Mixed osmolytes: The degree to which one osmolyte affects the protein stabilizing ability of another

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Abstract

Mixtures of organic osmolytes occur in cells of many organisms, raising the question of whether their actions on protein stability are independent or synergistic. To investigate this question it is desirable to develop a system that permits evaluation of the effect of one osmolyte on the efficacy of another to either force-fold or denature a protein. A means of evaluating the efficacy of an osmolyte is provided by its *m*-value, an experimental quantity that measures the ability of the osmolyte to force a protein to unfold or fold. An experimental system is presented that enables evaluations of the *m*-values of osmolytes in the presence and absence of a second osmolyte. The experimental system involves use of a marginally stable protein in 10 mM buffer (pH 7, 200 mM salt, and 34°C) that is at the midpoint of its native to denatured transition. These conditions enable determination of *m*-values for protecting and denaturing osmolytes in the presence and absence of a second osmolyte, permitting assessment of the extent to which the two osmolytes affect each other's efficacy. The two osmolytes investigated in this work are the denaturing osmolyte, urea, and the protecting osmolyte, sarcosine. Results show unequivocally that neither osmolyte alters the efficacy of the other in forcing the protein to fold or unfold—the osmolytes act independently on the protein despite their combined concentrations being in the multi-molar range. These osmolytes avoid altering one another's efficacy at these high concentrations because the number of osmolyte interaction sites on the protein is large and the binding constants are quite small. Consequently, the site occupancies are low enough in number that the two osmolytes neither compete nor cooperate in interacting with the protein.

Keywords: protein stability; protein folding; osmolytes; *m*-values; sarcosine

Common environmental stresses encountered by organisms tend to cause cellular water loss or gain. Vast numbers of organisms have evolved to handle water loss or gain by increasing or decreasing the intracellular levels of small organic molecules called osmolytes (Yancey et al. 1982). In many cases, the environmental stress is accompanied by additional stresses that threaten the stability of intracellular macromolecules. So in addition

to their ability to control cell water loss or gain, many osmolytes have been selected for their ability to stabilize cell components such as proteins (Hochachka and Somero 2002). In fact, it is common to find more than one intracellular organic osmolyte in the cells of organisms (Yancey et al. 1982; Burg 1995, 2000; Yancey 2005). The presence of mixtures of osmolytes inside cells raises a host of questions about their individual and/or combined roles in protecting the organism against the environmental stresses. The most immediate question is whether combinations of osmolytes are synergistic or independent in their ability to protect the cell (and organism) against potentially harmful stresses the organism encounters.

The mammalian kidney is a case in point. A primary function of the nephron is to concentrate salt and urea for

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excretion, but because urea is permeable to cell membranes, the concentrated urea diffuses and is also transported into adjacent cells (e.g., collecting duct cells), threatening the structure and function of intracellular proteins (Nakayama et al. 2000). Fortunately, protecting osmolytes can be increased in these cells (Garcia-Perez and Burg 1991; Burg 1995), and some of the osmolytes are known to counteract urea's negative effects on proteins (Yancey et al. 1982; Burg 1995; Baskakov et al. 1998; Bolen 2001; Yancey 2005). Given the essential roles of osmolytes in protecting the cell, it is important to determine how one osmolyte's effect on protein stability is affected by the presence of a second osmolyte.

There are three chemical classes of protecting osmolytes (Yancey et al. 1982), and while all of them have varying abilities to stabilize proteins, they also have different physical and chemical properties that may alter the function of a second osmolyte. Mello and Barrick (2003) were first to address this issue, showing experimentally that TMAO did not alter the ability of urea to denature barnase or notch ankyrin domain. Since these effects are not yet predictable, it will be necessary to investigate each pair of osmolytes to learn by experiment whether one affects the other's efficacy in stabilizing or destabilizing proteins. Both sarcosine (a zwitterion) and TMAO (uncharged, pH 7) are methylamines and have the potential to counteract urea's denaturing effect on proteins. In the work described here, we provide an experimental strategy to investigate how the individual effect of either urea or sarcosine on protein stability is affected by the presence of the other osmolyte.

