Bacterial Osmoregulation: A Paradigm for the Study of Cellular Homeostasis

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Running Title:

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CELLULAR HOMEOSTASIS

Cell biologists strive to understand how physiological processes occur *in situ*. New tools are providing impressive, high-resolution images of bacterial cell surfaces, interiors and molecular machines. Spectroscopic techniques are revealing how the sub-cellular locations, associations and conformations of macromolecules change over time. Cellular processes are being mathematically modelled. To fully understand how cells function we must also identify critical physical and chemical properties of the intracellular milieu, define their variability, and determine how they support or constrain cellular processes.

Cellular homeostasis encompasses the adjustment of physical and chemical properties of the intracellular milieu in response to physiological processes and environmental changes, and the consequences of those adjustments for cell function. Microbiologists describe the impacts of environmental variables on bacterial growth and survival. In contrast, dilute aqueous solutions of purified cellular components are traditionally used to elucidate cellular mechanisms. We must bridge the gap between these perspectives to understand cellular homeostasis. This requires that we analyze the intracellular milieu, accurately simulate it *in vitro* and reframe research questions.

Bacterial osmoregulation provides paradigms for the study of cellular homeostasis. Cells respond to osmotic pressure changes by significantly altering the compositions of the cytoplasm and the cytoplasmic membrane. These changes can be exploited as tools to elucidate the dependence of physiological processes such as protein-nucleic acid interactions and protein folding on properties of the intracellular milieu. Osmosensory transporters and mechanosensitive channels are central players in the osmoregulatory response. Analyses of their responses to physical and chemical parameters, *in vivo* and *in vitro*, are illustrating how cellular

processes respond to changes in the intracellular milieu. The following sections discuss these approaches, insights gained and future challenges.

The intracellular milieu is thermodynamically non-ideal Water is the predominant cellular constituent and most physiological processes occur in an aqueous environment. However the intracellular milieu is a concentrated aqueous milieu that differs fundamentally from the dilute aqueous solutions widely used to elucidate cellular mechanisms. Why haven't researchers chosen experimental systems that better simulate the intracellular milieu?

Consider a solution in which water (the most abundant constituent) is defined as the solvent and one or more inorganic ions or organic molecules, present in smaller amounts, are denoted solutes. The tendency of solute *s* to self-associate, bind to other molecules or participate in chemical transformations is determined by its activity (a_s). The activity of the solute is the product of its concentration (m_s) and its activity coefficient (γ_s). The activity coefficient of each solute is hard to predict, depending on its own concentration, the chemical nature of the solvent, and the concentrations and chemical natures of other solutes. However activity coefficients (γ) approach 1 as solute concentrations (m) approach zero. A dilute solution approximates the hypothetical solution, defined as thermodynamically ideal, in which activity coefficients are 1 and concentrations equal activities. Thus dilute solutions simplify experimental design and interpretation. For example most studies of ligand binding (e.g. applications of the Hill Equation) or enzyme kinetics (e.g. applications of the Michaelis Menten Equation) are designed to exploit the assumption that substrate concentration is a valid proxy for substrate activity. However the intracellular milieu and many real solutions are thermodynamically non-ideal. They are not dilute, so solute activities do not equal solute concentrations, and solutes occupy a significant fraction of solution volume (Fig. 1).

Osmoregulation: stress response and experimental tool Many properties of the intracellular milieu may affect cellular processes. They include the temperature, hydrostatic pressure, osmotic pressure (or osmolality), fractional volume occupancy (or macromolecular crowding), pH and K^+ activity. Absolute values for most of them are hard to determine but key properties can be varied systematically by modulating the osmolality of the bacterial medium. This "osmotic stress" approach (50) helps investigators to determine which cellular properties are relevant to particular phenomena. It is best understood in the context of the osmoregulatory response.

Water flows out of cells as their medium becomes more concentrated (an osmotic upshift) and into cells as their medium becomes more dilute (an osmotic downshift). Bacterial membranes have high water permeabilities so cellular hydration is altered within seconds of an osmotic shift. Bacteria respond to osmotic stress by accumulating or releasing electrolytes and small organic solutes, some denoted compatible solutes because they accumulate to high levels without disturbing cellular functions. Osmotic upshifts inhibit most membrane-based bioenergetic functions and macromolecule synthesis while activating existing osmoregulatory systems and inducing osmoregulatory genes. In well characterized systems, including *Escherichia coli* and *Corynbacterium glutamicum*, the response to an osmotic upshift is contingent on the availability of osmoprotectants, exogenous organic compounds like glycine betaine that can be taken up to serve as (or be converted to) compatible solutes. When an osmotic upshift occurs in the absence of osmoprotectants, the bacteria immediately accumulate K^+ and an organic anion such as glutamate. Compatible solutes synthesized from endogenous substrates accumulate more slowly in processes that may require protein synthesis. Available osmoprotectants are taken up immediately via existing or more slowly via newly synthesized

transporters. Compatible solute accumulation attenuates the K^+ glutamate response and more effectively restores cellular hydration, critical functions and growth. Osmotic downshifts abruptly strain the cytoplasmic membrane and increase turgor pressure. Mechanosensitive channels open to allow non-specific efflux of cytoplasmic solutes and avoid cell lysis. Bacterial osmoregulatory mechanisms have been extensively reviewed (4, 10, 53, 55, 84, 119).

The impact of growth medium osmolality on *E. coli* has been systematically analyzed (16, 17, 18, 19, 28, 67, 87). The following discussion focuses primarily on properties of cells cultivated in minimal, defined growth media at various steady state osmolalities. Additional information has been obtained by studying the impacts of osmotic shifts under conditions that do or do not allow bacteria to mount osmoregulatory responses (50, 69).

The cytoplasm is a concentrated, multi-component milieu comprised of charged and uncharged solutes: macromolecules as well as inorganic ions and small organic molecules. Regardless of the growth osmolality, approximately 0.5 g of water is bound per gram of cytoplasmic macromolecules (87), an amount comparable to the estimated hydration of protein molecules *in vitro*. The solute array and the quantity of free cytoplasmic water depend on the osmolality and the availability of osmoprotectants. At low osmolality phosphate, the predominant inorganic anion, is present at a concentration of approximately 10 mM. Most metabolites are maintained at comparable or lower concentrations. In the absence of osmoprotectants, the amount of free water fell from less than 5-fold to less than 2-fold higher than the amount of bound water and the turgor pressure decreased from approximately 3 atm to less than 0.5 atm as *E. coli* was cultured in minimal media with osmolalities ranging from 0.03 to 0.8 mol/kg (16). These observations reinforce the impression, based on scale drawings (34) and simulations (66), that most cytoplasmic water interacts with other cytoplasmic constituents (Fig.

1). Steady state levels of polyamines (putrescine and spermidine) in the cytoplasm decrease as K^+ glutamate and trehalose levels increase with increasing medium osmolality (16, 18, 19, 28, 67, 87). The total cytoplasmic concentration of K^+ increases from approximately 0.2 to over 0.7 M as the growth osmolality increases from 0.1 to 1.0 mol/kg. K^+ and putrescine are major counterions for nucleic acid phosphate at low osmolality. At high osmolality, the amount of K^+ exceeds that of nucleic acid phosphate and glutamate accumulates as K^+ counterion (the concentration of glutamate rising from approximately 0.05 to 0.50 M). The trehalose concentration rises from approximately 0.04 to 0.4 M.

Exogenous osmoprotectants fundamentally alter the composition of osmotically stressed *E. coli* cells (28) and extend the growth range to higher osmolalities. For example, glycine betaine or proline can replace K^+, glutamate and trehalose to become the predominant cytoplasmic solute in cells grown at high osmolalities. The cytoplasmic glycine betaine concentration rises as a direct function of growth medium osmolality to exceed 1.5 M in bacteria cultivated at an osmolality just over 2 mol/kg (18). Even though the amount of free water still decreases with increasing osmolality, the water content of glycine betaine-containing bacteria is more than 1.5-fold higher than that of K^+ glutamate-accumulating bacteria at high osmolality.

