In order to circumvent deleterious effects of hypo- and hyperosmotic conditions in its environment, *Corynebacterium glutamicum* has developed a number of mechanisms to counteract osmotic stress. The first response to an osmotic upshift is the activation of uptake mechanisms for the compatible solutes betaine, proline, or ectoine, namely BetP, EctP, ProP, LcoP and PutP. BetP, the most important uptake system, responds to osmotic stress by regulation at the level of both protein activity and gene expression. BetP was shown to harbor three different properties, i.e. catalytic activity (betaine transport), sensing of appropriate stimuli (osmosensing) and signal transduction to the catalytic part of the carrier protein which adapts its activity to the extent of osmotic stress (osmoregulation). BetP is comprised of 12 transmembrane segments and carries N- and C-terminal domains, which are involved in osmosensing and/or osmoregulation. Recent results on molecular properties of these domains indicate the significance of particular amino acids within the terminal 25 amino acids of the C-terminal domain of BetP for the process of osmosensing and osmoregulation.
contrast, *C. glutamicum* harbors only secondary systems for uptake of compatible solutes [5,7]. The proline carrier PutP does not respond to osmotic stress and takes up proline for anabolic needs. EctP, ProP and LcoP accept the compatible solutes betaine, proline and ectoine with different specificities. EctP seems to represent the ‘back-up system’, its activity being constitutively present. The most important system in *C. glutamicum*, in terms of activity, and, at the same time, the best studied transporter, is the glycine betaine carrier BetP. BetP responds to hyperosmotic stress both on the level of protein activity (see below) as well as gene expression. BetP is a member of the BCCT family of secondary transporters. It is a polytopic membrane protein consisting of 595 amino acids. It is predicted to span the membrane 12 times, and it contains hydrophilic domains of 55–62 amino acids at both the N- and the C-terminal end. Both terminal domains face the cytoplasm [8]. Recently, strep-tagged BetP was isolated and purified, and two-dimensional crystals have been obtained [9]. The crystals were analyzed by electron cryo-microscopy and projection maps were calculated to 7.5 Å. In the crystal, BetP monomers are associated forming a dimer of trimers. In each monomer 10–12 transmembrane α-helices can be distinguished as well as two pore-like features suggesting potential transport pathways. The projection map of BetP does not resemble projection structures of other secondary carriers.

BetP comprises several different functional properties. (a) Its catalytic function is betaine transport driven by the electrochemical Na⁺ potential. (b) As a regulatory function, BetP is able to efficiently adapt its catalytic activity to the extent of hyperosmotic stress. (c) BetP directly senses the extent of osmotic stress without the help of additional proteins or cofactors. The fact that one single carrier protein harbors these different kinds of functional properties was first shown for the osmoregulated transporter ProP of *E. coli* [10].

2. Catalytic properties of BetP

BetP couples the electrochemical Na⁺ potential to betaine flux via co-transport of two Na⁺ ions [11]. Extremely high steady state accumulation ratios of up to $4 \times 10^6$ (internal/external concentration) have been determined. When measuring steady state internal pools at low external betaine concentrations and under variation of the electrochemical Na⁺ potential, the chemical potential (betaine concentration gradient) and the electrochemical Na⁺ potential were found to be in equilibrium [11]. BetP was shown to be strictly specific for glycine betaine, the affinity of the external binding site of BetP for betaine in *C. glutamicum* is relatively high (Kₘ of 8 μM), whereas the Na⁺ affinity is low (Kₘ of 4 mM).
When induced at the level of expression and activated at the level of protein activity, BetP is, together with acetate and glucose uptake, one of the fastest uptake systems in C. glutamicum, characterized by $V_{\text{max}}$ values up to 110 μmol g cd⁻¹ min⁻¹ [11]. The high activity of BetP in comparison to the other osmoregulated carriers in C. glutamicum is one of the reasons why betaine uptake in general dominates the uptake of other compatible solutes, provided it is present in the environment.

