Review

Living with urea stress

Laishram R Singh[1], Tanveer Ali Dar[2] and Faizan Ahmad[2,*]

[1]1Division of Population Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA
[2]Center for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi 110 025, India

*Corresponding author (Fax, 91-11-2698-0164; Email, faizan_ahmad@yahoo.com)

Intracellular organic osmolytes are present in certain organisms adapted to harsh environments. These osmolytes protect intracellular macromolecules against denaturing environmental stress. In contrast to the usually benign effects of most organic osmolytes, the waste product urea is a well-known perturbant of macromolecules. Although urea is a perturbing solute which inhibits enzyme activity and stability, it is employed by some species as a major osmolyte. The answer to this paradox was believed to be the discovery of protective osmolytes (methylamines). We review the current state of knowledge on the various ways of counteracting the harmful effects of urea in nature and the mechanisms for this. This review ends with the mechanistic idea that cellular salt (KCl/NaCl) plays a crucial role in counteracting the effects of urea, either by inducing required chaperones or methylamines, or by thermodynamic interactions with urea-destabilised proteins. We also propose future opportunities and challenges in the field.

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1. Introduction

Natural selection is believed to be an unforgiving and relentless force in the evolution of life on earth. An organism that cannot adapt to a changing environment or an environment hostile to cell functions is at risk as a species. Thus, it is important to understand the mechanisms used by plants, animals and microorganisms in adapting to environments in the biosphere that would ordinarily denature proteins and enzymes or otherwise cause disruption of life-giving cellular processes. These hostile environments involve such stresses as extremes of temperature, pH, cellular dehydration, desiccation, high extracellular salt environments and even the presence of denaturing concentrations of urea inside cells (Yancey *et al.* 1982; Yancey 2003, 2004). In response to these harsh environmental stresses, organisms accumulate low molecular-weight organic compounds called osmolytes to protect their cells and macromolecular assemblies. Two defining characteristics of protective osmolytes are that they stabilise proteins against denaturing stresses (Santoro *et al.* 1992; Taneja and Ahmad 1994; Xie and Timasheff 1997a, b Foord and Leatherbarrow 1998; Kaushik and Bhat 1998; Anjum *et al.* 2000; Kim *et al.* 2003), and their presence in the cell does not largely alter protein functional activity (Myers and Jakoby 1975; Yancey *et al.* 1982; Wang and Bolen 1996). The basic premise is that the natural selection of protecting osmolytes is based upon selection at a particular molecular level which confers generic stabilisation to all proteins without altering their functional activity (Yancey 2003, 2004).

Within the three chemical classes of osmolytes, distinctions have been made according to the manner in which functional activity is maintained within the cell by particular osmolytes. This has resulted in classification of organic osmolytes either as 'compatible' or 'counteracting' in terms of their effects on the functional activity of proteins (Borowitzka and Brown 1974; Bowlus and Somero 1979; Yancey

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Abbreviations used: α-LA; α-lactalbumin; DMSO, dimethyl sulphoxide; GPC, glycerophosphoryl choline; Hsp, heat shock protein; MDCK, Madine–Darby canine kidney; mIMCD, murine inner medullar collecting duct; NMR, nuclear magnetic resonance; PCD, papillary collecting duct; PI, papillary interstitial; RNase-A, ribonuclease-A; TMAO, trimethylamine *N*-oxide

et al. 1982). Compatible osmolytes are those that stabilise proteins without substantively affecting protein functional activity (Borowitzka and Brown 1974; Bowlus and Somero 1979; Pollard and Wyn Jones 1979; Wang and Bolen 1996). Representatives of this class include certain amino acids (e.g. proline and glycine) and polyols (e.g. trehalose, sucrose and sorbitol), and the stresses that compatible osmolytes protect against include dehydration, high-salt environments and extremes of temperature (Yancey *et al.* 1982). Counteracting osmolytes consist of the methylamine class of osmolytes (trimethylamine *N*-oxide [TMAO], glycerophosphoryl choline [GPC] and betaine), which are believed to have the special ability to protect intracellular proteins against the inactivating effects of urea on proteins (Yancey and Somero 1979; Lin and Timasheff 1994). In contrast to compatible osmolytes, which do not largely affect the functional activity of proteins, counteracting osmolytes are believed to cause changes in protein function that are the opposite of the effects urea has on protein function (Somero 1986). Urea is a special case. It is a perturbing solute that inhibits enzyme activity, yet it is employed by several species as a major blood and intracellular osmolyte (Yancey and Somero 1979; Yancey *et al.* 1982). The selective advantage of a methylamine is that it stabilises proteins from denaturation by urea (Yancey and Somero 1979; Lin and Timasheff 1994; Wang and Bolen 1997) and offsets urea's functional effects, such that the kinetic character of the (enzyme-mediated) metabolic pathways is maintained to the same degree in shark cells as in cells that have neither solute (Hochachka and Somero 1984).