In addition to altering the complement of low molecular weight cytoplasmic solutes, osmotic stress alters the fraction of cytoplasmic volume (Φ) occupied by macromolecules. The nucleoid constitutes a distinct region within the cytoplasm (125). There is growing evidence that key cellular processes rely on a bacterial cytoskeleton (75) that may exist as a stable or dynamic matrix. Proteins and nucleic acids were estimated to occupy 30-40% of the volume of the non-nucleoid cytoplasm for *E. coli* cells cultivated in glycine betaine-containing LB medium (osmolality 0.4 mol/kg) (126). This degree of macromolecular crowding could be simulated by a

globular protein with a molecular weight close to 75 kDa at a concentration of 0.34 g/mL (126). The fraction of cytoplasmic volume occupied by macromolecules varied from approximately 0.15 to 0.28 as *E. coli* was cultured in minimal media with osmolalities varying from 0.1 to 1 mol/kg in the absence of osmoprotectants. In the presence of glycine betaine, Φ varied from approximately 0.18 to 0.28 as the medium osmolality varied from 1 to more than 2 mol/kg (50). Clearly a substantial fraction of cell volume is occupied by macromolecules, that fraction increases with growth osmolality and it decreases as compatible solutes accumulate.

THE OSMOTIC STRESS APPROACH

Osmotic stress is a useful tool for analyses of effects of cytoplasmic composition (including crowding) on such processes as molecular diffusion, protein-nucleic interactions and protein folding *in vivo* (50), and for metabolomics (60). Interpretation of such studies hinges on an understanding solvent-macromolecule interactions, summarized below.

Impacts of the cytoplasmic solvent on macromolecular structures, interactions and functions The surfaces of macromolecules include regions that are non-polar, polar but uncharged, or charged. For example, the water-accessible surface of an average, monomeric, native protein was estimated to be 57% non-polar, 24% polar and 19% charged (72). Nucleic acid duplexes are anionic polyelectrolytes, cylindrical molecules with a uniform, dense negative surface charge (3). The conformations, interactions and functions of these molecules are profoundly influenced by interactions of solutes with their surfaces.

Physiological processes depend on high affinity, stereospecific binding of particular molecules at limited numbers of sites on macromolecular surfaces. Tight ligand binding is stabilized by specific chemical interactions occurring at close range. Each macromolecule can exist in an array of conformations with various ligand affinities and functional properties. As a

result, ligand concentration can control macromolecular conformation and function, a phenomenon called chemosensing (Fig. 2A).

In addition to playing specific physiological roles, cytoplasmic solutes are solvent components. They interact weakly at a multitude of surface sites to affect the conformations, interactions and functions of macromolecules. Preferential interactions cause solutes to partition unequally between bulk water and the water of hydration surrounding each area on the surface of each macromolecule (108). A solute concentrates at a surface if it is preferred relative to water, or excluded from a surface if water is preferred. Absolute differences in solute concentration between the bulk solution and the surface are small, but the collective effects of preferential interactions are significant when averaged over entire macromolecular surfaces and populations. Preferential exclusion can significantly favour conformations and associations that minimize solvent exposed protein surface area. Preferential interaction effects are minimized when simple, dilute reaction mixtures are used *in vitro* but they are intrinsic to all processes occurring *in vivo*.

Preferential interaction effects are exquisitely sensitive to solute concentrations, and to the chemical natures of the solute(s) and the exposed macromolecular surfaces (89). Solutes may be excluded from water surrounding macromolecular surfaces by virtue of their size (steric exclusion), creating osmotic stress on a molecular level. They can thereby dehydrate and stabilize “closed” conformations of enzymes and ion channels (80). Ionic solutes exert particularly powerful and complex effects. Interactions of ions with macromolecules bearing high surface charge density are described in terms of the polyelectrolyte effect (89). Individual ion effects are ordered according to the empirical Hofmeister series (12, 20, 89). Anions are more potent than cations at the same concentration, and they increase the exposure of protein surfaces in the following order:

$$SCN^- > ClO_4^- > I^- > Br^- > NO_3^- > Cl^- > CH_3COO^- > HPO_4^{2-} > SO_4^{2-} \sim F^-.$$

Ions that increase the exposure of protein surfaces are called chaotropes whereas those that decrease surface exposure are called kosmotropes. For dilute (quasi-ideal) solutions, collective ion effects can be analyzed in terms of the ionic strength (I) (89).

Effects of uncharged solutes can also be considered in terms of a Hofmeister series. The following solutes increase exposure of macromolecular surfaces in the order (9, 31):

urea > proline > glycine betaine > trimethylamine oxide

Urea is strongly chaotropic whereas trimethylamine oxide (TMAO) is strongly kosmotropic (121). Bacteria commonly accumulate amino acids (e.g. proline), quaternary ammonium compounds (e.g. glycine betaine) and polyols (e.g. trehalose) as compatible solutes (93). These compounds stabilize native protein structures via preferential exclusion from the polypeptide backbone (9). Their impacts on protein and nucleic acid folding and solubility are also determined by favourable and unfavourable interactions with individual amino acid sidechains and nucleotide bases (9, 89, 98).

The cytoplasm includes a mixture of inorganic ions and metabolites, each present at a low concentration, that have diverse chemical properties and hence interact differently with macromolecular surfaces. The concentrations and activities of cytoplasmic solutes are difficult to define precisely because they interact strongly with water and each other. For example polyanionic RNA and DNA strongly influence the behaviour of other ionic species. Approximately one-half of cytoplasmic K^+ interacts with nucleic acids even though it is not bound at specific sites (15, 37, 67). Osmoregulatory solutes can powerfully influence properties of the cytoplasm because they can attain high cytoplasmic concentrations. They skew properties

of the cytoplasmic solvent by contributing as much to cytoplasmic osmolality as all other solutes combined.

In addition to preferential interaction effects, volume exclusion (or macromolecular crowding) may affect cellular processes by rendering the cytoplasm thermodynamically non-ideal. Crowding arises from the high concentrations of solutes, particularly macromolecules, present in the cytoplasm (Fig. 1). Crowding limits macromolecule diffusion while stabilizing compact molecular conformations and enhancing associations *in vitro* (122). Efforts are being made to assess whether the diffusion of molecules within cells is in accord with crowding theory and with measured effects of the osmolality on crowding in the bacterial cytoplasm (69). Molecules diffuse much more slowly in the cytoplasm than in dilute solutions, but diffusion remains fast on the time scale of physiological processes. Macromolecules diffuse at rates related to their sizes and intracellular locations. Crowding theories and estimates do not fully account for this behaviour, but these analyses are complicated by the small size of bacterial cells and the structural heterogeneity of the cytoplasm. Different results are obtained if bacteria are subjected to large osmotic upshifts and osmoregulatory responses are prevented, or they are cultivated at the same high osmolality for many generations. Macromolecular diffusion is much slower and more spatially restricted in the former than in the latter case, implying that solute accumulation and/or other adaptive processes offset structural consequences of osmotic stress (49, 70).

Significant changes in molecular volume and surface exposure occur when macromolecules associate or dissociate, and when proteins undergo conformational changes as they associate with specific, high-affinity ligands and/or catalyze reactions. Macromolecular crowding favours physiological processes that decrease the volumes of participating molecules. Preferential interactions favour or inhibit reactions according to the net redistribution of solute molecules

between the surfaces of macromolecules and the bulk solvent. The extent of each intracellular process is influenced by the many changes to macromolecule-solvent interactions that it invokes.

Protein-nucleic acid interactions K^+ glutamate strongly inhibits the association of proteins with DNA *in vitro*. This phenomenon can be explained in terms of the polyelectrolyte effect: the tendency for ions to redistribute around high charge-density polymers like DNA. In contrast, osmotically induced K^+ glutamate accumulation weakly affected the association of the lactose repressor and of sigma 70 RNA polymerase with DNA *in vivo* (92). Record and his colleagues developed a possible resolution to this paradox by analyzing the impact of osmotic stress on the cytoplasmic milieu and protein-DNA interactions (19, 87, 88). As outlined above, osmotic stress increases the cytoplasmic concentrations of K^+ glutamate and macromolecules in parallel, so that the cytoplasmic K^+ glutamate concentration correlates with the degree of macromolecular crowding (18, 19). K^+ glutamate and crowding may exert opposite perturbing effects on protein-DNA interactions, the K^+ glutamate inhibiting interactions that might otherwise be too strong due to the increased crowding in dehydrated cells. Both effects would be absent from bacteria that rehydrate more fully by accumulating preferentially excluded compatible solutes such as glycine betaine in lieu of K^+ glutamate (31). The maintenance of appropriate protein-nucleic acid interactions would therefore require adjustments to multiple, interwoven cytoplasmic properties.

Protein folding Researchers are also using the osmotic stress approach to compare the impacts of compatible solutes on protein folding and function *in vitro* and within *E. coli* cells (27, 33, 47). Recent studies target variants of eukaryotic cellular retinoic acid binding protein that can be specifically labelled with a fluorescent reporter group *in vivo* (44). The impacts of urea, proline and glycine betaine on these proteins were examined using *E. coli* strains that could or could not

accumulate the osmoprotectants when subjected to osmotic stress (45, 46). Proline was more effective than glycine betaine in preventing *in vivo* aggregation while proline and glycine betaine had similar abilities to block *in vivo* urea denaturation. Further work must account for the relative impacts of K^+ glutamate, trehalose, compatible solutes and macromolecular crowding on protein stability as well as the *in vivo* solute accumulation levels.