3. Osmoregulatory properties of BetP

Accumulation of compatible solutes must be adapted to the actual situation of the cell, depending on internal and external osmolality and consequently on turgor pressure. Besides, by variation of the biosynthesis rate, solute accumulation can be regulated by modifying the rate of uptake, the rate of efflux or both. BetP is able to instantly respond to osmotic stress, and its activity is adapted to the actual extent of stress. Two different features of osmoregulation, as far as it concerns hyperosmotic stress, are recognized (Fig. 2). As an osmoregulated uptake system it is more or less silent in the absence of hyperosmotic or in the presence of hypotonic stress. Upon an osmotic upshift, BetP becomes activated (activated state). Once the hyperosmotic stress has been compensated by accumulation of compatible solutes, uptake activity is reduced in order to prevent an overshoot of internal solute concentration (activity adaptation).

The time course of activation and activity adaptation under in vivo conditions is shown in Fig. 2 for BetP-mediated betaine uptake. The observed response time of BetP to an osmotic upshift is shorter than the time resolution of conventional kinetic experiments, i.e. less than 1 s. If the initial activity, measured immediately after the onset of osmotic stress, is plotted in dependence of the extent of osmotic stress, a kind of dose–response curve is obtained (Fig. 3). Without osmotic stress, BetP is virtually inactive. The threshold of activation is observed at about 300–400 mosM/kg, and BetP reaches an optimum activity around 1300 mosM/kg. This activation profile is very similar in vivo, i.e. in intact cells of C. glutamicum (Fig. 3A) and in vitro when BetP is reconstituted in proteoliposomes. The external osmolality was adjusted by NaCl (full circles), sorbitol (open circles), or glycerol (squares). Only non-permeable osmolytes induce an osmotic gradient and thus osmotic activation of BetP.

![Fig. 3. Profile of BetP activation by osmotic stress. (A) The external osmolality was adjusted by adding NaCl to cells of C. glutamicum and E. coli and betaine uptake activity was determined. In E. coli cells (strain MKH13, devoid of osmoregulated carriers), BetP was heterologously expressed. (B) Activation of betaine uptake by BetP reconstituted in proteoliposomes. The external osmolality was adjusted by NaCl (full circles), sorbitol (open circles), or glycerol (squares). Only non-permeable osmolytes induce an osmotic gradient and thus osmotic activation of BetP.](image-url)

![Fig. 2. Betaine uptake by BetP after osmotic upshift. Cells are suspended in buffer at an osmolality of 125 mosM/kg. At time zero, NaCl is added to reach final osmolalities of 125 mosM/kg (no addition, circles), 625 mosM/kg (triangles), 925 mosM/kg (squares), or 1325 mosM/kg (stars). At 40 min, the osmolality is increased to 1600 mosM/kg.](image-url)
Several conclusions can be drawn from these experiments: (a) BetP does not need additional effector components for its regulatory function. Its full functional competence observed both in E. coli and in proteoliposomes argues for BetP harboring all three properties, i.e. that of a transporter, an osmoregulator and an osmosensor; (b) the stimulus responsible for osmoregulation must be functional in all systems tested, C. glutamicum and E. coli cells, as well as proteoliposomes; (c) a different membrane environment of BetP leads to modulation of the activation profile. After betaine accumulation, the cell reaches osmotic compensation, and the uptake of compatible solutes decreases (Fig. 2). The apparent steady state reached is not a steady state typically observed in uptake systems, independent of whether it is explained on thermodynamic (balance of electrochemical potentials) or kinetic reasons (e.g. trans-inhibition). The fact that the steady state levels of solute accumulation depend on the extent of osmotic stress to be compensated [11], argues that the mechanism by which the steady state is defined is an intrinsic aspect of osmoregulation.

4. Osmosensory properties of BetP

The first event in a signal transduction cascade of osmotic stress response, an osmosensor relays a signal to the transport protein leading to osmotic activation or activity adaptation. BetP was shown to possess all three functions relevant for signal transduction, consequently, it should harbor sensor domain(s) and must be able to relay an appropriate signal to the catalytic (transport) domain regulating its activity.

