Osmosensor and Osmoregulator Properties of the Betaine Carrier BetP from Corynebacterium glutamicum in Proteoliposomes*

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The secondary glycine betaine uptake system BetP of Corynebacterium glutamicum was purified from Escherichia coli membranes in strep-tagged form after heterologous expression of the betP gene and was reconstituted in E. coli lipids. BetP retained its kinetic properties (V_max and K_m for betaine and Na^+) as compared with intact cells. The influence of driving forces (Na^+ gradient and/or electrical potential) on betaine uptake was quantified in proteoliposomes. BetP was effectively regulated by the external osmolality and was stimulated by the local anesthetic tetracaine. A shift of the optimum of osmotic stimulation to higher osmolalities was linearly correlated with an increasing share of stimulated by the local anesthetic tetracaine. A shift of regulatory properties of BetP may be related to the osmotic stress is a common challenge encountered by mammals, plants, fungi, and bacteria. Hypo or hyperosmotic stress causes water influx or efflux, respectively, from the cell, thereby changing its hydration, volume, and/or turgor pressure. As a response, cells have developed a variety of effective mechanisms to overcome water stress. Osmoregulation is thus a biological phenomenon of fundamental significance, e.g. salinity tolerance in plants, kidney physiology, microbial pathogenesis, and microbial biotechnology. It is necessary for the survival and growth of prokaryotic and eukaryotic microorganisms in their native habitats. The most widely distributed strategy of response to hyperosmotic stress is the uptake and/or biosynthesis of osmoprotectants, also called compatible solutes, e.g. glycine betaine, proline, ectoine, or trehalose (1, 2). To optimally adapt the response of the cell to the extent of osmotic stress, the transporters and biosynthetic enzymes involved in the stress response are regulated on the level of expression and activity. Signal transduction pathways exist that originate from the osmotic challenge of the cell and ultimately lead to the immediate (activity regulation) and long-term (expression regulation) responses of the cell. The very first step in signal transduction necessarily is related to osmosensing mechanisms at the cell membrane or in the cytoplasm (2). Our work on BetP of Corynebacterium glutamicum focuses on osmosensing processes at the cell membrane.

C. glutamicum, a GC-rich Gram-positive soil bacterium, is extensively used in amino acid production (3). It is equipped with four secondary carriers for the uptake of the compatible solutes betaine, proline, and ectoine (4). Two of them, BetP and ProP, are regulated by the external osmolality both on the level of activity and expression, EctP is constitutively synthesized but osmoregulated on the level of activity, and PutP is an anabolic proline carrier not involved in osmoregulation. The high affinity, Na^+-coupled glycine betaine uptake system BetP was biochemically analyzed in detail (5, 6), and the corresponding gene has been characterized (7). BetP is predicted to be a typical 12-transmembrane segment transporter carrying cytoplasmically exposed domains of 50–60 amino acids at its N- and C-terminal parts. Specific deletions in both terminal domains of BetP led to modulation of its response to osmotic stress and resulted in the uncoupling of the sensory form of the catalytic function, i.e. transport (6). These results were interpreted in terms of BetP comprising both functions of an osmosensor and an osmoe regulating this carrier at least to some extent, BetP seems to be a suitable candidate to study the kind of signals that are sensed by the membrane-embedded transporter.

In view of the complex situation within the intact cell while sensing osmotic stimuli and transducing the corresponding signals, to be able to define the putative stimulating parameters it is desirable to reduce this complexity. The suitability of a reconstituted system for this purpose has recently been shown for the osmoregulated ProP from E. coli (14). Proteoliposomes are an attractive approach for this goal mainly because of two aspects. First, putative additional components or

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Osmotic stress is a common challenge encountered by mammals, plants, fungi, and bacteria. Hypo or hyperosmotic stress causes water influx or efflux, respectively, from the cell, thereby changing its hydration, volume, and/or turgor pressure. As a response, cells have developed a variety of effective mechanisms to overcome water stress. Osmoregulation is thus a biological phenomenon of fundamental significance, e.g. salinity tolerance in plants, kidney physiology, microbial pathogenesis, and microbial biotechnology. It is necessary for the survival and growth of prokaryotic and eukaryotic microorganisms in their native habitats. The most widely distributed strategy of response to hyperosmotic stress is the uptake and/or biosynthesis of osmoprotectants, also called compatible solutes, e.g. glycine betaine, proline, ectoine, or trehalose (1, 2). To optimally adapt the response of the cell to the extent of osmotic stress, the transporters and biosynthetic enzymes involved in the stress response are regulated on the level of expression and activity. Signal transduction pathways exist that originate from the osmotic challenge of the cell and ultimately lead to the immediate (activity regulation) and long-term (expression regulation) responses of the cell. The very first step in signal transduction necessarily is related to osmosensing mechanisms at the cell membrane or in the cytoplasm (2). Our work on BetP of Corynebacterium glutamicum focuses on osmosensing processes at the cell membrane.