OSMOSENSORY MECHANISMS

Osmotic upshifts dehydrate cells and impair many cellular functions. Most transporters are inhibited, but osmosensory transporters are activated to forestall these effects by mediating the uptake of K^+ or osmoprotectants (23, 52) (Fig. 3). Osmotic downshifts cause rapid water influx that strains and could rupture the cytoplasmic membrane. Mechanosensitive channels open to release solutes, water follows and lysis is avoided (59). Thus key osmosensory systems reside in bacterial cytoplasmic membranes. Osmosensory transporters and mechanosensitive channels can detect and respond to osmotic shifts after purification and reconstitution in proteoliposomes (84). Efforts to understand how these systems sense and respond to osmolality changes are deepening our understanding of cellular homeostasis and providing tools for its study. They depend on background knowledge of macromolecule-solvent interactions, discussed above, and of membrane-solvent interactions, summarized below. More detailed discussions of membrane-solvent interactions appear elsewhere (8, 116).

Solvent effects on cytoplasmic membranes Solvent constituents undergo preferential interactions with the exposed surfaces of biological membranes as they do with the surfaces of proteins and nucleic acids. The nature of those interactions is determined by the chemical nature of the membrane surface, which is in turn a function of its phospholipid headgroup composition. Like those of nucleic acids, the anionic surfaces of bacterial membranes interact powerfully with

ions in the adjacent solution (58, 84). Most analyses of osmosensory mechanisms have been based on proteins from *C. glutamicum*, *Lactococcus lactis* or *E. coli*. The phospholipid headgroups in *C. glutamicum* and *L. lactis* are predominantly anionic (mostly PG and glycolipid, respectively) (29, 40, 78). In contrast, the *E. coli* membrane includes approximately 25 mole percent anionic phospholipids (phosphatidylglycerol (PG) and diphosphatidylglycerol, also known as cardiolipin (CL)), the remainder being mostly zwitterionic phosphatidylethanolamine (PE) (109). The proportions of cyclopropane fatty acids and CL increase (the latter at the expense of PE) with growth osmolality (95, 109) and CL is concentrated at the cell poles (71). Thus cell surface location can influence the nature of preferential interactions between the membrane and solvent components and the impact of lipid on membrane protein function (97).

Phospholipid membranes have unusual physical properties and they serve as a semi-permeable barrier between water-filled compartments (e.g. the cytoplasm and the periplasm or the external environment) (8, 116). Imposition of an osmotic gradient on a topologically closed membrane system with a membrane impermeant solute has multiple consequences. Osmotic gradients dissipate within seconds due to water flux via the phospholipid bilayer and the aquaporins (94, 112). Spherical, membrane-bounded systems shrink in response to an osmotic upshift and become non-spherical because the phospholipid bilayer is fluid but inelastic. Osmotic downshifts reverse these effects and strain the membrane. Osmotic shifts may also change phospholipid hydration and/or packing density (113, 114). Turgor pressure develops if the membrane is bounded by a wall. Recognition of these effects, combined with experimentation based on patch-clamping, led to the discovery that mechanosensitive channels open when membrane strain reaches a threshold value in cells subjected to an osmotic

downshock (65). Our understanding of the molecular basis for that phenomenon, based on crystal structures, diverse experiments and structural simulations, is reviewed elsewhere (55).

Critical membrane properties could be influenced by both preferential interactions and mechanical distortions. They include membrane thickness, the area per lipid molecule, headgroup spacing, surface charge density, hydrogen bonding among lipid and protein molecules and intrinsic strain. Intrinsic strain is a lateral pressure, exerted in the membrane plane, that increases towards the membrane core (8). It arises from the balance between the intrinsic tendency of each lipid monolayer to curve, accommodating the bulky acyl chains, and the requirement that lipid bilayers associate to segregate the acyl chains from water. This phenomenon has been invoked to explain effects of lipid composition on light-driven protein pumping via bacteriorhodopsin (106).

Osmosensing Transporters Osmoprotectant transporters ProP of *E. coli*, BetP of *C. glutamicum* and OpuA of *L. lactis* and *Bacillus subtilis* are serving as paradigms for the study of osmosensing (117). This review is not encyclopaedic as the properties of these proteins are discussed in detail elsewhere (53, 84, 118, 119, 123). ProP is broad in substrate specificity, transporting proline and glycine betaine with similar affinities, whereas BetP and OpuA are glycine betaine-specific. These transporters are osmosensors since chemically diverse, membrane impermeant solutes contribute to their activation as those solutes contribute to the osmolality of the external medium. Each transporter activates in cells and, after purification, in proteoliposomes (86, 100, 111). The mechanism(s) by which osmosensory transporters sense and respond to osmolality changes are not yet fully understood. They may be regulated by a solution or membrane property other than the activity of a specific ligand, and by a mechanism other than site-specific ligand binding (Fig. 2).

Transporter structures and transport mechanisms Researchers seek to understand how increasing osmolality alters the structures and activities of osmosensory transporters in the context of phospholipid membranes. It is also important to understand how increasing the osmolality decreases the activities of structurally similar transporters that are not osmosensors.

ProP is a H^+-osmoprotectant symporter and a member of the Major Facilitator Superfamily (MFS), BetP is a Na^+-osmoprotectant symporter and a member of the Betaine-Carnitine-Choline Transporter (BCCT) family, and OpuA is an ATP-Binding Cassette (ABC) transporter. Thus osmosensory transport is not associated with one evolutionary family or bioenergetic mechanism. All three transporters are likely to follow an alternating access mechanism (26, 32, 57, 76) (Fig. 4). According to that mechanism substrates bind within an outward-facing cleft between paired, intra-membrane helix bundles. The substrate may become occluded within a structural intermediate, then a conformational change exposes the binding site to the cytoplasm and allows substrates to exit (32, 57). Organic substrates may first bind weakly, perhaps via hydrogen bonds that involve specifically-positioned water molecules, and binding may then be optimized when substrates are occluded. Any osmosensory mechanism must ultimately accelerate one or more rate determining steps in the transport mechanism (Fig. 4).

Projection and crystal structures and biochemical data show that BetP is a homotrimer of 12 transmembrane helix (TM) subunits (91, 124) (Fig. 5A). Each subunit follows the LeuT fold (32), with helices 3-7 and 8-12 serving as the inverted repeat helix bundles that comprise the transporter core (Fig. 5B). The quaternary ammonium group of substrate glycine betaine is occluded at mid-membrane by a box comprised of aromatic residues associated with helices 4 and 8 (Fig. 5B). This structure adds significant insight into the transport mechanism shared by members of the LeuT structural family (32, 123). A break in the 3-fold symmetry of the trimer is

created by inter-subunit interactions of the extended, α-helical C-termini (cytoplasmic *in vivo*) which are osmoregulatory (77).

The structures of MFS transporters are fundamentally different from that of BetP. A homology model for ProP (Fig. 5C,D) is based on the three crystal structures for 12 TM MFS members that show cytoplasm-facing transporter conformations (48, 120). They include the well characterized H^+-lactose symporter, LacY (36), which is inhibited as ProP activates under osmotic stress (23) (Fig. 3). In each transporter TMs 1, 4, 7 and 10 line a pore to which flanking TMs 2, 5, 8 and 11 contribute. TMs 3, 6, 9 and 12 are outermost and not involved in pore formation. These TMs form interwoven N- and C-terminal six-helix bundles, linked by a long loop, that flank a central cleft. A substrate analogue is found in the cleft at mid-membrane in one LacY structure. Most polar and ionisable residues implicated in transport cluster deep in the C-terminal bundle of LacY and an analogous cluster is found in the N-terminal bundle of ProP (48). LacY is a monomer (36) whereas ProP from *E. coli* is a homodimer with an interface that includes TM 12 and an intermolecular, antiparallel α-helical coiled-coil formed by extended C-termini (24, 38, 61, 109) (Fig. 5E). At least one ProP orthologue is osmoregulated and lacks the coiled-coil domain (82, 109).

