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Effect of polyol osmolytes on ΔG_D, the Gibbs energy of stabilisation of proteins at different pH values

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Abstract

Thermal denaturation curves of lysozyme and ribonuclease-A were determined by measuring their far-UV circular dichroism (CD) spectra in the presence of different concentrations of five polyols (sorbitol, glycerol, mannitol, xylitol and adonitol) at various pH values in the range 7.0–1.9. The denaturation curve at each polyol concentration and pH was analysed to obtain values of T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m), and these ΔH_m and T_m values obtained at different pH values were used to obtain ΔC_p (constant-pressure heat capacity change) at each polyol concentration. Using values of ΔH_m, T_m and ΔC_p in the Gibbs–Helmholtz equation, $\Delta G_D°$ (Gibbs energy change at 25 °C) was determined at a given pH and polyol concentration. Main conclusions of this study are that polyols have no significant effect on $\Delta G_D°$ at pH 7.0, and they stabilise proteins in terms of $\Delta G_D°$ against heat denaturation at lower pH values. Other conclusions of this study are: (i) T_m at each pH increases with increasing polyol concentration, (ii) ΔH_m remains, within experimental error, unperturbed in the presence of polyols, and (iii) ΔC_p depends on polyol concentration. Furthermore, measurements of the far- and near-UV CD spectra suggested that secondary and tertiary structures of both proteins in their native and denatured states are not perturbed on the addition of polyols.

Keywords: Protein stability; Thermal denaturation; Protein structure; Polyol osmolyte; Ribonuclease-A; Lysozyme

1. Introduction

Considerable time in nearly all fields of biochemical sciences is devoted to improving protein stability, which is the result of a balance between the intramolecular interactions of protein functional groups and their interaction with solvent environment [1–3]. Naturally occurring osmolytes are co-solvents that are used to protect organisms from denaturation by harsh environmental stresses. These molecules stabilise proteins, not by interacting with them directly but by altering the solvent properties of the surrounding water and hence the protein–solvent interactions [3]. Their effect seems to be general for all proteins. They have no inhibitory or enhancing effects on biological activity under physiological conditions hence are called compatible osmolyte [2,4]. Stabilizing osmolytes are chemically diverse and include such chemical classes as polyols, certain amino acids and their derivatives, and methylamine compounds [4]. Among these chemical classes polyhydric alcohols (polyols) are among the most prevalent molecules used by nature to protect organisms against the stresses of high osmotic pressure and freezing [2,5]. Polyols belong to the class of compatible osmolytes [6–9]. They have also been found to be effective stabilisers of proteins and biological assemblies when added at high concentrations [3,10–14].

There are various mechanisms that have been used to explain the observation on the effect of osmolytes on the protein denaturation equilibrium, native (N) state ↔denatured (D)

Abbreviations: ΔG_D, Gibbs free energy change; ΔG_D°, Gibbs free energy change at 25 °C; RNase-A, ribonuclease-A; ΔC_p, constant-pressure heat capacity change; T_m, midpoint of thermal denaturation; ΔH_m, enthalpy change at T_m; CD, circular dichroism.

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state [15–18]. The most widely used mechanism is due to Timasheff [17]. According to this mechanism osmolytes stabilise N state because they are preferentially excluded from the protein surface, for the preferential exclusion increases the chemical potential of the protein proportionately to solvent exposed surface area. Thus, by Le Chatelier's principle, osmolytes favour the more compact state, i.e., the N state over the structurally expanded state, i.e., D state. Hence according to this mechanism ΔG_D, the Gibbs free energy change associated with the denaturation process, N state↔D state, should increase in the presence of osmolytes, for $\Delta G_D = -RT\ln([D]/[N])$, where square bracket represents concentration. The most recent mechanism of stabilisation of proteins by osmolytes is due to Bolen and co-workers [18]. According to this mechanism osmolytes stabilise N state because of their overwhelming unfavourable interaction with the peptide backbone. Thus, this "osmophobic effect" favours the N state over the D state of proteins. Hence, according to this mechanism ΔG_D should increase in the presence of osmolytes.