2. Urea is perturbing in action

In contrast to the usually benign effects of most organic osmolytes, the waste product urea is a well-known perturbant of macromolecules. Urea is a chaotropic agent that disrupts hydrophobic interactions responsible for the globular structure of proteins (Nozaki and Tanford 1963; Von Hippel and Schleich 1969; Yancey and Somero 1979; Yancey and Somero 1980). This loss of structure influences enzyme kinetic properties such as maximal velocity (V_{max}) and K_m (Yancey and Somero 1979, 1980) and alters the melting point transition temperature of proteins (Nozaki and Tanford 1963; Singh *et al.* 2008). Urea can also preferentially bind to proteins, dehydrating their exposed surfaces and promoting unfolding (Creighton 1991; Zou *et al.* 1998; Wu and Wang 1999). In addition, there are numerous non-specific effects that can influence protein function, such as solute-induced attenuation of hydrophobic interactions important in substrate–protein interactions or attenuation of electrostatic interactions between substrate and protein (Bolen and Fisher 1969). Urea could also result in cell membranes that are too fluid and unstable (Barton *et al.* 1999). In addition to a chaotropic nature, high urea concentration can also bring about post-translational modification of proteins either by carbamoylation or carbonylation near neutral pH; for insightful discussions, *see* reviews by Kraus and Kraus (2001) and Nystrom (2005). Here we review the literature pertaining to the methylamines' counteraction of the deleterious effects of urea on function and stability of proteins.

At the concentrations found in marine elasmobranches and mammalian kidneys, a wide variety of biological functions are inhibited or disrupted including alteration (usually inhibition) of enzyme kinetics (V_{max} or K_m), destabilisation of protein folding and assembly, inhibition of muscle contraction and tissue respiration, and death of exposed cells and organisms (Yancey 1985). For example, bony fish exposed to 400 mM urea in seawater begin dying as their blood urea exceeds 200 mM (Griffith *et al.* 1979). Occasionally, major effects of low urea concentration (e. g. 25 mM) (Hand and Somero 1982) are seen on proteins, but most begin above 100 mM. Intracellular urea in the cells has been shown to disrupt protein structures at the high concentrations found in some animals and marine life (MacMillen and Lee 1967; Hand and Somero 1982; Wolff and Balaban 1990; Withers and Guppy 1996). It has also been shown that intracellular urea concentrations are high enough to offer competitive inhibition of enzymes (Withycombe *et al.* 1965; Yancey and Somero 1978; Lushchak and Lushchak 1994).

Beyond its physiological effects on protein structure and function, hyperosmolar urea has been shown to acutely regulate multiple signalling events in renal medullar cells *in vitro*. In particular, a pathway exhibiting a hallmark of a receptor tyrosine kinase pathway is triggered by urea. This includes activation of phospholipase C-γ (Cohen *et al.* 1996), activation of phosphatidylinositol 3-kinase and its effectors Akt and p70 S6 kinase (Zhang *et al.* 2000), activation of Shc with recruitment of Grb2 (Zhang *et al.* 2000) and induction of immediate early genes (Cohen and Gullans 1993). Urea also exerts a pro-oxidant effect necessary for increased expression of the stress-responsive gene *Gadd153* (Zhang *et al.* 1999). However, little is known about the cellular aspects of hyperosmotic urea. Acute hyperosmotic urea shocks have been reported to induce apoptosis (Michea *et al.* 2000).

The perturbing action of urea would seem to disprove the compatible osmolyte hypothesis. However, the cells of all non-aestivating urea-accumulating animals examined so far (cartilaginous fishes, amphibia, mammalian kidneys) contain stabilising osmolyte, mainly methylamines (Yancey 1985; Garcia-Perez and Burg 1991; Wray and Wilkie 1995).