Intensive studies of ABC transporters, including multiple crystal structures, suggest that the substrate binds in a cleft between paired helix bundles and can access either membrane surface via their reorientation (26, 76). ABC transporters also include one or more external subunits or domains that bind substrates and deliver them to the integral membrane complex as well as two highly conserved nucleotide-binding subunits associated with its cytoplasmic surface (Fig. 5F). The nucleotide binding subunits couple transport to ATP hydrolysis. Crystal structures of substrate-binding OpuAC proteins and their homologues in substrate-free and substrate-bound

forms show that the quaternary ammonium group of glycine betaine (or a related substrate) is coordinated within a Trp box and other residues interact with the substrate carboxyl group (43, 101, 105, 115) (Fig. 5G). The structures of the membrane-integral OpuAB domains/subunits and the nucleotide binding OpuAA subunits are not known. The OpuAA subunits bear C-terminal Cystathionine β-Synthase (CBS) domains with an anionic, C-terminal tail implicated in osmoregulation (6, 64, 119).

What signal(s) do osmosensory transporters detect? In principle, an osmosensory transporter could detect any cellular property that changes with the osmolality, including turgor pressure, membrane strain (or tension) and membrane intrinsic curvature (116) as well as the water activity (or osmolality), the activities of individual solutes or groups of solutes or the crowding of macromolecules in the cytoplasm. The following discussion is based on the perspective that a single osmosensory mechanism may be shared among osmosensory transporters. Alternatively, different transporters may detect different physiological signals in different ways (117).

The composition of the solvent inside proteoliposomes (the lumen) can be varied without imposing osmotic gradients that alter the topology or tension of the bounding membrane (99, 118). ProP, BetP and OpuA can be activated by raising the lumenal salt concentration without imposing an osmotic shift on proteoliposomes (84). In addition, ProP activity correlates with the osmolality rather than with the magnitude or direction of osmotic shifts imposed on cells or proteoliposomes (21, 85). Thus activation of these transporters does not require changes to turgor pressure or in-plane membrane strain. Water activity is unlikely to determine transporter activity directly as water saturates all solvent-exposed sites on protein and membrane surfaces.

The compositions of the solvents outside and inside proteoliposomes have been varied in an effort to determine what properties consistently correlate with transporter activity.

Proteolipsomes shrink or swell when osmotic shifts are imposed with external, membrane-impermeant solutes, simultaneously concentrating or diluting all solutes present in the proteoliposome lumen. ProP and BetP assume outside-out orientations in proteoliposomes (23, 104) and their activities can only be detected by applying transport assays to topologically closed membrane systems. Thus proteoliposomes must be loaded with diverse solutions to examine the impact of solvent composition on the cytoplasmic transporter surface (21, 99). OpuA assumes a random orientation in proteoliposomes but a functional orientation can be imposed by applying ATP to only one membrane surface. Similar results have been obtained when solute uptake via OpuA was measured using proteoliposomes loaded with ATP and test solutes, or when ATP and test solutes were added to glycine betaine-loaded proteoliposomes and glycine betaine efflux via inside-out OpuA molecules was monitored (63, 111, 112).

A sigmoid relationship is observed between the initial rate of solute uptake (a_0) and assay medium osmolality in cells, cytoplasmic membrane vesicles and proteoliposomes (Fig. 3). This implies that transporter molecules are systematically converted from an inactive to an active conformation as the osmolality increases. Such data can be fit to an empirical relationship that implies no particular activation mechanism:

$$a_0 = A_{max} [1 + \exp (- (X-X_{1/2}) / B)]^{-1} \qquad \text{(Eq 1)}$$

where A_{max} is the uptake rate that would be observed at infinite osmolality, B is a constant inversely proportional to the slope of the response curve, X is the osmolality (also symbolized as Π/RT, where Π is the osmotic pressure, R is the Gas Constant, T is the temperature), and $X_{1/2}$ is the osmolality at which activity is half maximal (also symbolized as $\Pi_{1/2}/RT$) (22). In this relationship the osmolality (X) can be replaced with any property that varies in parallel with it

(e.g. the calculated concentration of a lumenal solute). Alternatively, such data have been fit to the Hill equation:

$$a_0 = A_{max} [1 + K^n_{ion} / [Ion]^n]^{-1} \quad \text{(Eq 2)}$$

where K_{ion} is the salt concentration required to attain half-maximal activity and *n* is a constant related to the slope of the curve, explicitly suggesting that transporter activation is a cooperative response to salt concentration (63). A_{max} values are variable because transporter purification, reconstitution and loading are intrinsically variable procedures. $X_{1/2}$ and K_{ion} values are independent of transporter quantity and more reproducible.

All tested membrane impermeant solutes have similar effects on activity when applied to attain the same osmolality at the external transporter surface. Differences in behaviour emerged when diverse solutes were applied to the cytoplasmic transporter surfaces (117). All three transporters activated as the concentrations of inorganic ions in the lumen increased (21, 99, 112). In contrast, small, kosmotropic organic solutes did not activate. Alkali cations can be grouped by physical properties, K^+, Rb^+ and Cs^+ forming a cluster distinct from Na^+ and Li^+. OpuA was activated similarly by K^+, Na^+, Li^+ or NH_4^+ chloride but not by sucrose. It was further activated by $MgCl_2$ and $BaCl_2$ but inhibited by RbCl and CsCl (6, 112). The ATPase activities of the OpuAA subunits were also stimulated by salts but not sucrose (41, 112). Internal K^+ phosphate, glutamate or chloride, Rb^+ or Cs^+ chloride activated BetP to varying degrees, whereas Na^+, NH_4^+ or choline chloride and organic solutes did not (99, 102). K^+ salts yielded the strongest stimulations. K^+, Na^+, Li^+ and Cs^+ chlorides activated ProP equally (21). Lumenal Poly(Ethylene)Glycols (PEGs) also activated ProP at constant K^+ phosphate concentration and this activation was PEG size-dependent (21). Effects of large non-electrolytes on BetP and

OpuA have not been reported and crowding effects have not been tested with the proteoliposome systems. PEGs contribute to volume exclusion but they are not ideal for crowding studies (73).

Even though the experimental systems discussed above do not fully simulate cytoplasmic conditions, it is challenging to disentangle the variables that influence transporter activity. Each solute will exert effects not related to osmosensing, particularly at the high concentrations required for these experiments. Each transporter appears to have a larger surface exposed to the cytoplasm than to the periplasm or exterior of the cell, possibly rendering each more sensitive to the composition of the solvent bathing the cytoplasmic than the external surface. No transporter that is not an osmosensor has been studied in this way as a control. By analogy, osmoregulatory sensor kinase MtrA of *C. glutamicum* was activated more by K^+ salts than by salts of other cations, suggesting that it may be a K^+ sensor. However sensor kinase proteins that were not osmoregulatory shared the same ion specificity (74).

Differing views of osmosensing have been derived from the observations summarized above, none of them consistent with all available data. Krämer and his colleagues conclude that BetP is a K^+-sensing chemosensor (99, 102, 104). This concept is appealing since K^+ uptake and K^+ glutamate accumulation are triggered by osmotic stress. However BetP activity does not correlate with cytoplasmic K^+ concentration under all conditions, suggesting that BetP may respond to an additional signal (11, 78, 79). Proteoliposome studies suggest that K_d for K^+ would be in the range 0.2 to 0.4 M (99). It is not clear whether a site with such a low affinity could form the basis for a regulatory mechanism and no K^+-specific osmoregulatory site has been reported. Perhaps macromolecular crowding, exerted *in vivo* but not *in vitro*, alters the transporter structures to enhance the K^+ affinity of a regulatory site. BetP, ProP and OpuA can be activated by raising the concentrations of cations other than K^+ in the proteoliposome lumen

and Rb^+ and Cs^+ no longer inhibit OpuA when its structure is altered (63). The effects of other salts could be attributed to an ion-binding site with weak specificity, K^+ being the physiologically relevant ion. K^+ does stimulate ProP activity in cells and membrane vesicles, but this probably results from maintenance of the protonmotive force under osmotic stress (23, 51, 62, 68). ProP activated as the osmolality increased at a K^+ concentration of only 35 μM (23) or as the concentrations of PEGs in the proteoliposome lumen increased at constant K^+ concentration (21).

Noting that OpuA activity correlated with the ionic strength and lumenal sucrose did not activate, Poolman and his colleagues conclude that OpuA is an ionic strength sensor (6, 63, 64). Analyses of OpuA activity in proteoliposomes included studies with mono- and divalent cation salts, but the concentrations of divalent cations were limited to 20 mM (63) because they profoundly affect the structure and function of phospholipid bilayers (5). Thus correlations of OpuA activity with the ionic strength and the osmolality would be similar. Ionic strength and ion concentration vary differently only for polyvalent ion salts, and effects of ion valency on ProP and BetP activity have not been reported. ProP activity clearly did not correlate with the ionic strength of the lumen when proteoliposomes were loaded with, and activated by, non-ionic PEGs (21). Correlation of transporter activity with ionic strength suggests an osmosensory mechanism involving electrostatic interactions because, in dilute solutions, increasing ionic strength weakens electrostatic interactions among functional groups on macromolecular surfaces. However K_{ion} for OpuA (Eqn 2) was approximately 0.2 M. Ionic strength calculations are unlikely to capture preferential interaction effects which may be dominant in this concentration range (89). It is possible that a lower K_{ion} would be observed if the lumenal solvent were crowded.

