and 4 °C. After dialysis, stock solution of each protein was filtered using a 0.22 μm Millipore filter. Concentrations of protein solutions were determined experimentally using molar absorbance coefficient (ε) values of 29,210 $M^{-1}\,cm^{-1}$ and 39,000 $M^{-1}\,cm^{-1}$ for *α*–LA [10] and lysozyme [11], respectively, at 280 nm. All optical measurements were performed in 0.05 M sodium cacodylate trihydrate buffer at pH 7.0. The concentrated solutions of each sugar (glucose, Glc; galactose, Gal; and fructose, Fru) and GdmCl were prepared in desired buffer solution. All the solutions contain 0.1 M KCl, and filtered using Whatman filter paper and degassed using vacuum pump. Concentrations of stock solutions of sugars (glucose and fructose) [12] and GdmCl [13] were determined by refractive index measurements. Since refractive index of galactose is not known, concentration of stock solution of galactose was determined by dissolving a known amount of it in the desired degassed buffer. Solutions of mixed monosaccharides were prepared by mixing previously prepared solutions of constituent monosaccharide solutions to yield the appropriate stoichiometric ratio. For example, for comparison with 1 M solution of sucrose, a solution containing 1 M glucose and 1 M fructose is prepared by mixing equal volumes of 2 M solutions of glucose and fructose.

2.2. Methods

2.2.1. Thermal denaturation measurements

Jasco 1500 (J–1500) spectropolarimeter equipped with a temperature controller (PTC–100) was used to measure thermal denaturation of both the proteins, α–LA and lysozyme. Thermal denaturations of each protein under all experimental conditions were monitored by following changes in ellipticity, θ, at 222 nm as a function of increasing temperature from 20 to 85 °C. The rate of heating was 1 °C/min, a suitable rate of heating that provides appropriate time for equilibration of protein samples. The concentration of protein solution used for CD measurements was 0.3 mg ml^{-1}. The raw CD data, θ_λ, were converted into mean residue ellipticity, $[\theta]_\lambda$ (degree cm^2 $dmol^{-1}$) at a given wavelength λ, using the relation:

$$[\theta]_\lambda = \theta_\lambda\, M_o/10lc \tag{1}$$

where θ_λ is the observed ellipticity in millidegrees at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration in mg ml^{-1}, and l is the path length in centimeter.

Thermal denaturations of lysozyme under all our experimental conditions were measured in the presence of 2 M GdmCl in order to obtain a complete transition curve within the measurable temperature range. Thermal denaturation profiles of both the proteins under all experimental conditions were measured in triplicate, and reversibility of heat–induced denaturation of a protein was checked in the presence of monosaccharide mixtures as described in Beg et al. [8].

2.2.2. Modeling of thermal denaturation curves

In our previous publications [8,9], the temperature dependence of the mean residue ellipticity at 222 nm, denoted here by $[\theta]_{222}$, measured in solutions of α–LA and lysozyme in the absence and presence of different saccharides, was analyzed according to a reversible two–state model introduced by Beg et al. [8], summarized here for convenience.

In the absence of added sugar, the temperature dependence of $\Delta G_{NU}{}^0$, Gibbs free energy of converting native (N) state to unfolded (U) state, is given by the integrated Gibbs–Helmholtz relation [14].

$$\Delta G^0_{NU}(T) = -RT\ln K^0_{NU}(T) = \Delta H_{T_m^0}(1 - T/T^0_m) - \Delta C_P[T^0_m - T + T\ln(T/T^0_m)] \tag{2}$$

where R denotes the molar gas constant, T the absolute temperature, $T_m{}^0$ denotes the temperature at which the protein is half–unfolded in the absence of sugar, $\Delta H_{T_m}{}^0$ the enthalpy change of unfolding at $T_m{}^0$, and ΔC_p the heat capacity change, assumed to be independent of temperature and sugar concentration. In the presence of a single sugar at molar concentration c_S, first–order excluded volume theory predicts that the equilibrium constant will be shifted according to

$$\Delta \ln K_{NU} \equiv \ln K_{NU}(T, c_S) - \ln K^0_{NU}(T) = \alpha c_S \tag{3}$$

where α is a temperature–independent constant that is a function of the