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Whereas immediate response and long-term adaptation to osmotic stress is relatively well characterized in several microorganisms, e.g. in E. coli, Salmonella typhimurium, and Bacillus subtilis (2, 10, 11), the mechanisms related to sensing osmotic stress are not well understood (2). This also holds true for eukaryotic cells, in which many osmolatory genes, proteins, and signal cascades have been identified, whereas the mechanisms by which the extracellular osmolality is sensed remains largely obscure (2, 12, 13). This in particular concerns both the physical parameters that may be actual stimuli in osmosensing as well as their interaction with the osmosensory proteins. In view of the well characterized properties of BetP and the possibility to discriminate between sensory and catalytic function of this carrier at least to some extent, BetP seems to be a suitable candidate to study the kind of signals that are sensed by the membrane-embedded transporter.

In view of the complex situation within the intact cell while sensing osmotic stimuli and transducing the corresponding signals, to be able to define the putative stimulating parameters it is desirable to reduce this complexity. The suitability of a reconstituted system for this purpose has recently been shown for the osmoregulated ProP from E. coli (14). Proteoliposomes are an attractive approach for this goal mainly because of two aspects. First, putative additional components or
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Liposome Preparation—E. coli phospholipids (polar lipid extract, Avanti, Alabaster, AL), t-α-Phosphatidyl-DL-glycerol (from egg yolk, Sigma), or a mixture of both were added to 100 mM potassium P, pH 7.5, 2 mM mercaptoethanol to a final concentration of 20 mg of phospholipids/ml. The lipids were solubilized with 1.5% N-Octyl-a-D-glucoside and were dialyzed three times against the same buffer to remove the detergent.

Preparation of Inverted Membrane Vesicles—Synthesis of BetP was induced in E. coli DH5αmcmer pASK-IBA5 betP with 200 μg of anhydrotetracycline/liter culture at an optical density of 1.5 at 600 nm. After 3 h of further growth cells were harvested by centrifugation, washed with potassium P, pH 7.5, 2 mM mercaptoethanol, and resuspended in the same buffer. Membrane vesicles were prepared by passage of the cell suspension through a French press (SLM Aminco; Colora, Lorch, Germany) and centrifuged at 19,000 × g for 15 min to remove unbroken cells. Membranes were isolated by centrifugation (214,000 × g for 30 min), washed with the same buffer, adjusted to 20 mg of protein/ml, and stored at −80 °C.

Purification of Strep-BetP—To an aliquot (typically 500 μl) of washed membrane vesicles of E. coli DH5αmcmer pASK-IBA5 betP, the same volume of double-concentrated buffer K (50 mM potassium P, pH 8.0, 10% glycerol, 2 mM mercaptoethanol, 1 mM EDTA) was added. While stirring on ice, Strep-BetP was extracted with N-dodecyl-a-n-maltoside or Triton X-100 that were added stepwise until a final concentration of 5% was reached. After stirring on ice for 30 min, the suspension was centrifuged for 20 min at 109,000 × g in a Beckman TLX ultracentrifuge (Beckman, Munich, Germany). The supernatant fraction containing solubilized Strep-BetP was purified by Strep-tagII/StrepTactin affinity chromatography (IBA, Göttingen, Germany) using a 2-ml column of StrepTactin resin. The column was pre-equilibrated with buffer K− (50 mM potassium P, buffer, pH 8.0, 10% glycerol, 2 mM mercaptoethanol, 1 mM EDTA) and 39 mM N-dodecyl-a-n-maltoside). The supernatant was diluted with four volumes of buffer K− before addition to the column to allow binding of proteins to StrepTactin. The flow rate was adjusted to 0.1 ml/min. The column was washed with three bed volumes of buffer K− before Strep-BetP was eluted with the same volume of buffer K− containing 5 mM desthiobiotin. The eluate was collected in 1–2-ml fractions, and the fractions with the highest protein concentration were used for reconstitution.