This author proposes that the activities of osmosensory transporters may be determined by their own hydration, in turn influenced by solute exclusion from their surfaces (117). Small solutes may be excluded from the tightly packed external surfaces of the intra-membrane helix bundles, both sterically and via preferential interactions. Large cytoplasmic molecules would be sterically excluded from cytoplasmic transporter surfaces. PEGs in the lumen of proteoliposomes activate ProP to an extent that correlates directly with their size (21). PEGs in similar size and concentration ranges increased the affinity of glucose for hexokinase, which is a bilobed, soluble enzyme similar in size to the membrane-integral domains of ProP, BetP and OpuA (90). PEGs are believed to be sterically excluded from the closed conformation of hexokinase in which the lobes close over the glucose-containing active site (90). The osmotic activation of ProP *in vivo* correlated with increased reactivities of cysteine residues inserted near the periplasm-proximal, but not the cytoplasm-proximal end of TM 1 (48). Such changes could reflect closure of the cytoplasm-facing cleft and concomitant opening of the periplasmic surface.

The concept that transporter activity could reflect transporter hydration can be understood by comparing ProP with other H^+ transporters. LacY and ProP are flexible and highly hydrated (1, 2, 48, 61). Many polar amino acid side-chains are buried in the membrane (Fig. 5C) and those that are not solvent-exposed must hydrogen bond with each other or intra-protein water. H^+-lactose symport is osmolality sensitive but lactose exchange, which does not require H^+ binding or release, is not (23). Water may serve as a cofactor in H^+-coupled lactose transport (2) as it participates in osmolality-sensitive, light driven H^+ pumping by bacteriorhodopsin (14, 56). High osmolality may activate ProP by removing excess water molecules that would interfere with H^+-symport, leaving water as a cofactor at key internal sites. Thus LacY and ProP would be designed to attain optimal hydration at low and osmolality, respectively. For a unifying

mechanism to encompass OpuA, such effects must be exerted on the intra-membrane helix bundles.

The role of the membrane and the structural mechanism of osmosensing Osmosensory transporters detect osmolality changes in the context of membrane phospholipid, and multiple observations suggest that phospholipid participates in osmosensing. The osmotic activation profile of OpuA was not affected by changing the acyl chain length, the configuration or the position of a double bond within the phospholipid fatty acids (112). However the osmolality required to activate OpuA, BetP or ProP increases with the proportion of anionic lipid in cells or proteoliposomes (54, 97, 103, 112). In addition cationic amphipath tetracaine stimulated the activities of BetP and OpuA at low osmolality (81, 100, 111, 112).

As detailed elsewhere, amino acid replacements and deletions within the CBS domains and the anionic tail of OpuAA (6, 7, 63), the long helical C-terminus of BetP (53, 77, 81, 103, 104) and the coiled-coil domain of ProP (24, 38, 61, 97, 109) alter their osmolality responses. These alterations change $X_{1/2}$ and/or alter the shape of the osmotic activation curve (e.g. reducing the amplitude of the response by raising activity at low osmolality). The C-terminal domains of OpuAA and BetP are proposed to serve as "osmosensing switches". Osmotically-induced changes to cytoplasmic ionic strength or K^+ concentration are believed to alter the association of these domains with other parts of each protein and/or the membrane surface (6, 7, 53, 63). Extensive biochemical evidence indicates that the C-terminus can interact with other parts of BetP and/or the membrane (77). Such switching would determine the proportions of these transporters in "inactive" and "active" conformations. The structure of BetP suggests a mechanism based on inter-subunit interactions (91), but no data yet demonstrates that the trimeric state is required for osmosensing or transport.

The osmotic activation of ProP depends on the membrane lipid composition, the position of the transporter on the cell surface and the C-terminal coiled-coil domain. The osmolality at which ProP activity is half maximal varies directly with the proportion of CL in the membrane of wild type bacteria and with the proportion of PG in the membrane of CL-deficient bacteria (97). Analogous behaviour was seen by varying the lipid proportions in proteoliposomes (97). The proportion of CL varies directly with the osmolality of the bacterial growth medium in wild type bacteria and the proportion of PG varies directly with the osmolality in CL-deficient bacteria (97). ProP concentrates at the poles of *E. coli* cells in a CL-dependent manner (96). These observations suggest that, physiologically, the osmotic activation profile of ProP is adjusted by regulating CL synthesis and locating ProP in the CL-rich membrane at the cell poles. Perhaps this mechanism places ProP in an anionic lipid environment like those of OpuA and BetP.

The coiled-coil domain of ProP is clearly implicated in the adjustment of ProP localization and the osmolality response but it is not an "osmosensing switch". Peptide replicas of the C-terminus of *E. coli* ProP form homodimeric, antiparallel α-helical coiled-coils *in vitro* (Fig. 5E), and such a structure appears to link ProP monomers *in vivo* (24, 38, 39, 127). Some amino acid replacements that disrupt the coiled-coil elevate $X_{1/2}$ and some prevent ProP localization at the cell poles (24, 97, 109, 110). However ProP variants that cannot form coiled coils are osmosensing transporters, as is at least one ProP orthologue that lacks the coiled-coil (109). Furthermore, some amino acid replacements within the membrane-integral domain of ProP also elevate $X_{1/2}$ or render ProP activity osmolality-insensitive (48, 61, 120). Thus osmosensing could occur directly at the level of the alternating access mechanism (48).

Osmosensing via membrane lipid provides an attractive addition or alternative to the mechanisms outlined above. The membrane could be an osmosensing antenna, providing a

large, solvent-exposed surface and thereby increasing sensitivity to solvent changes (83, 116). Key membrane properties such as surface charge density or intrinsic strain could be affected by interactions of solutes with the membrane surface, in turn altering transporter structure and function. Such effects may be mimicked by experimental and physiological variations in membrane phospholipid composition, and by treatment with membrane-perturbing amphipaths such as tetracaine. The failure of small organic kosmotropes to activate osmosensory transporters has been taken as evidence that activation is salt-specific. Alternatively, at the high concentrations used for these experiments, organic kosmotropes may increase membrane hydration via preferential exclusion. The rate of glycine betaine influx via BetP decreased as bacteria adapted to an osmotic upshift by accumulating that osmoprotectant (11). This observation could reflect rehydration of the membrane and/or the transporter. This concept could be tested by examining the impacts of diverse kosmotropic and chaotropic organic solutes on transporter activities in proteoliposomes.

CONCLUSION

Physiologists aim to elucidate cellular processes by isolating variables *in vitro*. Experimental systems based on dilute aqueous solutions do not encompass effects of preferential interactions, volume exclusion, or non-equivalence of solute concentration and activity that may be prominent *in vivo*. Experimenters are addressing these challenges by studying how protein-nucleic acid interactions, protein folding and osmosensing occur *in vitro* and *in vivo*. Such work has revealed that protein-nucleic acid interactions may be optimized via coordinated variations in cytoplasmic solvent composition and macromolecular crowding. Progress is being made towards elucidating osmosensory mechanism(s), but further work is required as consensus has not been reached regarding the signal(s) detected or the structural mechanism(s) of osmosensory transporters.

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LITERATURE ANNOTATIONS

(8): Explains the physical properties of membranes.

(9): Explains principles governing the impacts of preferential interactions with organic solutes (or osmolytes) on protein folding.

(21): Investigation of the impacts of lumenal solutes (salts and PEGs) in proteoliposomes on osmosensory transporter ProP.

(50): Application of the osmotic stress approach to elucidate the impacts of cytoplasmic properties on physiological processes, illustrated with effects on protein-nucleic acid interactions.

(63): Investigation of the impacts of lumenal salts in proteoliposomes on osmosensory transporter OpuA.

(58): Explains how interactions of solutes with macromolecules and membranes influence their structure and hydration.

(89): A comprehensive account of preferential interactions among solvent components and macromolecules.

(99): Investigation of the impacts of lumenal solutes in proteoliposomes on osmosensory transporter BetP.

(119): Bacterial osmotic stress responses.

(122): Explains the origins and consequences of macromolecular crowding for physiological processes.