A long list of possible stimuli is considered as being sensed by bacterial cells [1]. Mainly four different categories are discussed: (i) stimuli directly originating from the environment, e.g. external osmolality, ionic strength, or concentration of particular solutes; (ii) the same parameters may be relevant at the cytoplasmic site, too, since a change in internal water activity is the consequence of a change in external osmolality. Molecular crowding of cytoplasmic macromolecules may also be relevant; (iii) membrane-related parameters such as cell turgor and membrane strain may be important for carrier proteins; (iv) changes in the surrounding osmolality might also directly influence soluble and membrane-embedded proteins by altering their surface hydration and thus their conformation.

It has been shown for reconstituted BetP of C. glutamicum that an increase in the luminal K⁺ concentration, i.e. at the side where the hydrophilic domains are located, is sufficient to activate BetP. In these experiments, several possible triggers were excluded, namely changes of external solutes, as well as changes of internal solutes except K⁺, e.g. choline⁺, NH₄⁺ [8] and Na⁺ (unpublished observations), furthermore membrane strain and cell turgor, since proteoliposomes are lacking turgor pressure. By identifying an increase in luminal K⁺ as an activating stimulus, BetP has been converted from an osmosensor to a chemosensor. The internal threshold concentration of K⁺ necessary for activation of BetP in E. coli phospholipid liposomes was around 220 mM [12]. Furthermore, a strong influence of the membrane phospholipid composition on activation was observed. The higher the share of negatively charged phospholipids, the higher was the threshold of K⁺ necessary for activation.

Recently, data on sensing by and activation of BetP have been obtained which give a closer insight at the molecular level. The terminal hydrophilic domains of BetP, both at the N- and the C-terminal end, have been shown to strongly influence activation in intact C. glutamicum cells [14] (Fig. 4). Truncation of the N-terminal domain did not significantly change the catalytic activity of BetP, however, the activation profile was shifted to higher osmolalities, i.e. higher osmotic stress was required to activate this mutant form of BetP. Truncations at the C-terminal domain had a more drastic effect. When truncating 25 or 45 amino acids at the end of this domain, which consists of about 56 amino acids, deregulation of the protein was observed, whereas truncation of the terminal 12 amino acids led to partial deregulation only. These mutant forms were found to be constantly active in intact C. glutamicum cells even in the absence of osmotic stress, although at a somewhat reduced V_max. These results argue for the C-terminal domain being involved in osmosensing and may thus be interpreted in terms of locating the sensory input at this hydrophilic domain. Interestingly, they also indicate that the C-terminal

Fig. 4. Consequence of N- and C-terminal truncations of BetP on transport activity. Betaine uptake activity of wild-type BetP in C. glutamicum cells (circles) increases in response to increasing hyperosmotic stress. After truncation of 60 amino acids at the N-terminal hydrophilic domain of BetP, as indicated by an arrowhead in the upper panel, the activation profile is shifted to higher osmolalities (crosses). Truncation of 25 amino acids at the C-terminal domain leads to deregulation of BetP (squares). The increase at low Na⁺ concentration is caused by the dependence on Na⁺ as a co-substrate.
domain acts as an inhibitory element. In other words, this domain seems to be required to keep BetP in an inactive state in the absence of osmotic stress. If the C-terminal domain would function by activating BetP, a truncation should result in inactivation of the protein. Notably, these results have recently been confirmed using isolated wild-type and mutant forms of BetP reconstituted in proteoliposomes. BetP was activated in dependence of the luminal $K^+$ concentration, and the terminal truncations led to the same changes in the regulation pattern as observed in intact cells (unpublished observations).