An experimental measure of the effect of an osmolyte on protein stability is the slope (m -value, $d\Delta G/d[\text{osmolyte}]$) of a plot of the native to denatured free energy change as a function of osmolyte concentration. Because the m -value reflects the effect that a change in the concentration of the cosolute has on the stability of the protein, it is a good measure of the "efficacy" of the osmolyte in forcing the protein either to fold or unfold. In this study, m -values for urea, a denaturing osmolyte, were determined as a function of sarcosine concentration, and sarcosine m -values were determined as a function of urea concentration.

Sarcosine and urea change the stability of proteins in opposite directions, so evaluating their m -values requires a particular arrangement such that m -values can be obtained both for denaturation and forced-folding at identical pH, temperature, salt, and buffer conditions. Our strategy is to use a protein shown by Mello and Barrick (2003) to be poised near the midpoint of its native to denatured transition at a given temperature, pH, salt, and buffer. This arrangement of conditions is sufficient to allow determination of the m -value for forced-folding by sarcosine and the m -value for forced-unfolding by urea each in the absence of the other.

There is more than one way to obtain m -values, and a clear distinction between the methods is important in comparing and contrasting the results reported here. Independent and separately determined m -values for sarcosine- and urea-induced folding and unfolding can readily be obtained as fitting parameters by fitting the respective folding and unfolding cooperative transitions to the linear extrapolation method (LEM) (Greene and Pace 1974; Pace 1986; Santoro and Bolen 1988). This *direct* method for obtaining m -values can also be used in evaluating m -values for either sarcosine- or urea-induced folding or unfolding in the presence of several fixed concentrations of the opposing osmolyte. With each such fitting, one also obtains as a fitting parameter $\Delta G_{N \rightarrow D, x}^o$, the standard free energy change in the presence of x concentration of the other osmolyte. The slope ($d\Delta G_{N \rightarrow D, x}^o/d[\text{osmolyte}]$) of a plot of $\Delta G_{N \rightarrow D, x}^o$ as a function of the concentration of the other osmolyte gives an alternate and *indirect* determination of its m -value. The *direct* and *indirect* determinations of m -values provide a thorough evaluation of the efficacy of one osmolyte in stabilizing or destabilizing a protein in the presence of another.

As discussed above, our strategy involves a protein poised at the midpoint of its native to denatured transition at a convenient temperature, pH, buffer, and salt concentration. The 15-kDa protein Nank4–7* meets these requirements. This protein contains four tandem ankyrin repeat sequences and is a subfragment of the seven ankyrin repeat fragment, Nank1–7* of the *Drosophila* Notch Receptor (Zweifel and Barrick 2001a,b). By setting experimental conditions to 34°C, 10 mM sodium phosphate buffer (pH 7.0), and 200 mM NaCl, the system is poised at the midpoint of the native to denatured transition, allowing evaluation of the efficacies of counteracting osmolytes alone and in combination.

Results

Assay conditions

The most suitable experimental conditions (pH, temperature, salt, and buffer concentration) for our needs were determined by the two experiments depicted in Figure 1. Figure 1A shows how the melting temperature (T_m) changes as a function of pH, obtained from thermal denaturations of Nank4–7* at several different pH values. Within the tested range of pH the figure shows two regions, one encompassing the theoretical pI for our protein (pI = 5.4), where aggregation takes place and accurate determination of the T_m was not possible, and a second region from pH 5.5 to 9.5 where reversible thermal denaturation occurs. As can be seen from the figure, a maximum stability is observed in the pH range between 6.0 and 7.5, and we used pH 7 in the experiments reported here to take advantage of the stability being minimally affected by small changes in pH ($\delta T_m/\delta \text{pH}$ closest to zero).

Figure 1. Defining experimental conditions for Nank4-7*. (A) Change in the T_m for Nank4-7* as a function of pH in 10 mM sodium acetate, 10 mM sodium phosphate, 10 mM glycine, and 200 mM NaCl. From this experiment, we selected pH 7, where T_m is relatively constant with pH. (B) Change in the T_m for Nank4-7* as a function of the NaCl concentration in 10 mM sodium phosphate buffer (pH 7.0). The dotted line is meant to guide the eye and indicates a propensity of the curve to level off at higher salt concentrations. At 200 mM NaCl the system approaches a regime in which T_m is relatively insensitive to changes in salt concentration.

