Archives of Biochemistry and Biophysics 591 (2016) 7–17

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Testing the dependence of stabilizing effect of osmolytes on the fractional increase in the accessible surface area on thermal and chemical denaturations of proteins

Safikur Rahman [1], Syed Ausaf Ali, Asimul Islam, Md. Imtaiyaz Hassan, Faizan Ahmad*

Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, 110025, India

ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form
4 November 2015
Accepted 21 November 2015
Available online 12 December 2015

Keywords:
Osmolytes
Methylamines
Protein stabilization
Accessible surface area
Gibbs free energy

ABSTRACT

Here we have generated two different denatured states using heat- and guanidinium chloride (GdmCl) - induced denaturations of three disulfide bond free proteins (barstar, cytochrome-*c* and myoglobin). We have observed that these two denatured states of barstar and myoglobin are structurally and energetically different, for, heat-induced denatured state contains many un-melted residual structure that has a significant amount of secondary and tertiary interactions. We show that structural properties of the denatured state determine the magnitude of the protein stabilization in terms of Gibbs free energy change (ΔG_D°) induced by an osmolyte, i.e., the greater the exposed surface area, the greater is the stabilization. Furthermore, we predicted the *m*-values (ability of osmolyte to fold or unfold proteins) using Tanford's transfer-free energy model for the transfer of proteins to osmolyte solutions. We observed that, for each protein, *m*-value is comparable with our experimental data in cases of TMAO (trimethylamine-N-oxide) and sarcosine. However, a significant discrepancy between predicted and experimental *m*-values were observed in the case of glycine-betaine.

1. Introduction

Many proteins refold from their unfolded state to attain the native functional state. The correct folding of the protein polypeptide chain is determined not only by its amino acid sequence but also by the solvent environment. Cells are quite often exposed to widely fluctuating environmental conditions such as extremes of temperature, pH, cellular dehydration, desiccation, high extracellular salts, and even the presence of denaturing concentrations of urea inside the cell [1–3]. Protein folding in the cell is quite often challenged because of these environmental stresses. Failure to circumvent these hostile external environmental stresses may lead to various proteopathies, including protein misfolding, aggregation, amyloid formation and enhanced proteosomal degradation due to protein destabilization and eventually apoptosis [4,5]. Even the protein quality control system, including molecular chaperones, specialized intracellular proteases and accessory factors that regulate the activity of chaperones and proteases, may also get damaged due to the extreme stresses.

Interestingly, organisms or cells have a developed mechanism of accumulation of low molecular mass organic molecules called osmolytes which protect them against adverse environmental stresses. Osmolytes can be grouped into three chemical classes as polyols and sugars (e.g., trehalose, glycerol, inositols, etc.), amino acids (e.g., glycine, proline, etc.) and their derivatives (e.g., β-alanine, taurine, etc.) and combinations of methylamines (e.g., sarcosine, trimethylamine-N-oxide (TMAO), etc.) and urea [3,6]. Often, they are classified as compatible or counteracting based on their effects on both the stability and function of proteins [7]. Compatible osmolytes increase stability of proteins against thermal denaturation with little or no effect on their function near room temperature [8,9]. Representatives of this class include certain amino acids and polyols [8,9]. Counteracting osmolytes, on the other hand, are believed to have a special ability to protect intracellular proteins against inactivation and/or destabilization by urea [10–13]. That is, they cause changes that are opposite to the effect of urea on protein stability and function of the protein [1,9,14,15]. Organs like mammalian kidney and many animals like cartilaginous fishes and coelacanth that are rich in urea, employ these

* Corresponding author.
E-mail address: fahmad@jmi.ac.in (F. Ahmad).
[1] Present address: Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110 007, India.

http://dx.doi.org/10.1016/j.abb.2015.11.035
0003-9861/

counteracting osmolytes including glycine-betaine, glycerophosphorylcholine (GPC) and TMAO [6,8,10].

There are two defining characteristics of compatible osmolytes, namely, they stabilize proteins against denaturation by heat or chemicals [16–21], and their presence in the cell does not largely alter protein functional activity [3,9,18,22–24]. The mechanism of osmolytes-induced protein stability has been studied extensively [3,9,25–28]. It has been widely accepted that osmolytes stabilize or induce protein folding due to preferential hydration effect [26,27]. Bolen et al. [29,30] from their elegant series of experiments that measured the transfer-free energy of the amino acid side chains and backbone, showed that the preferential hydration effect on proteins is chiefly because of the unfavorable interactions of osmolytes with the peptide backbone in the denatured state while the total contribution of amino acid side chains is little. Their studies led to the conclusion that osmolytes-induced thermodynamic stability of proteins is due to shift in denaturation equilibrium, N (native state) ↔ D (denatured state), from right to the left.