3. Urea functions as a balancing osmolyte

Osmoconformers adapt to osmotic stress by accumulating one or more organic solutes. Organisms acquire these

osmolytes most economically by exploiting metabolic end-products (Hochachka and Somero 2002) and, therefore, urea has become a major balancing osmolyte in diverse ureogenic species (Griffith 1991). Urea is the primary nitrogenous waste product of most semiterrestrial and terrestrial amphibians (Shoemaker *et al.* 1992). The urea concentration in body fluid is generally 5–10 mM for hydrated frogs, but is sometimes as high as 30 or 50 mM. Table 1 in Withers and Guppy (1996) summarises the urea concentrations in various animals. In contrast, aestivating amphibians accumulate urea to a high concentration (McClanahan 1972; Jones 1980; Loveridge and Withers 1981;Etheridge 1990) because urea that is synthesised for ammonium detoxification cannot be excreted, as dehydrated or aestivating frogs are essentially anuric (Shoemaker *et al.* 1992; Yokota *et al.* 1985). The urea concentration is often higher than 100 mM in these aestivating amphibians and may even exceed 300 mM (table 1 in Withers and Guppy [1996]). Urea can assume physiological roles other than as a nitrogenous waste product. For example, it is a major osmolyte that maintains osmotic gradient for marine elasmobranch fishes (300–500 mM) (Yancey *et al.* 1982; Yancey 1994; Ballantyne 1997) and mammalian kidney (400–600 mM). It increases up to 3–4 M in xeric rodents under antidiuretic conditions (MacMillen and Lee 1967). Some frogs also accumulate large amounts of urea when acclimated to a high external salinity (e.g. *Xenopus laevis*, *Rana cancrivora* [Goldstein 1972; Funkhouser and Goldstein 1973; Romspert 1976; Katz and Hanke 1993]) or during aestivation (e.g. *Scaphiopus couchii*) by an accelerated rate of urea synthesis (Jones 1980; McBean and Goldstein 1970). The high concentration of urea in these frogs promotes a favourable osmotic gradient for water transfer between the frog and its environment, and so urea functions as a balancing osmolyte (McBean and Goldstein 1970; Jones 1980). Amphibians accumulate urea when exposed to a low water potential, a response that aids in maintaining proper hydration during saline adaptation and aestivation (Shpun *et al.* 1992; Jørgensen 1997). Urea is also important in amphibian hibernation, although relatively little is known about the winter physiology and water balance of these animals (Pinder *et al.* 1992). In principle, urea that accumulates in response to water deficit could also serve a cryoprotective function in hibernating amphibians (Costanzo and Lee 2005).

4. Origin of the counteraction hypothesis (urea: methylamine)

Although urea is a perturbing solute that inhibits enzyme activity and stability, it is employed by some species as a major osmolyte (Yancey and Somero 1979; Yancey and Somero 1980; Yancey *et al.* 1982;Yancey and Burg 1990). The answer to this paradox was the discovery of protective osmolytes such as betaine, TMAO, sarcosine and GPC in certain elasmobranches by Yancey and co-workers (Yancey and Somero 1979, 1980; Yancey *et al.* 1982) and later in other marine organisms and mammals (Yancey *et al.* 1982;Yancey and Burg 1990; Yancey and Siebenaller 1999). The optimal ratio of methylamines to urea for stabilising enzymes against the perturbing effects of urea is approximately 1:2, which is the ratio generally found in tissues containing high urea levels (Yancey and Somero 1980; Yancey 1988, 2003). It is believed that at this magic ratio the opposing effects of these two classes of solutes on protein stability and function are cancelled algebraically (Yancey 2003). This is referred to as the counteraction hypothesis.

For a number of enzymes from sharks and rays, mammalian kidney and non-urea-containing mammalian organs, Yancey and Somero (1980) found that urea alone generally increases K_m and decreases k_{cat}, whereas methylamine alone has the contrasting effect of decreasing K_m while increasing k_{cat}. When urea and methylamine are combined in a 2:1 urea:

Table 1. Enzymes tested for the counteraction hypothesis

Enzymes	Reference
Elasmobranch and mammalian glutamate dehydrogenase	Yancey and Somero 1980
Elasmobranch and teleost actomyosin ATPase	Yancey 1985
Elasmobranch lactate dehydrogenase	Yancey and Somero 1980
Arginosuccinase	Yancey and Somero 1980
Creatine kinase	Yancey and Somero 1980
Carbamoyl phosphate synthetase	Anderson 1981
Yeast alcohol dehydrogenase	Mashino and Fridovich 1987
Teleost glycine cleavage complex	Yancey 1992
Mammalian (renal) Ca-ATPase	Vieyra *et al.* 1991; Yancey 1985
Mammalian (renal) cAMP phosphodiesterase	Yancey 1992
Muscle Ca-ATPase	de Meis and Inesi 1988
Mammalian (renal) arginosuccinase	Yancey and Somero 1980

methylamine ratio, the effects of both solutes on K_m and k_{cat} offset one another, giving apparent k_{cat} and K_m values in the combined presence of urea and methylamine that are equal to k_{cat} and K_m determined in the complete absence of the two solutes (Yancey and Somero 1980). Opposing effects are also seen on protein stability, including thermal denaturation of bovine RNase-A (Yancey and Somero 1979), catalase (Mashino and Fridovich 1987) and lysozyme (Arakawa and Timasheff 1985), renaturation rate of elasmobranch and mammalian lactate dehydrogenase (Yancey and Somero 1979), and unfolding of bovine glutamate dehydrogenase with thiol reagent. Recently, betaine at 2.5 M was found to reverse the large increase in the dielectric increment and relaxation time of bovine serum albumin in 5 M urea (Bateman *et al.* 1992). One cell line study found that urea and betaine in growth medium penetrate Madine–Darby canine kidney (MDCK) cells, such that the intracellular contents can be manipulated (Yancey and Burg 1990).

Yancey and Somero's counteraction hypothesis is elegant in its simplicity, but the extent to which it holds as a general mechanism for proteins in urea/methylamine-containing cells is unclear (Mashino and Fridovich 1987). To be completely effective and general in its action, the counteracting osmolyte methylamines would be expected to offset the effects of urea on any protein, regardless of whether that protein evolved in the presence of these two solutes. At this point, most studies on the effects of urea, methylamine and urea/methylamine mixtures on k_{cat} and K_m have focused on enzymes from kidney or cartilaginous fishes, enzymes that have evolved in the presence of methylamines and urea (Burg *et al.* 1996; de Meis and Inesi 1988; Yancey and Somero 1978; Yancey and Somero 1980). Only a very small number of studies have been conducted on enzymes that have not evolved in the presence of methylamine or urea (Yancey and Somero 1978; Yancey and Somero 1980; Mashino and Fridovich 1987), so the question of whether the counteraction hypothesis is general in its effects has not been extensively explored. Of the small number of enzymes studied, a significant fraction of these do not exhibit counteraction (Yancey and Somero 1978; Yancey and Somero 1980; Mashino and Fridovich 1987).

5. Mechanism of urea–methylamine compensation

The initial explanation for osmolyte properties was proposed by Clark and Zounes (Clark and Jounes 1977) and Wyn Jones and co-workers (Wyn Jones *et al.* 1977). Both groups realised that amino acids and methylamine solutes are structurally similar to the Hoffmeister series, a ranking of anions and cations which, since 1888, have been found to affect the solution phenomena in the same way: ions to the left generally stabilise, salt out and enhance catalysis of the macromolecular system while those to the right do the opposite (Collins and Washabaugh 1985; Woolverton *et al.* 1990).

A long-standing hypothesis to explain the effect of osmolytes in bulk solution and in the neighbourhood of proteins invokes the phenomenon of surface tension (Arakawa and Timasheff 1982). Hydrogen bonding between water molecules results in a high surface tension at aqueous interfaces. Substances (such as methylamines) that increase surface tension will be in a lower concentration at the interface than in the bulk solution, while those that lower surface tension will be in a higher concentration at the interface. If a protein or other large molecule is present in solution, then small molecules that lower surface tension should accumulate adjacent to the protein, while those that raise it are conversely excluded. However, as Bolen (2004) points out, the behaviour of a protein in the presence of urea and TMAO is exactly the opposite of the predictions from surface tension effects. Urea, which increases the surface tension of water, should stabilise rather than denature proteins, while TMAO, which lowers surface tension of water, should favour denaturation. This is the reverse of what is observed to occur. Recently, molecular dynamical modelling of the ternary mixture protein/water/urea/TMAO (Bennion and Daggett 2003) suggests that water structure is enhanced by TMAO. TMAO was able to strengthen and shorten water–water, and water–urea H-bonds, the effect of which was to deny urea access to the peptide backbone of the protein, and hence limit unfolding. In addition, TMAO interacting with protein side chains assisted in ordering the hydration layer around the protein, further stabilising it.