Figure 1B shows the T_m dependence on NaCl concentration at pH 7, obtained from temperature denaturations at different salt concentrations. Nank4-7* becomes more stable with increasing salt concentration, and stability becomes maximal at high salt. In fact, with a NaCl concentration change from 0 to 50 mM the ΔT_m is six times that occurring when the salt is increased from 200 to 250 mM. Given that the protein is less sensitive to small salt fluctuations at high salt, 200 mM was used in the work described. For Nank4-7*, the use of 10 mM sodium phosphate buffer (pH 7.0), 200 mM NaCl, and 34°C provides the desired conditions of a 50:50 mixture of native:denatured protein in which forced-folding induced by sarcosine and forced-unfolding by urea can be studied in the presence and absence of the second osmolyte.

Direct and indirect m -values

In order to determine the interdependences of the urea/sarcosine pair, we performed a series of urea denaturation/

sarcosine forced-folding experiments with Nank4-7*. The observed molar ellipticities of Nank4-7* are plotted as a function of sarcosine and urea concentrations (see Fig. 2). The experiments consisted of urea-induced denaturations in the presence of several sarcosine concentrations (0, 0.6, 0.9, and 1.5 M) and of sarcosine-induced folding of Nank4-7* in the presence of several urea concentrations (0, 0.6, 0.9, and 1.5 M). The squares represent results with only one of the osmolytes present, with the open squares depicting sarcosine-induced folding of the protein in the absence of urea, and the semi-filled squares representing urea-induced denaturation of Nank4-7* in the absence of sarcosine. At the experimental conditions chosen, the titrations represented by the squares start with the ensemble near 50% folded, i.e., $[F]/[U] = 1$.

The solid lines in Figure 2 represent urea-induced denaturations performed in the presence of fixed sarcosine concentrations ranging from 0 (squares) to 1.5 M (inverted triangles). The dashed lines represent sarcosine-induced folding in fixed urea concentrations ranging from 0 (squares) to 1.5 M (inverted triangles). The whole set of curves is the result of a simultaneous fitting of all the data to the LEM (see Materials and Methods). The slopes and intercepts of the baselines were shared parameters in this fitting, while the m -values and $\Delta G_{N \rightarrow D,x}^o$ parameters were allowed to vary. This procedure enabled evaluation of the m -value and $\Delta G_{N \rightarrow D,x}^o$ directly from the fitting of each curve, and we refer to these m -values as *direct* m -values.

The other parameters evaluated from the fits shown in Figure 2 are values for $\Delta G_{N \rightarrow D,x}^o$, the free energy changes

Figure 2. Comparisons of urea-induced denaturation of Nank4-7* at fixed sarcosine concentrations: 0 M (half filled squares), 0.6 M (half filled circles), 0.9 M (half filled triangles), 1.5 M (half filled inverted triangles); and sarcosine-induced Nank4-7* folding at fixed urea concentrations: 0 M (open squares), 0.6 M (open circles), 0.9 M (open triangles), 1.5 M (open inverted triangles). Observed molar ellipticity values of Nank4-7* at 228 nm are plotted as a function of sarcosine or urea concentrations in 10 mM sodium phosphate buffer (pH 7.0) and 200 mM NaCl at 34°C. The lines (solid lines for urea denaturations, dashed lines for sarcosine forced folding) represent a global fitting of all the data sharing pre- and post-folding and unfolding baselines.

in the limit of zero denaturant but fixed concentrations, x , of sarcosine, and the free energy changes in the limit of zero sarcosine at fixed concentrations, x , of urea. These $\Delta G_{N \rightarrow D, x}^o$ data are plotted in Figure 3. The slopes of these plots ($d\Delta G_{N \rightarrow D, x}^o/d[\text{sarcosine}]$ and $(d\Delta G_{N \rightarrow D, x}^o/d[\text{urea}])$) give *indirect* m -values for sarcosine and urea respectively.

The m -values for urea and sarcosine obtained by the *direct* method are shown in Figure 4A. The data show that the directly determined m -value for urea-induced denaturation of Nank4-7* in the absence of sarcosine, U0S, is identical to the m -values directly determined for urea in the presence of 0.6, 0.9, and 1.5 M sarcosine. Similarly, the *direct* m -value for sarcosine-induced folding of the protein in the absence of urea, S0U, is the same as the m -values determined in the presence of 0.6, 0.9, and 1.5 M urea. Figure 4B compares the *direct* m -values S0U and U0S with the corresponding *indirect* m -values obtained using $\Delta G_{N \rightarrow D, x}^o$ evaluated in the limit of zero concentration of sarcosine and of urea. The results of Figure 4, A and B, show unequivocally that neither urea nor sarcosine alters the efficacy of the other in forcing the protein to either fold or unfold.

Discussion

Protecting osmolytes such as TMAO and sarcosine are remarkable in their ability to protect proteins against denaturation due to a variety of stresses. Their ability to counteract the denaturing effect of urea is shown by the degree to which a fixed concentration of the protecting osmolyte shifts the midpoint of the denaturation transition ($C_{1/2}$) to higher values. Because our system is poised to allow *direct* determination of m -values for denaturation

Figure 3. Determinations of *indirect* m -values for sarcosine and urea. The m -values are inferred from the slopes of the plots of $\Delta G_{N \rightarrow D, x}^o$ vs. $[x]$. The values for $\Delta G_{N \rightarrow D, x}^o$ were obtained from linear extrapolation method (LEM) fits of data in Fig. 2. (Diamonds) $\Delta G_{N \rightarrow D, x}^o$ values obtained from the urea-induced denaturations of Nank4-7* in fixed concentrations (x) of sarcosine. The slope of this plot yields the *indirect* m -value for sarcosine. (Circles) $\Delta G_{N \rightarrow D, x}^o$ values obtained from sarcosine-induced foldings of Nank4-7* in fixed concentrations (x) of urea. The slope of this plot yields the *indirect* m -value for urea.

Figure 4. Comparisons of the m -values obtained from sarcosine forced-foldings and urea denaturations. (A) m -values directly obtained from LEM fits of urea-induced unfolding in the presence of 0, 0.6, 0.9, and 1.5 M sarcosine (U0S, U0.6S, U0.9S, and U1.5S, respectively) and LEM-determined m -values from fits of sarcosine-induced folding in presence of 0, 0.6, 0.9, and 1.5 M urea (S0U, S0.6U, S0.9U, and S1.5U). Within the errors of measurements, there are no differences in m -values for the UxS series, nor for the SxU series. (B) Comparison of the m -values determined directly and indirectly. (Dark gray bars, fit urea and fit sarc) *Indirect* m -values obtained as discussed in the text; (white bars) *direct* m -values obtained from fitting the urea-induced unfolding data shown in Fig. 2 in the absence of sarcosine (U0S), and fitting the sarcosine-induced folding data shown in Fig. 2 in the absence of urea (S0U). Comparison of the indirect and the direct m -values shows they are the same within error (fit urea vs. U0S, and fit sarc vs. S0U).

and forced-folding in the absence and presence of the second osmolyte, and provides data for the *indirect* evaluation of m -values for both osmolytes, sufficient data are obtained to define how the efficacy of one osmolyte in forcing a protein to fold or unfold depends on the presence or absence of the second osmolyte. In our view, since osmolytes are distinct in their energetic contributions to protein stability (Auton and Bolen 2005), once the dependences of the m -values for both osmolytes are established as

a function of the second osmolyte for any given globular protein, the same relationships should hold for any globular protein.

For the system under study, our results show that the effect of sarcosine on the stability of the protein (its *m*-value) is the same regardless of the absence or presence of different concentrations of urea. Similarly, the urea *m*-value is unaltered by the absence or presence of different concentrations of sarcosine (Fig. 4A,B). Provided that the stability of a protein in the presence of urea and TMAO ($\Delta G_{(urea,TMAO)}$) is given by Equation 1,

$$\Delta G_{(urea,TMAO)} = \Delta G_{N \rightarrow D}^o - m_{urea}[urea] - m_{TMAO}[TMAO] \quad (1),$$

Mello and Barrick (2003) were able to show that urea and TMAO have no effect on the other's ability to stabilize or denature a protein. It remains to be seen whether other urea/protecting osmolyte pairs follow the pattern observed for urea/TMAO and urea/sarcosine.

The collective concentrations of the osmolytes become high in these experiments, so it may come as a surprise that *m*-values are not changed in mixtures of osmolytes. What might be the molecular rationale for such behavior? Because the *m*-values for the two osmolytes remain unchanged in the presence or absence of the other, it is clear that the observed results cannot be explained by the two osmolytes interacting and canceling each other's effects on the protein. Our view is that an answer to the above question lies in the nature of the interactions of osmolytes and the native and denatured species (Auton and Bolen 2005; Street et al. 2006). The binding of osmolytes (such as urea and sarcosine) to proteins is typically very weak and has been discussed extensively by Schellman (Schellman 1978, 2002, 2003; Schellman and Gassner 1996). The average intrinsic association constant for urea to protein sites is favorable and typically $\sim 1.2 \text{ M}^{-1}$ (negative ΔG), a constant of 1.0 shows no preference for water over osmolyte ($\Delta G = 0$), and a constant < 1.0 shows a preference for water over osmolyte (positive ΔG) (Schellman 2002). We expect the average intrinsic association constant of sarcosine to the protein to be < 1.0 , giving a positive ΔG of interaction. Small binding constants mean that for the denatured or native protein only a small fraction of the possible binding sites for urea or sarcosine is occupied even when urea and/or sarcosine concentrations are quite high. Accordingly, there is no effective competition between the two osmolytes for sites on the protein; each one can exert its effect independently within the osmolyte concentration ranges investigated.

Finally, it is well known that there are several examples of tissues in which a number of different protecting osmolytes coexist (Yancey 2005). Cells of the renal inner medulla are rich in osmolytes, with sorbitol, glycine betaine, myo-inositol, taurine, and glycerophosphochol-

ine accumulating in response to osmotic stress (Burg 1995). The independence of the action of the pair, sarcosine and urea, determined in this study raises the question of whether the actions of coexisting protecting osmolytes in tissues such as kidney are independent or synergistic. This important issue is currently under study using the methods described.

Materials and methods

Chemicals

NaCl was obtained from Fisher Scientific; Na_2HPO_4 , $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and sodium acetate were obtained from Mallinkrodt; and glycine came from Sigma. Ultrapure urea was purchased from USB and sarcosine from Fluka. Prior to use, urea and sarcosine solutions were treated with activated carbon (Aldrich) and filtered through 0.22- μm sterile filters (Millipore Millex GP), and their molar concentrations were determined refractometrically.

Protein expression and purification

The C-terminal His₆-tagged Nank4-7* construct used in this study contains four tandem ankyrin repeat sequences and was expressed and purified as described previously (Zweifel and Barrick 2001a; Mello and Barrick 2003).

pH and salt sensitivity studies

Temperature scans from 5°C to 65°C for the pH and NaCl sensitivity studies were performed using a Jasco J-720 spectropolarimeter at 222 nm with a temperature scan slope of 1°C/min. Nank4-7* was present at 0.18 mg/mL, and a 1-mm cuvette completely filled and sealed was used to avoid concentration of the sample by evaporation at higher temperatures. The pH studies were performed in 10 mM sodium acetate, 10 mM NaH_2PO_4 , 10 mM glycine, and 200 mM NaCl. The salt sensitivity studies were performed in 10 mM sodium phosphate buffer (pH 7.0).

Equilibrium denaturations/forced folding

All scans were performed in a Jasco J-720 spectropolarimeter. The buffer used contained 10 mM phosphate buffer and 200 mM NaCl at 34°C. Nank4-7* was present at 0.18 mg/mL. Two stock solutions were prepared containing the same protein concentration, one with the protein in buffer and the other with the protein in buffer and high osmolyte (urea or sarcosine) concentration. The two solutions were then mixed to achieve the desired osmolyte concentration. The samples were allowed to equilibrate for 10 min in the instrument at the desired temperature (34°C) before a 5-min time course measurement was performed. All measurements were performed at 228 nm to minimize the strong absorption by sarcosine at lower wavelengths. Data were analyzed by the linear extrapolation method (LEM) (Pace 1986; Santoro and Bolen 1988) by fitting the data to the equation $y = a + [k/(1 + k)](b - a)$, where $a = N_i + N_s \cdot x$, $b = D_i + D_s \cdot x$, $k = \exp[-(\Delta G + m \cdot x)/RT]$, y is the CD signal, N_i and N_s are, respectively, the intercept and slope of the folded baseline, D_i and D_s are for the denatured

baseline, and x is the osmolyte concentration. The data were fitted using the program Origin 7 (OriginLab Corporation).

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