It has not been elucidated on a mechanistic level, how the increase in internal $K^+$ concentration causes BetP activation. Several observations can be put together to a hypothetical model. The structure of the heterologously expressed C-terminal domain of BetP was found to be influenced by the presence of amphiphilic surfaces. CD-spectroscopy of this domain consisting of 56 amino acids revealed a random structure which was only slightly shifted to a more structured conformation by addition of detergents below their critical micellar concentration. The peptide, however, adopted a 90% $\alpha$-helical structure in the presence of detergent micelles [15]. It was furthermore shown by resonant mirror spectroscopy that the C-terminal domain binds strongly to lipid monolayer surfaces. As expected, the binding capacity depended on the ionic strength of the buffer used. Surprisingly, the binding affinity was decreased at high $K^+$ concentrations in the range of several 100 mM, but not to a comparable extent by other cations, such as Na$^+$, neither did it depend on the kind of anions used [15]. On the basis of these observations a model can be suggested where (i) the state of binding of the C-terminal domain of BetP to the membrane surface determines the state of activity of the carrier, and (ii) the terminal domain changes its conformation and thus its binding state in dependence of the surrounding $K^+$ concentration. The $K^+$ and membrane-surface dependent conformational change is then supposed to be responsible for activation of BetP. This model would also explain the observed dependence of the activation threshold on the kind and charge of phospholipids present in the membrane.

In contrast to the BetP activation after an osmotic upshift, not much is known concerning stimuli and signal transduction responsible for downregulation of BetP activity after osmotic compensation (activity adaptation). In accordance with the view that the adapted state is not equivalent to the ‘ground state’ before osmotic activation (see above), $K^+$ could be ruled out as a stimulus for this regulation in intact \textit{C. glutamicum} cells, since the internal $K^+$ concentration was found not to change during activity adaptation (unpublished observations). Also a change in external parameters could be excluded. For this kind of regulatory process, stimuli related to membrane properties (turgor or membrane strain) could be possible candidates. Consequently, these studies in intact cells have to be extended to proteoliposomal systems in order to define the responsible stimuli.

Finally, it should be pointed out that sensory events are certainly of crucial importance for expression regulation of the \textit{betP} gene, too. The membrane-embedded sensor elements of the two-component regulatory system must be able to perceive stimuli related to osmotic stress and must convert them into appropriate signals. It is, however, unclear whether these stimuli are related to those which are perceived by BetP in the course of regulation at the level of protein activity.

5. Conclusions and perspectives

The simplicity of the proteoliposomal system was the basis for detailed studies leading to the identification of major factors involved in osmosensing and osmoregulation. These results, however, have to be taken with some caution with respect to their general significance. First, they have been obtained in proteoliposomes which differ from the situation of intact cells in many aspects. Under in vivo conditions, a high concentration of macromolecules (proteins) in the surrounding hydrophilic spaces is present as well as membrane proteins in the lipid phase, the composition of lipids is more complex, and last but not least, the cell wall is present which gives rise to turgor pressure. Second, the influence of the physical state of the membrane and/or the membrane surface seems to be more important than has been considered so far. Studies carried out with BetP of \textit{C. glutamicum}, and in particular with OpuA of \textit{L. lactis}, indicate the involvement of the lipid phase on regulatory processes of the embedded transporter protein [16,17]. Moreover, the fact that turgor is absent in proteoliposomes does not necessarily rule out its influence on regulating osmoreactive carriers in intact cells. It should also be taken into account that mechanosensitive efflux channels in fact seem to be regulated by a change in the lateral membrane pressure [18]. Third, there is direct evidence that a multiplicity of stimuli may be relevant for controlling osmoregulated carrier proteins. Even when only the influence of solutes from the cytoplasmic space is considered, the three best studied systems, namely ProP of \textit{E. coli} [19], OpuA of \textit{L. lactis} (van der Heide et al., 2000) and BetP of \textit{C. glutamicum} [8] were found to respond to ions and/or solutes in general in the former cases, and specifically to $K^+$ in the latter. It should also be noted that only activation upon osmotic stress has been studied in detail so far, but not activity adaptation of osmoregulated carrier proteins after stress compensation. Consequently, more detailed investigations in particular also in intact cells are required to elucidate possible additional stimuli which may play a role in the control of osmoregulated transport proteins. Finally, it should be emphasized that another aspect has also not been studied in much detail so far, namely the question of whether the oligomeric state of these transporters may be relevant for osmosensing and/or osmoregulation. This issue has been brought up by studies of \textit{E. coli} ProP, which
contains a coiled-coil domain and thus a putative protein–protein interaction site [20,21]. The recent finding that BetP seems to be a trimer in its native state [9], adds further ideas in this direction, since monomer interaction may be an element of osmoregulation, too.

References