A number of groups have shown that thermodynamic compensation can explain the counteraction without invoking interaction between the solutes (Lin and Timasheff 1994; Wang and Bolen 1997; Baskakov *et al.* 1998). The generally held belief of the mechanism of counteraction is that urea shifts the denaturation equilibrium, native conformation $\leftrightarrow$ denatured conformation towards the right and methylamines have the opposite effect on this equilibrium. Therefore, a 2:1 ratio of [urea]:[methylamine] keeps the denaturation equilibrium unperturbed (i.e. equilibrium in the absence of either co-solute).

6. The urea–methylamine system brings about partial compensation

In vitro studies were carried out to see the effects of these solutes on the denaturational equilibrium of proteins (Yancey and Somero 1979; Lin and Timasheff 1994; Burg 1995). Most studies carried out earlier used T_m (melting temperature) as a measure of protein stability and could provide data on partial or little counteraction of proteins and enzymes by the molar ratio 2:1 (urea:methylamine). There is always a lag of 1–3°C in T_m in the presence of 2 M

urea and 1 M TMAO (*see* figure 2 in Burg [1995], *see* figure 2 in Lin and Timasheff [1994]); Yancey and Somero [1979]). Measurements of T_m of RNase-A have also shown that the best ratio for a perfect compensatory effect is 1:1.35 (Burg 1995). Various functional activity measurements have shown that complete compensation of methylamine:urea works at 1:1 (Bagnasco *et al.* 1986; Baskakov *et al.* 1998; Qu and Bolen 2003).

A list of enzymes tested for the counteraction hypothesis and reported to counteract at the 2:1 urea:methylamine ratio is given below (table 1). Unfortunately, none of the enzymes reported here was found to show perfect compensation at the said ratio.

7. Counteraction is protein dependent

Counteraction does not always occur, i.e. a few urea-inhibited enzymes are not restored by TMAO (Hand and Somero 1982; Mashino and Fridovich 1987; Yancey 1992; Yancey and Somero 1980). This may require a different kind of adaptation, i.e. urea insensitivity or urea-requiring activity in some elasmobranch proteins (Yancey 1985) or perhaps some uncompensated inhibition is tolerable in non-limiting reactions. Aestivators that use urea may not need methylamines, since metabolism needs to be suppressed (Hand and Somero 1982; Yancey *et al.* 1982). Furthermore, in apparent contradiction to the counteracting osmolytes hypothesis, urea (1.0 M) and three different methylamines (TMAO, betaine, and GPC) all have similar and partially additive inhibitory effects on aldose reductase, an enzyme that is important in the renal medulla for catalysing the production of sorbitol from glucose (Burg and Peters 1997). All of them substantially decrease both the Michaelis constants (K_m) and V_{max}. There are several other instances in which counteraction has not been observed. These exceptions show that (i) TMAO does not counteract the perturbing effects of urea on enzyme–substrate interactions of A4-lactate dehydrogenase with pyruvate, or of glyceraldehyde-3-phosphate dehydrogenase with glyceraldehyde 3-phosphate (Yancey and Somero 1980); (ii) TMAO does not counteract the effects of urea on phosphofructokinase activity and structure (Garcia-Perez and Burg 1991); and (iii) betaine does not counteract the K_m effects of urea on uricase (Yancey 1992). Counteraction has also been found to be more complex in certain systems including contraction of skinned shark muscle fibres (Altringham *et al.* 1982) and various aspects of mitochondrial respiration. Less commonly observed is the urea activation and TMAO inhibition of mitochondrial respiration (Ballantyne and Moon 1986; Anderson 1990) and of certain enzymes (Yancey and Somero 1980). Perhaps TMAO makes some enzymes too rigid or too aggregated for proper functioning, while urea may restore flexibility or disaggregation of functionality.