KEY WORDS

osmotic stress

compatible solute

preferential interactions

macromolecular crowding

protein-nucleic acid interactions

osmosensing transporter

ABSTRACT

To understand physiological processes we must learn how cells control their own physical and chemical properties, a phenomenon known as cellular homeostasis. The dilute solutions usually used for experiments do not simulate the cytoplasm, where solute concentrations and activities are not equivalent, components experience preferential interactions and macromolecular crowding may dominate critical processes. These cellular properties can be varied systematically by varying the osmolality and simulated *in vitro*. Cells dehydrate and the cytoplasm becomes more crowded as the osmolality increases. They respond by accumulating potassium glutamate or compatible solutes to high levels; macromolecular crowding decreases and the accumulated solutes alter cytoplasmic chemistry in different ways. This osmotic stress approach is revealing how cytoplasmic properties modulate protein-nucleic acid interactions and protein folding. Proteins ProP, BetP and OpuA are osmosensory transporters because they sense the osmolality and respond by mediating compatible solute accumulation *in vivo* and in proteoliposomes. They differ in structure and energy coupling mechanism but may share an alternating access transport mechanism involving paired intra-membrane helix bundles. Each is an oligomer with extended, cytoplasmic carboxyl terminal domains that are regulatory but structurally unrelated. Each was exposed to diverse solvents *in vitro* to study osmosensing. On this basis researchers propose that BetP is a K^+-sensing chemosensor, OpuA responds to cytoplasmic ionic strength and ProP responds to effects of solute exclusion on its own hydration. Each transporter may represent a different osmosensory mechanism or each of the proposed mechanisms may reflect a different perception of a single, unifying mechanism.

MINI-GLOSSARY

Chemosensor	A *chemosensor* is a protein that detects the concentration of a specific chemical (or ligand) by binding that ligand at one or a few sites on the protein surface.
Compatible Solute	A *compatible solute* can attain high cytoplasmic levels without disrupting cellular functions (121). Glycine betaine and proline are compatible solutes.
Macromolecular Crowding	*Macromolecular crowding* is the tendency of macromolecules to influence biochemical equilibria (e.g. folding, interactions of macromolecules) or reaction rates (e.g. enzyme activities) by occupying space in a solution (122).
Ideal Solution	In an *ideal solution* the activity coefficient (γ_s) of every constituent is 1 and the activity of each solute (a_s) equals its concentration (c_s).
Osmosensor	An *osmosensor* is a protein that detects changes in water activity (direct osmosensing) or resulting changes in cell structure or composition (indirect osmosensing) and directs osmoregulatory responses.
Osmotic Pressure	The *osmotic pressure* is equal to the hydrostatic pressure that would be required to prevent water from flowing across a semipermeable membrane into an aqueous solution of a membrane-impermeant solute.
Preferential Interactions	*Preferential interactions* cause solutes to partition unequally between bulk water and the water of hydration surrounding each area on the surface of each macromolecule (108).
Turgor Pressure	*Turgor pressure* is the hydrostatic pressure difference that balances the osmotic pressure difference between the interior and exterior of walled cells.

ACRONYMS

ABC transporter, ATP-Binding Cassette transporter

BCCT family, Betaine-Carnitine-Choline Transporter family

CBS domain, Cystathionine-β-Synthase domain

CL, cardiolipin (otherwise known as diphosphatiylglycerol)

MFS, Major Facilitator Superfamily

PE, phosphatidylethanolamine

PEG, poly(ethyleneglycol)

PG, phosphatidylglycerol

TM, Transmembrane helix

SUMMARY POINTS

1. Cells control their own physical and chemical properties, a phenomenon known as cellular homeostasis. Researchers must characterize the intracellular milieu and simulate it *in vitro* to fully understand cell physiology.
2. Dilute aqueous solutions do not simulate the cytoplasm, where the concentration and activity of each solute are not equal, and preferential interactions among components and macromolecular crowding may dominate critical processes.
3. It is hard to measure solute activities and macromolecular crowding *in vivo* but these properties can be varied systematically by varying the osmotic pressure (the osmotic stress approach). As the osmotic pressure increases cells dehydrate and the cytoplasm becomes more crowded. *E. coli* cells respond by accumulating potassium glutamate or compatible solutes to high levels. Macromolecular crowding decreases and the accumulated solutes alter cytoplasmic chemistry in different ways.
4. The osmotic stress approach is revealing how macromolecular crowding and preferential interactions modulate protein-nucleic acid interactions and protein folding *in vivo*. It is also being used to assess the overall significance of crowding for *in vivo* processes.
5. Proteins ProP from *E. coli*, BetP from *C. glutamicum* and OpuA from *L. lactis* represent distinct transporter families and energy coupling mechanisms. They are osmosensory transporters because they sense the osmotic pressure and respond by mediating compatible solute accumulation *in vivo* and in proteoliposomes. The activity of each transporter increases as a sigmoid function of the osmotic pressure when membrane impermeant solutes are added to the external medium.

6. Researchers aim to understand osmosensing by elucidating structure-function relationships for ProP, BetP and OpuA *in vivo* and *in vitro*. Each likely functions via an alternating access mechanism involving paired intra-membrane helix bundles. Each is an oligomer with extended, cytoplasmic carboxyl terminal domains. These C-terminal domains are regulatory but their structures are unrelated.
7. To study osmosensing, ProP, BetP and OpuA are exposed to complex, concentrated solutions *in vitro*. They respond differently and with specificity to lumenal solutes in proteoliposomes. Researchers propose that BetP is a K^+-sensing chemosensor, OpuA is regulated by cytoplasmic ionic strength and ProP responds to effects of solute exclusion on its own hydration. Each transporter may represent a different osmosensory mechanism or each proposal may represent a different perception of a single osmosensory mechanism.

FUTURE ISSUES

1. Most studies of physiological mechanisms have been based on dilute aqueous solutions in which solute concentrations approximate solute activities while preferential interactions and volume exclusion are minimized. How, and to what degree, has this practice distorted our understanding of cell physiology?
2. When deprived of osmoprotectants, do all bacteria respond to osmotic upshifts by excreting putrescine while accumulating K^+ glutamate and compatible solutes of biosynthetic origin? Do the listed solutes fully account for trans-membrane charge balance in all bacteria? What do osmoregulatory K^+ transporters sense and how are they regulated? Do osmoregulatory putrescine transporters exist?
3. Osmosensory transporters serve as paradigms for the study of cellular homeostasis and researchers do not yet agree on the signal(s) to which they respond. Are these transporters chemosensors that respond to the concentration of specific chemical species such as K^+? Do they detect a collective solution property such as the ionic strength? Do they respond to osmotic stress acting at a molecular level, via steric solute exclusion? Does the membrane act as an osmosensing antenna?
4. How do the structures and kinetic mechanisms of osmosensing transporters change to accelerate osmoprotectant transport and mediate compatible solute accumulation to a level appropriate at each osmotic pressure.

SIDEBAR: SOLUTION PROPERTIES

The **osmotic pressure** of an aqueous solution (Π, atm) is:

$$\Pi = -(RT/V_w^{1}) \ln a_w \quad (1)$$

R is the Gas Constant (0.082054 L atm mol^{-1} K^{-1}), T is the temperature (degrees Kelvin, where 25ºC is 298ºK) and V_w^{1} is the partial molar volume of water (0.01801 L mol^{-1}). Π is inversely proportional to the water activity (a_w, unitless) which is 1.0 for pure water and falls towards zero as solutes are added.

The **osmolality** or osmotic pressure at a particular temperature is:

$$\text{Osmolality} = \Pi/RT = -V_w^{1} \ln a_w \quad (2)$$

The units of osmolality are Osmolal or osmoles per kg of solvent (water). The **osmolarity** is the number of osmoles per liter of solution (Osmolar). The osmolality can be measured with a freezing point or vapour pressure osmometer (107). The **osmolarity** is more challenging to measure; it can be approximated by summing the concentrations of osmotically active solutes.

The **excluded volume fraction** (Φ, the fraction of solution volume from which macromolecules exclude one another) can be calculated as:

$$\Phi = \Sigma c_i v_i \quad (3)$$

where c_i is the mass concentration of the i^{th} macromolecule (grams of macromolecule per mL of solution), v_i is its partial specific volume (mL of macromolecule per gram of macromolecule) and Φ is summed over all macromolecules. Most proteins have a partial specific volume of approximately 0.7 mL/g (13). Φ is a measure of macromolecular crowding.

The **ionic strength** (I) is a measure of the total ion content of a dilute solution (89):

$$I = \tfrac{1}{2} \Sigma (c_i z_i^2) \quad (4)$$

where c_i is the concentration of the i^{th} ion species, z_i is its net charge and the sum extends over all ions present. For example, the ionic strength of 0.1 M Na_2SO_4 is 0.3 M.