Reconstitution of Strep-BetP—Purified Strep-BetP was reconstituted as described previously (22). The phospholipids (5 mg/ml) were extruded (Liposofast; Avestin, Ottawa, Canada) by applying 13 extrusion cycles (0.4 μm polycarbonate filter; Poretics, Livermore, CA), partially solubilized with 0.5% Triton X-100, and stirred at room temperature for 1 h. At this stage the buffer was added in varying amounts to keep the final ratio of phospholipid:protein at 30 (w/w). To remove the detergents, Bio-Beads preswashed with distilled water were added at a Bio-Bead (wet weight, filter-dried) to Triton X-100 ratio of 5 and a bead/dodecylmaltoside ratio of 10 (w/w). The mixture was kept under gentle stirring at room temperature for 1 h, and the same amount of fresh Bio-Beads were added. After 1 h of gentle stirring the double amount of fresh Bio-Beads was added. The mixture was kept overnight at 4 °C and subjected to gentle stirring before the supernatant was removed. The turbid suspension was centrifuged for 20 min at 440,000 × g at 15 °C and washed three times in 100 mM potassium P, pH 7.5. The pellet was resuspended in a small volume of the same buffer and stored at −80 °C. Before use the proteoliposomes had to be extruded again. A small volume was diluted to 500 μl, extruded, and sedimented by ultracentrifugation as described above. After appropriate dilution, the pellet was used for transport assays.

Synthesis of [14C]Glycine Betaine—Synthesis of [14C]glycine betaine by oxidation of [14C]choline (Amersham Pharmacia Biotech) using choline oxidase was performed as described earlier (7, 23).

Transport Assay—Proteoliposomes prepared in 100 mM potassium P, buffer, pH 7.5, were diluted 200-fold into potassium-free buffer (25 mM or 100 mM Tris/Mes, pH 7.5) containing 15 μM [14C]glycine betaine, at least 30 mM NaCl, and about 0.1 μM valinomycin to create an outwardly directed K+ diffusion potential. To establish hypersomotic conditions, typically 250 mM NaCl or 500 mM sorbitol were added. Buffer osmolality was measured with an osmometer (OSMOMAT 030; Gonotec, Berlin, Germany). At various times intervals an aliquot of the reaction mixture was filtered through 0.22-μm nitrocellulose filters (GS; Millipore, Eschborn, Germany). The filters were washed with 2.5 ml of 100 mM LiCl and counted (LS-6500; Beckman, Munich, Germany). In a series of filtrations using washing buffers of varying osmolality we have confirmed that the routine application of 100 mM LiCl in the washing procedure did not lead to loss of liposomal contents (data not shown).

Silverstaining, Western Blot Analyses, and Protein Determination—

1 The abbreviations used are: PCR, polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid.
Samples from different steps of the purification procedure were analyzed by SDS-polyacrylamide gel electrophoresis (24). Proteins were separated on 12.5% polyacrylamide gels and detected with silver (25). Specific detection of Strep-BetP was achieved by Western blot analysis (26) using 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid)/NaOH, 10% methanol, pH 11, as electrode buffer. After blocking with bovine serum albumin the membrane was probed with strepavidine alkaline phosphatase conjugate (1:5000; Amersham Pharmacia Biotech). Binding of the conjugate was detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium assay (Sigma). Protein determination was performed using the Bradford method (27) or the amido black method (28) with bovine serum albumin as standard.

**RESULTS**

**Synthesis, Solubilization, Purification, and Reconstitution of BetP**—To facilitate protein purification and detection, Strep-tagII was attached to the N terminus of BetP, yielding Strep-BetP. In view of the fact that modifications of the N-terminal extension have previously been shown in *C. glutamicum* to potentially lead to changes in the functional and regulatory properties of BetP (6), we first studied the function of Strep-BetP in *C. glutamicum*. After homologous expression of the strepbetP fusion gene in *C. glutamicum*, transport assays proved that the regulation of betaine uptake activity by changes in external osmolality were virtually identical to that reported for the wild type protein (results not shown). Heterologous expression of strepbetP in *E. coli* cells led to membrane insertion of Strep-BetP. For solubilization, both dodecylmaltoside and Triton X-100 proved to be effective. After functional reconstitution into proteoliposomes, however, dodecylmaltoside-solubilized Strep-BetP resulted in higher specific transport activity and increased protein stability (results not shown).