In an attempt to further understand the urea–methylamine compensation on protein stability, our laboratory has recently tested different proteins with varying physico-chemical properties. We demonstrated that at a 2:1 (urea: methylamine) ratio, the counteracting effect on the Gibbs energy of stabilisation (ΔG_D^o) is protein specific; α-lactalbumin (α-LA) is perfectly compensated by the counteracting effect of TMAO and sarcosine. However, this is not true in the case of lysozyme and RNase-A. Based on the *m*-value measurements, we showed that one of the most probable causes for this protein-dependent compensatory effect may be that the change in the fractional exposure ($\Delta\alpha_i$) of protein groups is different in different proteins. Recently, the effects of sarcosine (Holthauzen and Bolen 2007) and TMAO (Mello and Barrick 2003) on the urea-induced denaturation of proteins and vice versa were investigated and values of m_u and m_{MA} were reported. The results reported by Mello and Berrick (2003) suggest that for a perfect compensation [urea]:[TMAO] ratios are 2.37:1 and 1.07:1 for Nank 1–7* and barnase, respectively (*see* Singh *et al.* 2008). These results suggest that 1 M TMAO can reverse the effects of 2.37 and 1.07 M urea on Nank 1–7* and barnase, respectively. In support of our prediction, the heat-induced denatured states of RNase-A and lysozyme were found to be entirely different from α-LA. The heat-induced denatured state of α-LA is highly compact (Griko 2000; Griko *et al.* 1994), whereas RNase-A and lysozyme have open denatured states (Privalov *et al.* 1989). In summary, we now understand that the urea–methylamine system is inefficient in maintaining protein stability and function. It brings about partial or little compensation; in addition, the compensation is protein specific.

8. Urea–non-methylamine counteraction systems

Interestingly, several reports exist in the literature on the counteraction of urea by other non-methylamine osmolyte systems but none holds true in general. It has been shown that glutamate can counteract the effect of urea on glutaminyl-tRNA synthetase from *Escherichia coli* (Mandal *et al.* 2003). Myoinositol, the strongest protein stabiliser, also counters the effect of urea on some proteins (Gerlsma 1968; Shifrin and Parrott 1975). However, it could not offset urea inhibition of two mammalian renal enzymes (Yancey 1992). A non-natural compound dimethyl sulphoxide (DMSO), used as a cryoprotectant, was found in one study to protect Na^+ K^+-ATPase from urea inactivation (Mirsalikhova 1978). Heat shock protein 72 (Hsp72), a molecular chaperone that is abundant in the renal papilla, was demonstrated to counteract urea-mediated inhibition of lactate dehydrogenase and β-galactosidase (Neuhofer *et al.* 1999, 2005; Neuhofer and Beck 2005). These studies

provide evidence which indicates that there is some other urea-counteracting system in addition to the urea–methylamine system.

9. Salt counteracts the effect of urea in many mammalian cells

Bento *et al*. (1998) evaluated the effects of NaCl and urea, individually and in combination, on the viability of murine inner medullar collecting duct (mIMCD) cells. Exposure to hyperosmolar NaCl or urea caused comparable dose- and time-dependent decreases in cell viability, such that 700 mosmol/kg H_2O killed more than 90% of the cells within 24 h. Surprisingly, cells exposed to a combination of NaCl + urea were significantly more osmotolerant, e.g. 40% cells survived 900 mosmol/kgH_2O. MDCK cells but not human umbilical vein endothelial cells also exhibited a similar osmotolerance response. In another study, it was found that cell death was strongly increased after adaptation to 250 mM urea. This effect was reversible, dose dependent and, interestingly, blunted by 125 mM NaCl (Colmont *et al.* 2001). In another communication on a reverse experiment, it was reported that pretreatment with urea (200 mM for 30 min) protected IMCD cells from the apoptotic effects of hypertonic stress (200 mosmol/kg H_2O). The protective effect of urea was dose dependent and was effective even when applied for a short time (less than an hour) following NaCl exposure. However, a protective effect was not observed in non-renal 3T3 cells. Pretreatment with hypertonic NaCl protects MDCK cells against high urea concentrations (Muller *et al.* 1998).

The mechanism by which NaCl offsets the harmful effects of urea in cells has been assigned to Hsp27 and Hsp70. In fact, hyperosmotic stress induces Hsp27 and Hsp70 in many cells including MDCK, papillary interstitial (PI), papillary collecting duct (PCD), IMCD. While organic osmolytes protect against high salt concentrations, Hsps appear not to be involved in this process (Alfieri *et al.* 2004). However, Hsp27 and Hsp70 are expressed at high levels in the renal papilla and their abundance changes appropriately with the diuretic state (Medina *et al.* 1996; Muller *et al.* 1998), suggesting a protective function against the adverse effects of high solute concentrations present during antidiuresis. The functional significance of Hsp70 and Hsp27 for the cells has been demonstrated; forced expression of Hsp70 protects MDCK cells against the detrimental effects of high urea concentrations (Neuhofer *et al.* 2001). This effect is at least partially attributable to the chaperoning activities of Hsp70, since urea is a potent protein-destabilising agent and Hsp70 counteracts the urea-mediated decrease in activity of several enzymes (Neuhofer and Beck 2005). Urea also induces apoptosis (Bento *et al.* 1998; Zheng *et al.* 2000; Colmont *et al.* 2001), while Hsp70 prevents the execution of the apoptotic pathway by several mechanisms, including inhibition of Apaf-1 and cytochrome c release from mitochondria (Beere *et al.* 2000). In agreement, targeted disruption of the tonicity-inducible *Hsp27* and *Hsp70* genes in mice is associated with renal papillary apoptosis after stimulation of the renal concentrating mechanism (Shim *et al.* 2002). These results are also in agreement with the observation that inhibition of NaCl-induced Hsp27 and Hsp70 expression in MDCK cells is associated with higher caspase-3 activity after urea exposure of mock transfected cells (Neuhofer *et al.* 2005).

10. Requirement of salt for maintaining perfect osmotic balance in urea–methylamine systems *in vivo*

The question of urea:methylamine counteraction is especially germane in the case of organisms surviving under high concentrations of extracellular salt. In mammalian kidneys, medullar fluid similarities can increase up to 3800 mosmol l^{-1} (equivalent to ~2.0 M NaCl) during antidiuresis and decrease up to 170 mosmol l^{-1} during duresis (Haussinger and Lang 1992). In marine elasmobranches (sharks, skates and rays) the intracellular osmolalities are equal to that of sea-water (Prosser 1973; Pang *et al.* 1977), which means that their body cells will experience an osmotic pressure that is equal to the major ions present in the sea-water (i.e. Na^+ = 470.2, K^+ = 9.9, Ca^{2+} = 10.2, Mg^{2+} = 53.6, Cl^- = 548.3 mmol l^{-1}) (Prosser and Brown 1961). Because animal cells cannot maintain any substantial osmotic pressure across their body membranes, the intracellular and extracellular osmolality have necessarily to be equal. The major intracellular solutes are urea, NaCl, GPC, betaine, TMAO, sorbitol, inositol, etc. One role of intracellular organic osmolytes is to balance osmotically high levels of salt in the extracellular fluid. It may, however, be noted that the total concentration of intracellular osmolytes (GPC, betaine, TMAO, sorbitol, inositol and urea) measured is not substantial. The mean sum measured by nuclear magnetic resonance (NMR) imaging is 85.8 μmol g^{-1} wet weight and the mean sum by chemical analysis was 76.7 mmol kg^{-1} wet weight (Burg 1995). This suggests that either other unidentified solutes that maintain the osmotic balance must be present in the cells (Beck *et al.* 1984; Schmidt-Nielsen 1997) or the total NaCl and KCl concentration is much larger than the total putative concentration of the salts. There are conflicting reports on the intracellular NaCl concentration (Bulger *et al.* 1981; Beck *et al.* 1984; Schmidt-Nielsen 1997). The strong evidence for the requirement of salt for osmotic balance inside the cells indicates that putatively NaCl may play a crucial role in bringing about a perfect compensation of protein stability at a urea:methylamine ratio of 2:1. Therefore, if we translate the same *in vitro*, the issues of the counteraction hypothesis may be resolved.