[1] Editor – please note that there should be bars over the Vs in equations 1 and 2, and the sentence following eqn. 1.

Figure Legends

Figure 1: **Cross section through an *Escherichia coli* cell A**. Macromolecules are shown at a magnification of ×1,000,000. The cell wall and flagellum are at the top and the nucleoid is at the bottom with the cytoplasm in between. **B**. A small region within the cell is shown at a higher magnification (5,000,000X). This image shows the crowding of small molecules between the larger ones. Small organic molecules are pink, phosphate ions are yellow and orange, chloride ions are green and water molecules are turquoise. The origins of these illustrations are described elsewhere (34, 35). Permission from Springer Science + Business Media pending.

Figure 2: **Chemosensing versus Osmosensing** Many proteins are sensors, their conformations changing between an "off" and an "on" state in response to a change in the environment. Chemosensors detect specific molecules. The proportion of chemosensor molecules in the "on" conformation increases in proportion to the activity of a specific chemical (the ligand) which binds to a specific site (or small number of sites) on the protein surface. Osmosensors detect changes in water activity (direct osmosensing) or resulting changes in cell composition or structure (indirect osmosensing). Some osmosensors may change their conformations in response to solvent changes. The proportion of osmosensor molecules in the "on" conformation would then increase when the sensor surface was exposed to a suitably altered solvent (depicted here by a change from a white to a shaded background). In either case sensing can involve a change in protein shape (pictured) or oligomeric state (e.g. ligand- or solvent-induced dimerization). Adapted with permission from *Microbiology and Molecular Biology Reviews,* volume 63, pages 230-262. Copyright 1999 American Society for Microbiology.

Figure 3: **LacY activity decreases as ProP activity increases under osmotic stress** Cytoplasmic membrane vesicles (ghosts) were prepared from *E. coli* cells expressing both *proP*

and *lacY*. Proline (red symbols) and lactose (blue symbols) uptake rates were measured in media supplemented with sucrose to adjust the osmolality (and hence the water activity). The data for ProP and LacY were fit to equation (1) and the equation $v_0 = V_{max} \exp [-k (\Pi/RT)]$, respectively, to produce the regression lines. Adapted with permission from *Biochemistry,* volume 47, pages 8176-8185. Copyright 2008 American Chemical Society.

Figure 4: **Alternating Access Mechanism of Membrane Transport** *Top*: According to the alternating access mechanism, substrates bind within an outward-facing cleft between paired, intra-membrane helix bundles. The substrate may become occluded within a structural intermediate. A conformational change exposes the binding site to the cytoplasm and allows substrates to exit (32, 57). *Bottom*: Kinetic mechanism for ion symport. This mechanism is particularly well established for MFS member LacY (36). I^+ is H^+ for ProP (23, 62) and Na^+ for BetP (30). The coupling stoichiometry (n) is 2 Na^+ per glycine betaine (30).

Figure 5: **Structures of Osmosensing Transporters** The structures of BCCT Family Member BetP from *C. glutamicum*, MFS member ProP from *E. coli* and the periplasmic binding protein component of ABC transporter OpuA from *B. subtilis* (OpuAA) are illustrated. Unless otherwise indicated the protein backbones are colored according to amino acid side-chain polarity: red for acidic residues aspartate and glutamate, blue for basic residues arginine, lysine and histidine, green for polar residues serine, threonine, cysteine, asparagine and glutamine and yellow for non-polar residues.

BetP: The crystal structure of trimeric BetP as viewed from the cytoplasm (A) and of a single BetP subunit as viewed from the membrane plane with the cytoplasmic surface down (B) ((91), PDB ID 2WIT). In A the three BetP subunits are coloured black, grey and by amino acid. B shows a single subunit with residues from the N-terminus through the end of TM 2 as strands

and residues 313-324 as a trace to reveal glycine betaine (space-filling, CPK colouring) occluded within the substrate-binding site. Aromatic residues contributing to the binding site are shown as purple sticks (Trp 189, Trp 194, Tyr 197 and Trp 374) (see G for comparison).

ProP: A homology model of ProP as viewed from the membrane plane with the cytoplasmic surface down (C) and from the cytoplasm (D) ((25, 120), PDB ID 1Y8S). The arrow in C marks the position of a substrate analogue in the homologous crystal structure of LacY ((2), PDB ID 1PV7). The stars in C and D mark the C-terminal amino acid of the model (residue 452, of 500 ProP residues)). E shows the structure of a homodimeric peptide corresponding to residues 468-497 of ProP, determined by nuclear magnetic resonance (NMR) spectroscopy (127). This homodimeric, antiparallel α-helical coiled-coil links ProP dimers *in vivo*. The dimer interface also involves TM 12 (38, 61).

OpuA: A schematic representation of transporter OpuA (**A**) and the crystal structure of periplasmic binding protein OpuAC from *B. subtilis* (**B**). In **A**, two cytoplasmic ATP-binding OpuAA subunits are blue, two transmembrane OpuAB domains/subunits are yellow and two external, substrate-binding OpuAC domains/subunits are red. In *B. subtilis* OpuAA, OpuAB and OpuAC are separate subunits and, as in other ABC transporters, there may be a single OpuAC subunit per OpuAB dimer. In *L. lactis* OpuAB and OpuAC are domains of the same protein and OpuAC may function as a homodimer in which two, seven TM bundles flank a betaine binding site (42). **B** shows a crystal structure of OpuAC from *B. subtilis*, coloured by amino acid residue, in complex with glycine betaine (space filling, CPK colouring). The binding pocket includes 3 tryptophan residues (Trp 72, Trp 178 and Trp 225, shown as purple sticks) that are arranged in a prism-like geometry and coordinate the trimethylammonium group of glycine betaine via cation-π interactions ((43), PDB ID 2B4L).