Several changes, with respect to the original procedure for application of StrepTactin columns, had to be introduced to obtain pure Strep-BetP protein. An increased NaCl concentration of 200 mM was necessary in all buffers during the purification procedure, and the concentration of desthiobiotin for elution of Strep-BetP had to be raised to 5 mM. The presence of glycerol significantly improved the protein stability; addition of 10% glycerol to the column buffers was found to be optimal (data not shown). From 1 liter of *E. coli* cell culture we obtained 1.3 g of dry biomass, which, after purification, led to 600 µg of Strep-BetP protein. The protein band eluted from the affinity column with an apparent molecular mass of about 55 kDa was identified as Strep-BetP by Western blot analysis (Fig. 1).

Functional reconstitution of Strep-BetP into *E. coli* phospholipids was optimized with respect to a number of parameters. Although dodecylmaltoside proved to be superior for solubilization of BetP, the use of Triton X-100 at a ratio of 1 g of detergent/1 g of phospholipid and an absolute concentration of 0.5% (w/v) was optimal (data not shown) for detergent treatment of the pre-formed liposomes (see “Experimental Procedures”). Furthermore, the lipid/protein ratio during reconstitution turned out to be critical; optimal reconstitution activity was at a lipid/protein ratio of 30:1 (w/w). Under these conditions a fraction of 64 ± 14% of the total amount of BetP applied was reproducibly found to be incorporated in the proteoliposomal membrane after reconstitution (data not shown).

**Kinetic and Energetic Properties of Strep-BetP in Proteoliposomes**—As observed in intact cells (5, 6, 29) also in proteoliposomes BetP proved to be specific for glycine betaine. Neither proline nor ectoine were accepted as substrates (Fig. 2). Fig. 2 also shows that betaine uptake in proteoliposomes containing reconstituted BetP was very fast. Because determination of a true zero value for the filter background was possible by extrapolating the data obtained with proline or ectoine as substrates (Fig. 2), initial uptake rates could be calculated, and thereby the basic kinetic parameters of reconstituted BetP were derived (Fig. 3). The apparent *K*ₐ for betaine was determined to 3.6 ± 0.5 µM, which is somewhat lower than the corresponding value in cells of *C. glutamicum* (8.6 ± 0.4 µM) or *E. coli* (12.8 ± 1.2 µM) (7). The *V*ₐₐₜₚₕ was 2.3 mmol of betaine/mg of protein/min. The apparent *K*ₐ for the cosubstrate Na⁺ was determined to 15.0 ± 1.5 mM and thus was somewhat higher than the *K*ₐ in cells of *C. glutamicum* (4.1 ± 0.4 mM) or *E. coli* (6.1 ± 1.2 mM) (7).

Both the Na⁺ gradient and the electrical potential have been identified as driving forces for betaine transport in *C. glutamicum* (5). Proteoliposomes offer an optimal measuring system for a detailed characterization of the influence of single components of the driving forces, because the conditions on both sides of the membrane can be varied (Fig. 4). To measure the dependence of betaine transport on the chemical Na⁺ potential (gradient), the *K*⁺ concentrations inside and outside the proteoliposomes were adjusted to 175 mM K⁺ in the presence of valinomycin, and a Na⁺ gradient across the membrane was established by fixing internal [Na⁺] at 1 mM and varying external [Na⁺] between 10 and 100 mM (Fig. 4A). The results show that betaine uptake can, in principle, be driven by a chemical potential only, although at low rates only. The influence of the electrical potential was investigated by establishing different K⁺ diffusion potentials. While the Na⁺ concentration

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**Fig. 1. Purification of Strep-BetP.** Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis and stained with silver (lanes 1–6), and Strep-BetP was immunodetected with strepavidin alkaline phosphatase conjugate (lane 7). Lane 1, induced cells (15 µg protein); lane 2, membrane vesicles (15 µg); lane 3, solubilized membrane proteins (15 µg); lane 4, flow through (15 µg); lane 5, last washing fraction (0.1 µg); and lanes 6 and 7, elution fraction (1 µg).