11. Magic of osmotic gradient or intracellular salt in urea–methylamine compensation of proteins

The counteracting osmolyte hypothesis suggests that a mixture is more beneficial to cells than urea or methylamine alone, since the latter might overstabilise proteins. Second, the hypothesis predicts that urea-counteracting osmolytes should be regulated specifically in response to intracellular urea and not external NaCl or osmotic pressure *per se*, to maintain a roughly constant ratio to urea inside the cells (Yancey and Somero 1979; King and Goldstein 1983). The second prediction is the major cause of a flaw in the theory. The fact that salts are critical for filling the osmotic gap in those organisms cannot be denied. It would be worthwhile to preferentially exclude cellular salt NaCl/KCl at higher concentrations from the protein surface and hence stabilise proteins. Furthermore, osmotic pressure correlates well with protein stabilisation. It may be noted that urea counteractants – methylamine osmolytes – are methylated glycine derivatives. *In vitro* studies show that most methylation reactions are K^+-dependent, with little betaine accumulating below 0.4 M K^+; at this potassium concentration sarcosine was the major product. K^+ concentrations above 0.4 M enhanced glycine and betaine synthesis from sarcosine and *N*, *N*-dimethylglycine (Robertson *et al.* 1992). This observation suggests that control of betaine synthesis is modulated by intracellular K^+; this cation may also function as an intracellular signal for osmoregulation. In support of this, many studies on cell lines indicate that urea and NaCl have a counterbalancing effect on the growth and survival of cells (Bento *et al.* 1998; Neuhofer *et al.* 1999; Zheng *et al.* 2000; Colmont *et al.* 2001). Mechanistically, urea induces both an apoptotic and a proapoptotic effect on cells and NaCl, on the other hand, could reverse its effect by inducing many antiapoptotic genes (Bento *et al.* 1998; Zheng *et al.* 2000; Colmont *et al.* 2001). In another study, *E. coli* (Chambers and Kunin 1985) were found to use trace amounts of human urinary osmolytes (including betaine) to grow effectively in the presence of salts and urea. Betaine alone did not provide protection, but it did in combination with salts. Another issue to be addressed here is what triggers methylamine accumulation inside the cells. As mentioned earlier, significant progress has been made on the effect of urea inside the cells leading to cell death. However, not a single report exists in stress response biology to indicate that urea stress induces specific genes required for methylamine biosynthesis and accumulation. It, therefore, appears that either osmotic gradient or intracellular ionic salts are the key determinants of what type and how much of methylamine will be accumulated in the cells. There are various reports in the literature that osmotic pressure or NaCl induces many genes required for the stress response in many organisms (Muller *et al.* 1998; Neuhofer *et al.* 1999; Neuhofer *et al.* 2001; Neuhofer *et al.* 2005). All these findings suggest that evaluation of osmotic gradient of organisms or the intracellular ionic salt concentration will answer the paradox of urea–methylamine compensation or mechanism of urea counteraction by other non-methylamine systems.

We propose that cellular salt KCl/NaCl might be a crucial candidate that has the magic of counteracting the stability of many enzymes while assisting in bringing about complete compensation of the urea–methylamine system. There are at least three possible ways by which NaCl can rescue proteins from urea denaturation and bring about urea counteraction in the presence or absence of methylamines. One mechanism may be that NaCl *per se* perhaps helps in counteraction by inducing Hsps (molecular chaperones). It is a common fact that hyperosmotic salt stress induces Hsps in cells. Reports demonstrate that Hsp72 and Hsp27 or Hsp70 may be the chaperones involved, depending on the species (Muller *et al*1 998; Neuhofer *et al.* 1999; Neuhofer *et al.* 2001; Neuhofer and Beck 2005; Neuhofer *et al.* 2005). The second possibility is that NaCl induces genes required for methylamine synthesis in the presence of urea. There are reports that hyperosmotic urea and NaCl induce distinct programmers of osmolyte accumulation (Garcia-Perez and Burg 1991). Therefore, the concentration of accumulated salt will determine how much methylamine is accumulated keeping in mind that it has to maintain the magic ratio of 2:1 (urea:methylamine). The third possibility is the thermodynamic effects of the interaction of NaCl with the macromolecules. The salts KCl and NaCl also act like protein stabilisers and are excluded from the protein surface at higher concentrations (Robinson and Jencks 1965). Therefore, understanding the chaperone effect of cellular salt in protein stabilisation and folding in the presence of urea may unveil many interesting insights of protein folding *in vivo*.

12. Conclusion

In summary, we now understand that the urea–methylamine system is highly restrictive in maintaining the stability and functioning of many proteins; rather, it brings about partial compensation of protein stability. It appears in nature that apart from the urea–methylamine system, there exist at least two more systems to counter the effects of urea on protein stability and function. The urea–NaCl system seems to work in general and the urea–molecular chaperone (Hsp) system seems to be highly restrictive. Consideration of the osmotic gradient or intracellular salt may resolve the issues of the protein dependency of counteraction and the partial compensatory effects of urea–methylamine. Further research needs to focus on these dimensions.

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