Although, osmolytes-facilitated protein stability has been deeply studied, most of these studies have been carried out on proteins having disulfide bond cross links or intrinsically unstructured proteins. Furthermore, to the best of our knowledge, no earlier studies compared osmolyte-induced thermodynamic stability of a protein obtained from heat- and GdmCl-induced denaturation, as it is widely known that these two modes of protein denaturation may give structurally two different denatured states [31–33]. To mimic the intercellular state of disulfide bonds containing proteins, we have used three different disulfide free proteins. We report that, for such a protein, the osmolyte-induced stabilizing effect in terms of Gibbs free energy change (ΔG_D°), obtained from the GdmCl-induced denaturation is larger than that obtained from the heat-induced denaturation. Furthermore, we have theoretically predicted the *m*-value (ability of osmolyte to refold or unfold protein) and found a good correlation between theoretical and experimental *m*-values of all GdmCl-denatured proteins.

2. Materials and methods

2.1. Materials

Commercial lyophilized preparation of horse heart myoglobin (Mb) and bovine cytochrome-*c* (b-cyt-*c*) were purchased from Sigma Chemical Co (St. Louis, MO). Barstar (from *Bacillus amyloliquefaciens*) was expressed in *Escherichia coli* and purified by the method published by Khurana and Udgaonkar [31]. GdmCl was purchased from MP Biomedical Inc. TMAO, Sarcosine and Glycine-betaine (GB) were obtained from Sigma Chemical Co (St. Louis, MO). These and other chemicals, which were of analytical grade, were used without further purification.

2.2. Preparation of protein solutions

Mb and b-cyt-*c* were oxidized by dissolving them in 0.1 and 0.01% potassium ferricyanide solutions, respectively. These solutions were then dialyzed against several changes of 0.1 M KCl solution (pH 7.0) at 4 °C. Barstar was also dialyzed in cold against 0.1 M KCl at pH 7.0. The concentrations of stock solutions of proteins were determined using molar absorption coefficient, ε ($M^{-1}cm^{-1}$) values of 171,000 at 409 nm for Mb [34], 106,000 at 409 nm for b-cyt-*c* [35] and 23,000 at 280 nm for barstar [31]. All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl.

2.3. Spectroscopic determination of free energy change associated with GdmCl- and heat-induced denaturations

GdmCl-induced denaturations of b-cyt-*c*, Mb and barstar in the absence and presence of different concentrations of each methylamine were monitored by following changes in $[\theta]_{222}$ (molar ellipticity at 222 nm) at pH 7.0 and 25 °C. The unfolding transitions of these proteins were reversible in the entire [g] (molar GdmCl concentration) range in the presence of each co-solute. Using a non-linear least-squares method, the entire data (y(g), [g]) of each denaturant-induced transition curve were analyzed for ΔG_D°, m_g and C_m using the relation [36],

$$y(g) = \frac{y_N(g) + y_D(g) \times e^{\left[-\left(\Delta G_D^0 + m_g[g]\right)/RT\right]}}{1 + e^{\left[-\left(\Delta G_D^0 + m_g[g]\right)/RT\right]}} \quad (1)$$

where y(g) is the observed optical property at [g], y_N and y_D are optical properties of the native and denatured protein molecules under the same experimental conditions in which y(g) was measured, ΔG_D° is the value of Gibbs free energy change (ΔG_D) in the absence of denaturant, m_g is the slope $(\partial \Delta G_D/\partial[g])_{T,P}$, R is the universal gas constant, and T is the temperature in Kelvin. It should, however, be noted that the analysis of each GdmCl-induced transition curve was done assuming that unfolding is a two-state process and [g]-dependencies of y_N(g) and y_D(g) are linear (*i.e.*, $y_N(g) = a_N + b_N$ [g] and $y_D(g) = a_D + b_D$ [g], where a and b are [g]-independent parameters, and subscripts N and D represent these parameters for the native and denatured protein molecules, respectively).

Heat-induce-denaturation studies were carried out in Jasco spectropolarimeter, Model J-1500-150 (JASCO Corporation, Japan), equipped with Peltier-type temperature controller, at a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. The change in secondary structure of the protein with increasing temperature was followed by measuring the far-UV CD at 222 nm. About 650 data points (data point at 0.1 °C interval) of each transition curve were collected. After denaturation, the sample was immediately cooled down to measure reversibility of the process. Each heat-induced transition curve was analyzed for T_m and ΔH_m using a non-linear least-squares analysis according to the relation:

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

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 temperature T (Kelvin), and R is the gas constant. In the analysis of each heat-induced 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) [37,38]. The value of the temperature-independent constant-pressure heat capacity change (ΔC_p) was determined from the slope of the linear plots of ΔH_m versus T_m, using the relation:

$$\Delta C_p = \left(\frac{\delta \Delta H_m}{\delta T_m}\right)_p \quad (3)$$

Using values of T_m, ΔH_m and ΔC_p, the value of ΔG_D at any temperature T, $\Delta G_D(T)$ was estimated using the Gibbs–Helmholtz equation: