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Counteraction of the deleterious effects of urea on structure and stability of mammalian kidney proteins by osmolytes

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ABSTRACT

Owing to the urine concentrating mechanism of kidney cells, urea concentration is very high (3.0–5.0 M) in mammalian kidneys which may denature many kidney proteins. Methylamines are known to counteract the deleterious effects of urea on structure, stability and function of proteins at 2:1 molar ratio of urea to methylamines. It is known that mammalian kidney cells also contain stabilizing osmolytes, non-methylamines (*myo*-inositol and sorbitol). A question arises: Do these non-methylmine osmolytes have ability to counteract the deleterious effects of urea on kidney proteins? To answer this question, we took two kidney proteins, namely, sheep serum albumin and Human carbonic anhydrase II. We measured their thermodynamic stability ($\Delta G^0_{N\leftrightarrow D}$, the Gibbs free energy change in absence of GdmCl (guanidinium chloride) associated with the equilibrium, native (N) state ↔ denatured (D) state) from the GdmCl-induced denaturation curves in the presence of different concentrations of urea and each kidney osmolyte individually and in combination. For both proteins, we observed that (i) glycine betaine and *myo*-inositol provide perfect counteraction at 2:1 molar ratio of urea to osmolyte, i.e., denaturing effect of 2 M urea is 100% neutralized by 1 M of glycine betaine (or *myo*-inositol), and (ii) sorbitol fails to refold urea denatured proteins.

1. Introduction

Micro-organisms, plants and animals living under different harsh environmental stress conditions such as very high and low temperatures, high pressure, high salts concentration, high urea concentration, have evolved a common mechanism to cope up with these stressful conditions. This mechanism involves accumulation of low molecular weight organic compounds collectively called osmolytes [1–6]. Osmolytes are divided into three groups: amino acids and their derivatives, sugars and polyols, and methyl ammonium compounds. These osmolytes are traditionally called stabilizing osmolytes, for they increase T_m (midpoint of denaturation) of proteins. That is, they have ability to shift the heat-induced denaturation equilibrium, native (N) state ↔ denatured (D) state from right to the left [7,8].

The renal inner medulla plays a key role in concentration and dilution of urine [9–11]. During antidiuresis, cells of renal medulla are exposed to high concentration of urea [12,13]. It has been reported that urea concentration is 1 M or more in mammalian kidney and it may reach up to 5 M in some desert rodents [14]. These concentrations of urea are enough to induce protein denaturation. It has also been reported that under such denaturing urea concentration, cells of renal medulla contain large amounts of methylamines (glycine betaine (GB) and glycerophosphocholine) [15]. In vitro and in vivo experiments have also shown that structure, stability and function of proteins are preserved by the opposing effects of urea and methylamines when molar concentration ratio of methylamine to urea is 1:2 [16,17]. Hence, methylamines are also called counteracting osmolytes. Another role of methylamines is presumably to balance the osmotic pressure of extracellular NaCl, which is normally high in the renal inner medulla [18,19].

It has been reported that, owing to urinary concentrating mechanism, cells in the renal inner medulla have high extracellular salts [20,21] which would cause cells to shrink. To protect cells of renal medulla, organisms accumulate *myo*-inositol and sorbitol, for it has been reported that with an increase in extracellular NaCl concentration, the concentration of these polyols increases in the kidney cells [18,22]. It is particularly interesting to note that it is not known whether, as observed for methylamines, these kidney osmolytes

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(non-methylamines) have the ability to counteract the deleterious effect of urea on cellular proteins of renal medulla. To answer this question, we have measured thermodynamic stability of two mammalian kidney proteins, namely, sheep serum albumin (SSA) and human holo-carbonic anhydrase II (HCAII) in the presence of urea and each of osmolytes (*myo*-inositol, sorbitol and GB) alone and in combination. We found that (i) each osmolyte alone stabilizes proteins against guanidinium chloride (GdmCl)-induced denaturation, (ii) urea alone favours GdmCl-induced denaturation of proteins, and (iii) in the osmolyte-urea mixture, both *myo*-inositol and GB bring about perfect counteraction of urea's effect at a molar ratio of 1:2 ([osmolyte]:[urea]), while sorbitol fails to counteract the deleterious effect of urea.

2. Materials and methods

2.1. Materials

Consumables used were of high analytical grade. SSA was extracted and purified from kidneys of sheep killed for food by the Slaughter House, Ghazipur, New Delhi, India. pET-15d vector for human carbonic anhydrase II (HCAII) was purchased from Novagen, Wisconsin, USA. Ampicillin and isopropyl−D-1-thiogalactopyranoside (IPTG) were obtained from Sigma (Saint Louis, USA). Ni-sepharose beads, Sephacryl S-100, Hi Trap DEAE FF and Superdex 200 pg columns were purchased from GE Healthcare, Sweden. 0.22 μm syringe filter was obtained from Millipore Corporation (USA). Sorbitol, *myo*-inositol, and glycine betaine (GB) were obtained from Sigma Chemical Co. Electrophoresis reagents were procured from Merck (Darmstadt, Germany). Urea and GdmCl were ultra pure samples from MP Biomedicals, USA.

