International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effect of Dextran 70 on the thermodynamic and structural properties of proteins

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ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form 20 April 2015
Accepted 22 April 2015
Available online xxx

Keywords:
Protein folding
Macromolecules
Macromolecular crowding

ABSTRACT

Biological macromolecules are known to evolve and function under crowded intracellular environments that comprises of a wealth of soluble and insoluble macromolecules like proteins, nucleic acids, ribosomes and carbohydrates *etc*. Crowded environment is known to result in altered biological properties including thermodynamic, structural and functional aspect of macromolecules as compared to the macromolecules present in our commonly used experimental dilute buffers. In this study, we have investigated the effect of Dextran 70 on the thermodynamic and structural properties of three different proteins (Ribonuclease-A, lysozyme and holo α-lactalbumin) at different pH values. We discovered that Dextran 70 has a protein-independent effect in terms of protein stability and structure.

1. Introduction

Most solvent environments used traditionally to study basic biological processes including protein folding, enzyme activity, structural allostery, *etc*. were performed under highly dilute conditions as compared to the highly crowded intracellular environment wherein proteins perform their biological functions [1,2]. Indeed, the cell interior is known to be densely populated due to the presence of soluble and insoluble macromolecules (proteins, nucleic acids, ribosomes and carbohydrates *etc.*) [1,3,4], which together make the intracellular environment "crowded" or "volume-occupied" rather than "concentrated" [3,5–7]. These macromolecules collectively occupy ~10–40% (a substantial fraction of the intracellular space) of the total fluid volume, restricting the volume available to other macromolecules present. Crowded environment therefore, results in altered biological processes including thermodynamic, functional and structural properties of macromolecules as compared to the macromolecules present in dilute buffers. Thus, it is important to perform studies under conditions that mimic the environment of the crowded intracellular milieu to have a more realistic insight of the *in vivo* scenario. Nowadays availability of synthetic crowding agents (like Ficoll, Dextran *etc.*) has made it possible to investigate the effect of macromolecular crowding on the properties of macromolecules.

Effect of macromolecular crowding on protein structure and stability has been widely investigated [8–12]. It has been known that macromolecular crowding mainly acts on the less compact, unfolded state rather than the more compact native state [5,13–15]. In addition, crowding has been demonstrated to increase native state structure, stability [8,10,11,16–18], and even induce shape changes in certain proteins [19,20]. Recently, Ficoll 70 has been shown to have different effects on different proteins in terms of thermodynamic stability at low pH where proteins are unstable [18]. In the light of the fact that cellular interior consists of different types of macromolecules (differing in shapes and/or sizes) that collectively make the cellular interior crowded, it is important to investigate the effect of other crowding agent (that has different properties than that of Ficoll 70) on the structure and stability of the three proteins in order to draw generalized conclusions.

In the present study, we investigated the effect of Dextran 70 on the structure and stability of three proteins (Ribonuclease-A, lysozyme and holo α-lactalbumin) and compared it with that of Ficoll 70 [18]. Indeed, Dextran 70 is a flexible, long-chain polymer of D-glucose with sparse, short branches and is better modelled as a rod-like particle. In contrast, Ficoll 70 is a compact, highly crosslinked and branched copolymer of sucrose and epichlorohydrin that behaves more like a semirigid sphere. Both crowding agents are widely accepted as test systems for isolating effects of macromolecular crowding created by macromolecules found in the biological setting where proteins normally perform their function [21]. We discovered that Dextran 70 confers a stabilizing effect on

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http://dx.doi.org/10.1016/j.ijbiomac.2015.04.051
0141-8130/

all the three proteins in terms of thermodynamic stability suggesting that Dextran 70 has a protein-independent effect on proteins. We also found that Dextran 70 has a stronger stabilizing effect on protein structure and stability relative to the effect of Ficoll 70.

2. Materials and methods

2.1. Materials

Commercially lyophilized preparations of Ribonuclease-A (RNase-A; from bovine pancreas), lysozyme (from chicken egg white) and holo alpha-lactalbumin (holo-LA from bovine milk) were purchased from Sigma Chemical Co. Sodium cacodylic acid and Dextran 70 were also obtained from Sigma Chemical Co. Potassium chloride, sodium acetate and glycine were obtained from Merck. Guanidinium chloride (GdmCl) was the ultrapure sample from MP Biomedicals. These and other chemicals, which are of analytical grade, were used without further purification.

2.2. Analytical procedure

RNase-A, lysozyme and holo-LA solutions were dialyzed extensively against 0.1 M KCl at pH 7.0 in cold (~4 °C). Protein stock solutions were filtered using 0.22 μm millipore syringe filter. Concentration of the protein solutions was determined experimentally using ε, the molar extinction coefficient values of 9800 M^{-1} cm^{-1} at 277.5 nm for RNase-A [22], 39,000 M^{-1} cm^{-1} at 280 nm for lysozyme [23] and 29,210 M^{-1} cm^{-1} at 280 nm for holo-LA [24]. The concentration of GdmCl stock solution was determined by refractive index measurements [25]. All solutions for optical measurements were prepared in the appropriate degassed buffer. For various pH ranges, the buffers used were 0.05 M glycine-HCl buffer (pH 3.0), 0.05 M acetate buffer (pH range 4.0–5.0) and 0.05 M cacodylic acid buffer containing 0.1 M KCl (pH range 6.0–7.0). Special care was taken to mix all solutions due to the high viscosity of Dextran 70. Since pH of the protein solution may change on the addition of co-solvents, pH of each solution was also measured after the denaturation experiments. It should, however, be noted that no corrections were made for the possible effect of co-solvents on the observed pH of protein solutions.

2.3. Thermal denaturation studies

Thermal denaturation studies were carried out in a Jasco V-660 UV/visible spectrophotometer equipped with a Peltier-type temperature controller at a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Each sample was heated from 20 to 85 °C. The change in absorbance with increasing temperature was followed at 287 nm for RNase-A, 300 nm for lysozyme and 295 for holo-LA. About 650 data points of each transition curve were collected. Measurements were repeated three times. The protein concentration used for thermal denaturation was 0.5 mg ml^{-1} and denaturation of each protein sample was performed in presence of varying Dextran 70 concentrations, ranging from 0 to 300 g l^{-1} at different pH values (pH 3.0–7.0). After denaturation, the protein sample was immediately cooled down to measure reversibility of the reaction. Each heat-induced transition curve was analyzed for T_m (midpoint of denaturation) and ΔH_m (denaturational enthalpy change at T_m) using a non-linear least squares method according to the relation:

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

where $y(T)$ is the optical property at temperature T (Kelvin), $y_N(T)$ 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.* $y_N(T) = a_N + b_N T + c_N T^2$ and $y_D(T) = a_D + b_D T + c_D T^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients) [26]. A plot of ΔH_m versus T_m at each concentration of Dextran 70 gave the value of ΔC_p, the constant-pressure heat capacity change. The value of ΔG_D at any temperature T, $\Delta G_D(T)$, was estimated with the help of the Gibbs–Helmholtz equation (Eq. (2)) with values of ΔH_m, T_m and ΔC_p.

$$\Delta G_D(T) = \Delta H_m\left(\frac{T_m - T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ \ln\left(\frac{T}{T_m}\right)\right] \quad (2)$$

2.4. Circular dichroism (CD) measurements

CD measurements were made in a Jasco J-810 spectropolarimeter equipped with a Peltier-type temperature controller with six accumulations. Protein concentration used for the CD measurements was 0.5 mg ml^{-1}. Cells of 0.1 and 1.0 cm path lengths were used for the measurements of the far and near-UV spectra, respectively. Necessary blanks were subtracted for each measurement. All readings for native state structural analysis were procured at 20 °C.

3. Results

To investigate for the effect of Dextran 70 on the thermodynamic stability of proteins, we carried out heat-induced denaturation studies of the three proteins in the presence of different Dextran 70 concentrations (0, 50, 100, 200 and 300 g l^{-1}) at different pH values (7.0, 6.0, 5.0, 4.0 and 3.0) by following the changes in absorbance at 287 nm for RNase-A, 300 nm for lysozyme and 295 nm for holo-LA as a function of temperature. Denaturation of each protein was reversible in the entire range of [Dextran 70], the molar concentration of Dextran 70 at all the pH values except for holo-LA. In case of holo-LA, we observed visible precipitation at pH 5.0 and pH 4.0 in the presence of Dextran 70 concentrations greater than 200 g l^{-1}. Fig. 1 shows representative heat-induced denaturation profiles of RNase-A, lysozyme and holo-LA at physiological pH (left panel) and lower pH (right panel), respectively. In the case of lysozyme, complete transition curves could not be obtained in the measurable temperature range at the given pH values. In order to bring down transition curves in the measurable temperature range, 1.5 M GdmCl was added to the samples. Therefore, the transition curves shown in Fig. 1 for lysozyme are the curves obtained in the presence of GdmCl. Each denaturation curve of a protein at a given [Dextran 70] was analyzed for T_m and ΔH_m and using a nonlinear least-squares method that involves fitting the entire data of the transition curve to Eq. (1) with all eight free parameters (a_N, b_N, c_N, a_D, b_D, c_D, ΔH_m and T_m). Table 1 shows values of T_m and ΔH_m of all the three proteins in the absence and presence of different [Dextran 70] at all the pH values. It is seen in Table 1 that the T_m of all the proteins remains unperturbed in the presence of Dextran 70 at physiological pH in contrast to the increase in T_m observed at lower pH values.

Table 1 also shows ΔC_p values obtained from the analyses of the plots of ΔH_m versus T_m at 5 different pH values in the absence and presence of different [Dextran 70]. Using the measured values of T_m, ΔH_m and ΔC_p, ΔG_D° values (the value of ΔG_D at 25 °C) in the absence and presence of Dextran 70 were also estimated using Eq. (2). The values of ΔG_D° estimated in such manner are also presented in Table 1. It is seen in Table 1 that similar to the effect of Dextran 70 on T_m, the ΔG_D° values are also not significantly perturbed at physiological pH.