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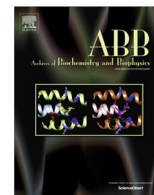

Osmolyte mixtures have different effects than individual osmolytes on protein folding and functional activity

Marina Warepam, Laishram Rajendrakumar Singh *

Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110 007, India

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ABSTRACT

Osmolytes are small organic molecules accumulated by organisms under stress conditions to protect macromolecular structure and function. In the present study, we have investigated the effect of several binary osmolyte mixtures on the protein folding/stability and function of RNase-A. For this, we have measured ΔG_D^o (Gibbs free energy change at 25 °C) and specific activity of RNase-A mediated hydrolysis of cytidine 2′–3′ cyclic monophosphate in the presence and absence of individual and osmolyte mixtures. It was found that the osmolyte mixtures have different effect on protein stability and function than that of individual osmolytes. Refolding studies of RNase-A in the presence of osmolyte mixtures and individual osmolytes also revealed that osmolyte mixtures have a poor refolding efficiency relative to the individual osmolytes.

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Introduction

Environmental stresses such as water stress, salt stress, cold and heat stress constantly challenge the stability and function of intracellular macromolecules [1–3]. One general mechanism of adaptation against these stresses is the accumulation of small molecular weight compounds known as osmolytes [1–3]. In general these osmolytes are grouped into three different chemical classes: polyols (e.g., sorbitol, sucrose, trehalose) commonly found in terrestrial plants, insects, polar fishes; free amino acids (e.g., proline, glycine, alanine) and their derivatives (e.g., taurine, ectonine, octopine) in numerous prokaryotes and methyl ammonium compounds (e.g., trimethylamine N-oxide, glycine betaine, sarcosine) in marine elasmobranches [1]. Based on functional activity they are grouped as (i) compatible osmolytes (that have no significant effect on enzyme activity e.g., most amino acids and polyols) [1,2,4] and (ii) non-compatible (that affects the enzyme activity e.g., all methylamines, lysine, arginine, histidine) [2,3,5]. The basic premise of accumulation of osmolytes is that they do not largely alter the enzyme's functional activity even when present at a very high concentration (except some osmolytes like proline) [4] up to several millimolars [2,3].

The effect of osmolytes on protein stability and function has been largely investigated. It is well known that the ability of the osmolytes to fold or stabilize proteins is due to its unfavourable interactions with the peptide backbones exposed upon protein denaturation leading to preferential hydration of the protein molecules [6,7]. It has also been shown that (i) osmolytes can force folding of unstable, intrinsically disordered and many mutant proteins [8–10] and (ii) help to inhibit/reverse protein aggregation [11–13]. Therefore, osmolytes have been promising potential therapeutics for a large number of human diseases caused due to protein conformational disorders [14]. It is to be noted that almost all these developments were derived based on studies of the effect of individual osmolytes on proteins. In contrast, the cellular osmolytic pool generally consists of mixture of several osmolytes rather than individuals at a given stress condition [15–20]. Thus, results on the effect of individual osmolytes on protein folding bear less resemblances with that of the actual *in vivo* conditions. Therefore to have a more realistic protein folding insight *in vitro* by osmolytes, it is important to investigate protein folding and functional activity studies in the presence of different osmolyte mixtures (not with individual osmolytes). In the present study, we have investigated the effects of several binary osmolyte mixtures (found in the marine elasmobranches and mammalian kidney cells) on the activity and stability of Ribonuclease-A (RNase-A)[1]. We discovered that osmolyte mixtures have different effects than those of their individuals in protein stability and function. Osmolyte mixtures have poor protein folding ability (induces folded proteins that have low

* Corresponding author. Fax: +91 11 27666248.
E-mail address: lairksingh@gmail.com (L.R. Singh).

[1] *Abbreviations used:* C_m, the midpoint of denaturation; CD, circular dichroism; GdmCl, guanidinium chloride; ε, molar absorption coefficient; K_m, Michaelis constant; k_{cat}, catalytic constant; RNase-A, Ribonuclease-A; TMAO, trimethylamine N-oxide.

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catalytic activity). The study indicates cross talks between/among osmolytes might help to regulate enzyme function *in vivo*.

Materials and methods

Commercially lyophilized preparation of RNase-A (type III-A) was purchased from Sigma Chemical Co. Betaine, sorbitol, TMAO, sarcosine, cytidine 2′–3′ cyclic monophosphate (C > p) and 8-anilino-1-naphthalene sulfonic acid (ANS) were also obtained from Sigma. Guanidinium chloride (GdmCl) was purchased from MP Biomedicals. These chemicals, which were of analytical grade, were used without further purification.

RNase-A solution was dialyzed extensively against 0.1 M KCl at pH 7.0 and 4 °C. Protein stock solution was filtered using 0.22 μm millipore filter paper. The protein gave a single band during polyacrylamide gel electrophoresis. Concentration of the protein solution was determined experimentally using molar absorption coefficient, ε (M^{-1} cm^{-1}) value of 9800 at 277.5 nm [21]. The concentration of GdmCl stock solution was determined by refractive index measurements [22]. All solutions for optical measurements were prepared in the degassed 0.05 M cacodylic acid buffer containing 0.1 M KCl. Since pH of the protein solution may change on the addition of cosolvents, pH of each solution was also measured after the denaturation and activity experiments. It was observed that the change in pH was not significant.

Activity measurement of native RNase-A

RNase-A activity was measured using cytidine 2′–3′ cyclic monophosphate (C > p) as the substrate (0.25 mg ml^{-1}) following the procedure described by Crook et al. [23]. In order to see the effects of osmolytes on the activity of RNase-A, the enzyme (0.035 mg ml^{-1}) was titrated with the desired concentration of osmolytes and incubated overnight. The reaction for RNase-A mediated hydrolysis of C > p in the absence and presence of individuals and their binary osmolyte mixtures at a given concentration was followed by measuring absorbance at 292 nm for 1 h in Jasco V-660 UV/Vis spectrophotometer equipped with a Peltier-type temperature controller (ETCS-761). It should be noted that enzymatic reactions in the absence or presence of osmolytes were observed to be completed in 1 h. From each progress curve, the initial linear portion (usually first 60 s) was used as a measure of specific activity.

GdmCl-induced denaturation measurements

GdmCl-induced denaturation of native RNase-A in the presence and absence of individual osmolytes and their binary mixtures were followed by measuring changes in the $[\theta]_{222}$ (mean residue ellipticity at 222 nm) as a function of GdmCl concentration at 25 °C and pH 7.0 using a J-810 (Jasco spectropolarimeter) equipped with a Peltier-type temperature controller (Jasco PTC-424S). Protein concentration used for circular dichroism was 0.5 mg/ml. It should be noted that each protein solution at each concentration of GdmCl was kept for adequate time (overnight) to allow equilibration. The CD signal was converted into mean residue ellipticity (deg cm^2 $dmol^{-1}$) using the relation,

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

where θ_λ is the observed ellipticity (millidegrees) at the wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), l is the path length (centimetres). The optical transition data were converted into ΔG_D, the Gibbs energy change for unfolding using the relation,

$$\Delta G_D = -RT \ln\{(y - y_N)/(y_D - y)\}, \tag{2}$$

where R is the universal gas constant, T is the temperature in Kelvin, y is the observed optical property and y_N and y_D are, respectively, the optical properties of the native and denatured protein molecules under the same experimental condition in which y has been determined. We then use linear extrapolation method for the analysis of ΔG_D^o as described previously [24,25], using the following equation

$$\Delta G_D = \Delta G_D^o - m_d[\text{Gdmcl}], \tag{3}$$

where ΔG_D^o is the value of ΔG_D at 0 M denaturant, and m_d gives the linear dependence of ΔG_D on the [GdmCl].

The reversibility of each of the GdmCl-induced denaturation curve was measured by diluting the protein samples at high GdmCl concentrations (5.5 M) down to the respective C_m values. It was observed that the optical property of the protein solution at C_m from the denaturation and renaturation experiments were similar.

Activity measurement of refolded RNase-A

For refolding experiments, heat-induced (85 °C) denatured RNase-A was cooled down in the presence of osmolytes to reach 25 °C using a dry bath (Indogenix). Refolding of the GdmCl-induced (5.5 M) denatured RNase-A was carried out by diluting the denatured protein to a ratio of l:100 using the cacodylic acid buffer (pH 7.0) that contains desired concentration of the osmolytes and kept overnight for equilibration. For the measurement of the specific activity of the refolded RNase-A, we used the same procedure described under the activity measurement of native RNase-A. All enzyme assays were carried out at least three times.

Thermal-induced denaturation measurements

Thermal-induced denaturation of native RNase-A and refolded RNase-A (from GdmCl, 5.5 M denatured state) in the presence and absence of binary osmolyte mixtures were followed by measuring changes in the $[\theta]_{222}$ (mean residue ellipticity at 222 nm) as a function of temperature (25–85 °C) at pH 7.0 using a J-810 (Jasco spectropolarimeter) equipped with a Peltier-type temperature controller (Jasco PTC-424S) with a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Protein concentration used for circular dichroism was 0.5 mg/ml. After denaturation, the sample was cooled down to measure reversibility. The reversibility was checked by comparing the optical property of the protein before and after round of denaturation and was found to be identical (data not shown). Each heat-induced transition curve was analysed for T_m (the midpoint of heat denaturation) using a non linear least-squares analysis equation [26],

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m/R(1/T - 1/T_m)]} \tag{4}$$

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 the denatured protein molecules, 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 $y_D(T) = a_D + b_DT + c_DT^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients.

Structural measurements

Far- and near-UV CD spectra of native, refolded (obtained from GdmCl-induced denatured state) and denatured state of RNase-A

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