2.2. Protein sample preparations

SSA was purified from sheep kidneys as described by Dar et al. [23]. HCAII was cloned, expressed and purified as described by Wahiduzzaman et al. [24] who have used 0.5 mM $ZnSO_4$ in the culture medium during over expression of the protein. The protein concentration of SSA and HCAII was determined experimentally using values of 49,915 M^{-1} cm^{-1} [25] and 50,420 M^{-1} cm^{-1} [24] for molar absorption coefficient at 280 nm (ε_{280}) of SSA and HCAII, respectively. For each experiment, a fresh 10.0 M urea stock solution of the denaturant in 10 mM Tris-HCl buffer (pH 7.4) was prepared. For GdmCl-induced denaturation studies, 7.0 M stock solution of the denaturant was prepared in the same buffer. Stock solution concentration of each denaturant was determined by the refractive index measurements [26]. All solutions used for optical measurements were degassed in order to avoid air bubbles.

2.3. Circular dichroism (CD) measurement

The far-UV CD measurements (250–200 nm) of SSA and HCAII were carried out in Jasco-1500 CD spectropolarimeter which was attached with Peltier-type temperature controller (PTC-517). Since urea and GdmCl show very high HT voltage below 210 nm, experiments in the presence of these denaturants were carried out from 250 to 210 nm. The scan speed, response time and number of accumulations were fixed at 100 nm min^{-1}, 1 s, and 5, respectively. Each spectrum was corrected for the contribution of the blank solution. The observed CD signal at wavelength λ (θ_λ, the ellipticity (millidegree) at wavelength λ) was converted into concentration-independent quantity, [θ]λ, the mean residue ellipticity (deg cm^2 $dmol^{-1}$) using the relation,

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

where M_o is the mean residue weight of the protein, l is the path length of the cell in centimetres, and c is the protein concentration in mg ml^{-1}. For CD measurements, 0.15 and 0.30 mg ml^{-1} of SSA and HCAII were used, respectively.

2.4. Reversibility of GdmCl-induced denaturation

Reversibility of denaturation induced by GdmCl in the presence of each kidney osmolyte was checked by following a procedure described earlier [27]. Briefly, for each denaturation experiment, a known amount of buffer, and required amounts of osmolyte and concentrated GdmCl solution (in the same buffer) were mixed followed by the addition of a known amount of protein stock solution. This protein solution was incubated overnight, a time long enough to complete the denaturation. For each renaturation experiment, protein was denatured first. To denature the protein, a known amount of protein was mixed with known amounts of concentrated GdmCl and osmolyte solutions (both in the same buffer), and this solution was incubated for 6 h, a time long enough to denature the protein. This denatured protein solution was diluted with required amount of the buffer, and solution was incubated for 12 h, a time long enough to complete renaturation of the protein. An observation of identical far-UV spectra of the protein from both denaturation and renaturation experiments is taken as a criterion for reversibility of denaturation in the presence of osmolytes.

2.5. Data analysis

As shown in Results section, GdmCl-induced denaturation of SSA in the absence of stabilizing osmolytes (*myo*-inositol, sorbitol and GB) and in the presence and absence of urea is a two-step process, i.e., N state ↔ X (intermediate) state ↔ D state. That is, there exists a stable intermediate (X) between N and D states. In the analysis of such a transition curve, data were separated into two consecutive transition curves, i.e., N state ↔ X state (transition I) and X state ↔ D state (transition II), and each transition curve was analyzed separately for stability parameters [28–30]. Values of Gibbs free energy change ($\Delta G_{N\leftrightarrow I}$), associated with the equilibrium, N state ↔ X state (transition I) and $\Delta G_{I\leftrightarrow D}$, Gibbs free energy change, associated with the equilibrium, X state ↔ D state (transition II) were determined using relations,

$$\Delta G_{N\leftrightarrow I} = -RT\ln(y\text{-}y_N)/(y_X\text{-}y) \quad (2)$$

$$\Delta G_{I\leftrightarrow D} = -RT\ln(y\text{-}y_X)/(y_D\text{-}y) \quad (3)$$

where y is observed optical property, y_N, y_X and y_D respectively are optical properties of the protein molecules in the native, intermediate and denatured states under the same experimental condition in which y has been measured, R is the gas constant, and T is temperature in Kelvin.

If the dependence of both $\Delta G_{N\leftrightarrow I}$ and $\Delta G_{I\leftrightarrow D}$ on [GdmCl] is linear, it is then described by relations,

$$\Delta G_{N\leftrightarrow I} = \Delta G^0{}_{N\leftrightarrow I} - m_I[\text{GdmCl}] \quad (4)$$

where $\Delta G^0{}_{N\leftrightarrow I}$ is the value of $\Delta G_{N\leftrightarrow I}$ in the absence of GdmCl, and m_I is the slope ($\partial\Delta G_{N\leftrightarrow I}/\partial$[GdmCl]. The midpoint of denaturation, C_{mI} is equal to $\Delta G^0{}_{N\leftrightarrow I}/m_I$), and

$$\Delta G_{I\leftrightarrow D} = \Delta G^0{}_{I\leftrightarrow D} - m_{II}[\text{GdmCl}] \quad (5)$$

where $\Delta G^0{}_{I\leftrightarrow D}$ is the value of $\Delta G_{I\leftrightarrow D}$ in the absence of GdmCl, m_{II} is the slope ($\partial\Delta G_{I\leftrightarrow D}/\partial$[GdmCl]). The midpoint of denaturation, C_{mII} is equal to $\Delta G^0{}_{I\leftrightarrow D}/m_{II}$.

As shown below, GdmCl-induced denaturation of SSA in the presence of stabilizing osmolytes is a one-step process, N state ↔ D