Recently, we studied the effect of trimethylamine N-oxide on the thermal denaturations of several proteins at different pH values [19]. The main conclusion of this study is that this osmolyte increases $\Delta G_D°$ (value of ΔG_D at 25 °C) of proteins at pH values above its pK_a (pK_a=4.66±0.10 [20,21]), and it decreases $\Delta G_D°$ of proteins at pH values below its pK_a. Since polyol osmolytes are nonionizable molecules, it is expected that, as predicted by all mechanisms of stabilisation of proteins [15–18], they should increase $\Delta G_D°$ of proteins at all pH values. In order to see whether this is indeed true, we have measured thermal denaturations of lysozyme and RNase-A in the absence and presence of five polyols (sorbitol, glycerol, xylitol, adonitol and mannitol) at different pH values in the range 7.0–1.9. We report for the first time that $\Delta G_D°$ of each protein is, within experimental errors, unchanged in the presence of all five polyols at pH 7.0, and it increases with decreasing pH. We also report that polyols have no effect on the secondary and tertiary structures of the two end states of the equilibrium N state↔D state.

2. Materials and methods

Commercial lyophilised preparations of RNase-A (type III-A) and hen egg white lysozyme were purchased from Sigma Chemical Co. D-Sorbitol, D-glycerol, D-mannitol, D-adonitol and D-xylitol were also obtained from Sigma Chemical Co. Guanidinium chloride (GdmCl) was the ultra pure sample from Schwarz/Mann. These and other chemicals, which were of analytical grade, were used without further purification.

RNase-A and lysozyme solutions were dialysed extensively against 0.1 M KCl at pH 7.0. Protein stock solutions were filtered using 0.45 μm millipore filter paper. Both proteins gave single band during polyacrylamide gel electrophoresis. Concentration of the protein stock solution was determined experimentally using ϵ, the molar absorption coefficient (M^{-1} cm^{-1}) values of 9800 at 277.5 nm for RNase-A [22] and 39,000 at 280 nm for lysozyme [23]. All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl. For various pH ranges, the buffers used were 0.05 M glycine hydrochloride buffer (pH range 1.9–3.5) and 0.05 M cacodylic acid buffer (pH range 5.0–7.0). It may be noted that a known amount of the unbuffered protein stock solution was used to prepare solutions at different pH values. Since pH of the protein solution may change on heating or on the addition of GdmCl, pH of each solution was also measured after the denaturation experiment. It was observed that the change in pH was not significant. It should, however, be noted that no corrections were made for the possible effect of co-solvents on the pH of protein solutions.

2.1. Thermal denaturation measurements

Thermal denaturation studies were carried out in a Jasco J-715 spectropolarimeter equipped with a peltier type temperature controller (PTC-348 WI) with a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Change in CD at 222 nm of the protein solution (concentration range 0.3–0.5 mg/ml) was measured in the temperature range 20 to 85 °C. About 650 data points of each transition curve were collected. After denaturation, the sample was immediately cooled down to measure reversibility of the reaction at different temperatures. It was observed that data from the renaturation experiments fell on the denaturation curve. All solution blanks showed negligible change in ellipticity with temperature and were, therefore, neglected during the data analysis. The raw CD data were converted into $[\theta]_\lambda$, the mean residue ellipticity (deg cm^2 $dmol^{-1}$) at a given wavelength λ using the relation,

$$[\theta]_\lambda = \theta_\lambda M_o / 10lc \quad (1)$$

where θ_λ is the observed ellipticity (millidegrees) at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the pathlength (cm). Each heat-induced transition curve was analysed for T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m) using a non-linear least-squares analysis according to the relation,

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m/R(1/T - 1/T_m)]} \quad (2)$$

where $y(T)$ is the optical property at temperature T K, $y_N(T)$ and $y_D(T)$ are the optical properties of the native and denatured protein molecules at T K, respectively, and R is the gas constant. In the analysis of the transition curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and denatured protein molecules (i.e., $y_N(T)=a_N+b_NT+c_NT^2$ and $y_D(T)=a_D+$