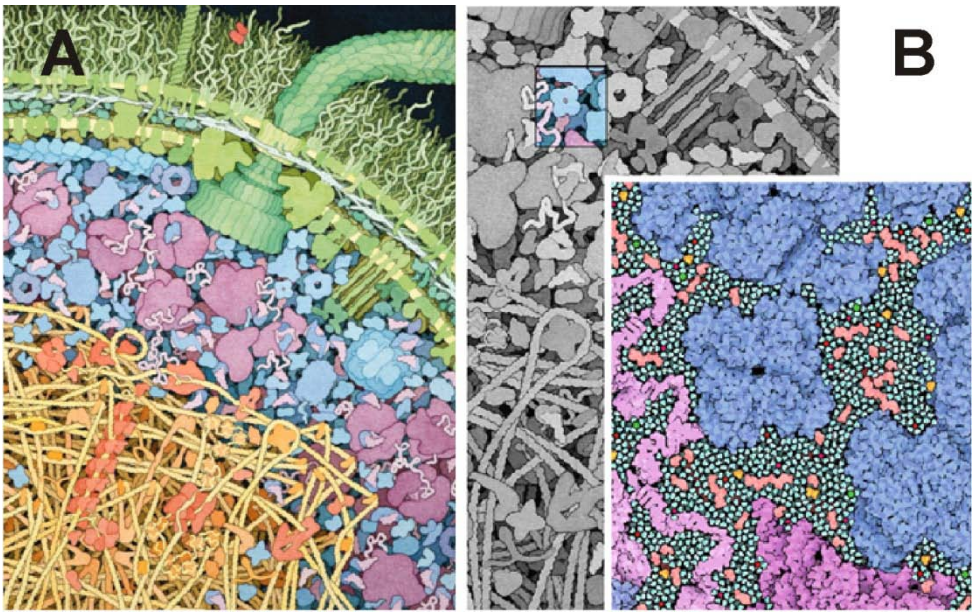


Figure 1

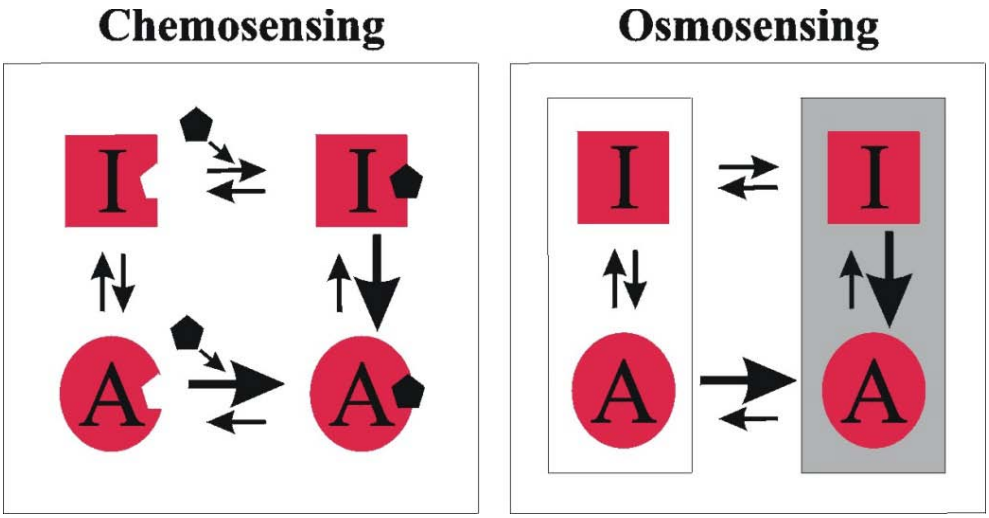


Figure 2

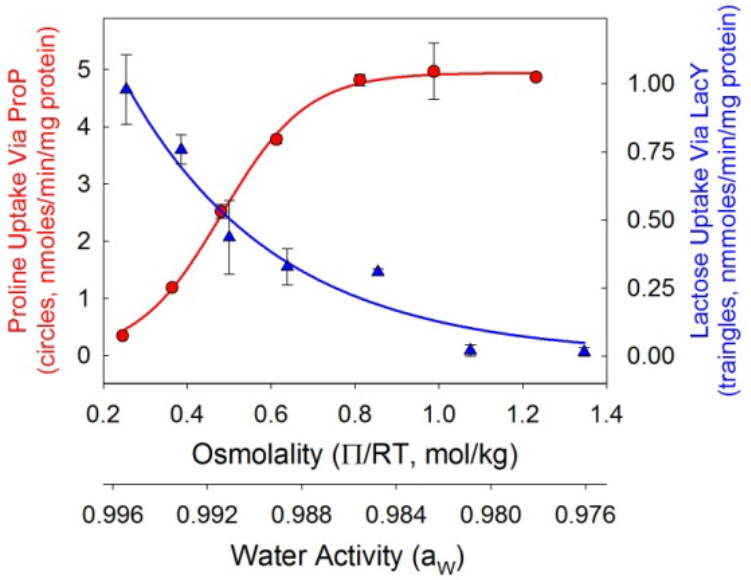
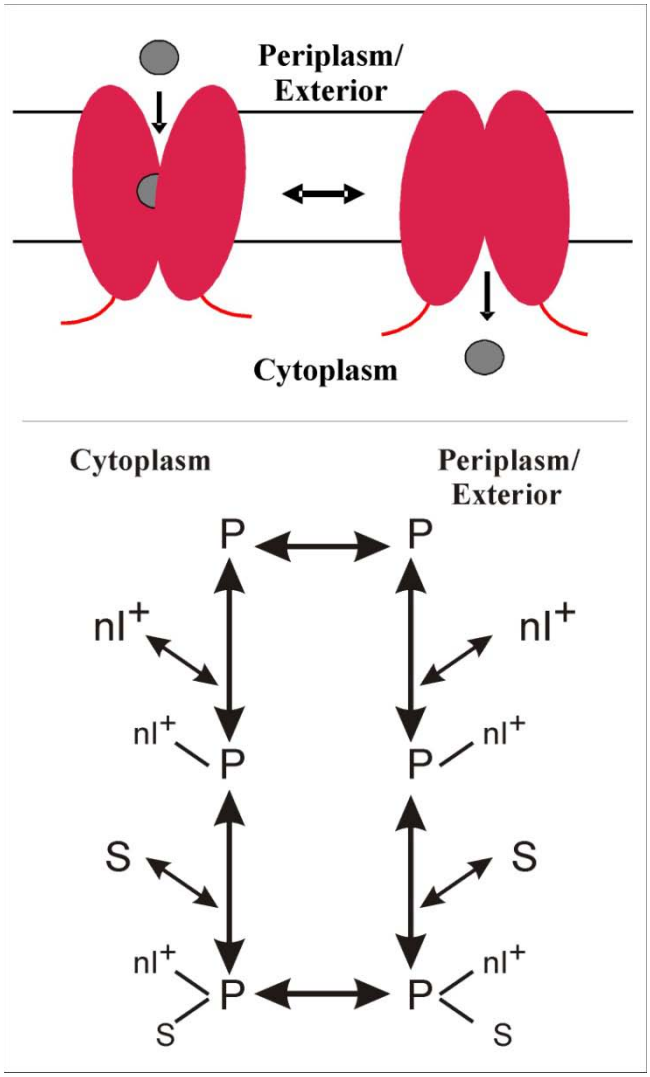

Proline Uptake Via ProP
(circles, nmoles/min/mg protein)
Lactose Uptake Via LacY
(traingles, nmmoles/min/mg protein)
0
1
2
3
4
5
0.00
0.25
0.50
0.75
1.00
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Osmolality (Π/RT, mol/kg)
0.996
0.992
0.988
0.984
0.980
0.976
Water Activity (a_W)

Figure 3


Periplasm/
Exterior
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Cytoplasm
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P
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Figure 4

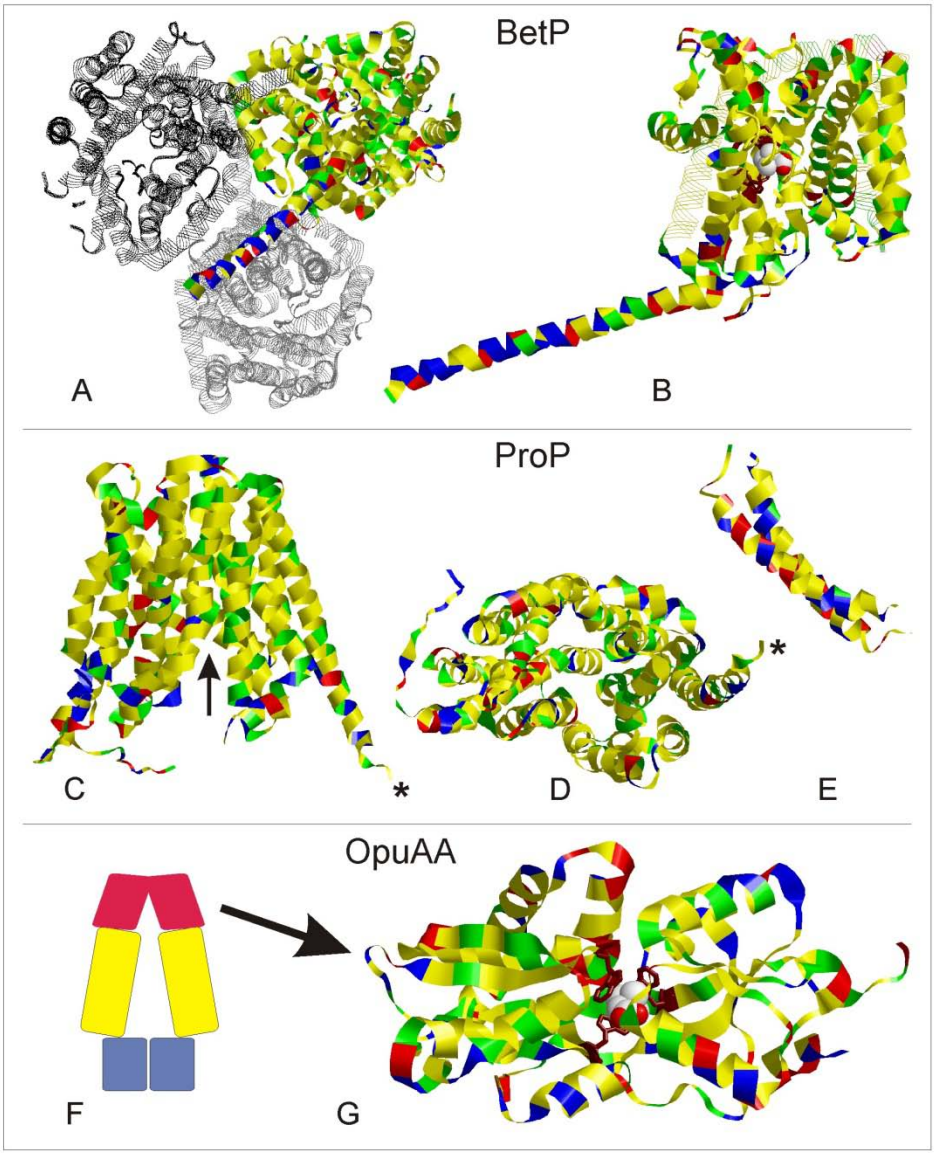

Figure 5