**Fig. 2. Transport specificity of Strep-BetP in proteoliposomes.** 15 µM of 14C-radiolabeled compatible solutes were added to proteoliposomes, glycine betaine (solid circles), proline (squares), or ectoine (open circles). For establishing hyperosmotic conditions, 250 mM NaCl was added to the uptake buffer.
inside and outside the proteoliposomes was kept at 50 mM, interior \([K^+]\) was fixed at 175 mM whereas exterior \([K^+]\) was varied from 175 to 1.75 mM. A linear dependence of the initial uptake rate on the applied electrical potential was observed at membrane potentials between 80 and 110 mV (Fig. 4B). To combine both driving forces, the \([K^+]\) gradient was kept constant (internal \([K^+]\), 175 mM and external \([K^+]\), 5.5 mM, corresponding to 90-mV electrical potential in the presence of valinomycin), whereas the \([Na^+]\) gradient was varied (internal \([Na^+]\), 1 mM and external \([Na^+]\), 10 to 100 mM, corresponding to 60- or 120-mV chemical potential) (Fig. 4C). Under these conditions, the same relative increase of betaine uptake due to the increasing chemical \([Na^+]\) potential was observed as found in the first experiment applying a \([Na^+]\) gradient only (cf. Fig. 4, A and C), however, at a strongly increased basal activity due to the driving force of the electrical potential.

Activity Regulation of BetP by Osmolality—Betaine transport was shown to be regulated by the osmolality of the surrounding medium both when BetP was inserted in \(C.\) glutamicum and in \(E.\) coli membranes (6, 7). In the reconstituted system consisting of pure BetP protein inserted into \(E.\) coli phospholipids we observed a strict dependence of betaine uptake activity on the extent of osmotic stress applied (Fig. 5). Addition of both nonionic sorbitol and ionic NaCl led to activation of BetP; however, the extent of activation by NaCl was significantly higher than that observed for sorbitol. Activation by glycerol was not observed, proving that only membrane-impermeable solutes are able to exert osmotic stress in the proteoliposomal system. The maximum activity of betaine uptake in proteoliposomes was reached at an external osmolality of about 700 mosmol/kg. Interestingly, even at zero transmembrane osmotic gradient, a low but significant uptake activity of BetP was observed.

Although the complexity of the reconstituted system is drastically reduced compared with intact cells the question concerning the nature of the signal triggering the immediate response of BetP to a change in osmolality is still not easy to address. One way of studying this problem is the application of membrane-active reagents like local anesthetics, which in low concentrations are supposed to change the physical state of the membrane only without directly affecting the inserted proteins (30–32). For this purpose tetracaine was added to proteoliposomes containing reconstituted BetP under isosmolar condi-
Uptake of [14C]glycine betaine into proteoliposomes is shown. The internal buffer of the proteoliposomes (100 mM potassium Pi, pH 7.5) had an osmolality of 220 mosmol/kg. The external buffer basically contained NaCl (33), whereas those of C. glutamicum contain mainly phosphatidyl glycerol (87%) and minor amounts of other phospholipids (5% phosphatidyl inositolmannoside, 3% phosphatidyl inositol, 2.5% phosphatidic acid, and 1% diphosphatidyl glycerol) (34). Unfortunately, no stable liposomes could be prepared from lipid extracts of C. glutamicum cells, although various extraction procedures have been applied (results not shown). This may be due to the high content of mycolic acids in the outer membrane of this organism, which, in contrast to other Gram-positive bacteria, possesses a bilayer structure in its cell wall. (35, 36). To mimic C. glutamicum plasma membranes as well as intermediate compositions between the E. coli and the C. glutamicum membrane type we prepared proteoliposomes from pure E. coli phospholipids, pure phosphatidyl glycerol, and from mixtures of the two lipids. In these proteoliposomes of different composition, betaine uptake rates were determined as dependence of increasing external osmolality (Fig. 7A). For a better comparison the maximum values obtained with proteoliposomes made from different lipid mixtures were each normalized to 100%. Although the absolute activity of reconstituted betaine uptake decreased with increasing amounts of phosphatidyl glycerol in the proteoliposomes, the results show that the optimum of osmotic stimulation of BetP-mediated betaine uptake shifts to higher values of osmolality as more phosphatidyl glycerol is included in the proteoliposomal membrane. To properly correlate these findings to the physiological situation, the recalculated data that were previously obtained for BetP inserted in E. coli and C. glutamicum membranes are shown in Fig. 7B (6, 7). The observed shift of osmotic activation of betaine transport from BetP in C. glutamicum was 1% in all experiments.
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glutamicum to BetP in E. coli membranes turns out to be closely similar to the shift observed in proteoliposomes, the phospholipid composition of which is changed accordingly. This indicates that, in fact, the phospholipid surrounding is a major factor for the regulatory properties of BetP in dependence of osmotic stress.

**DISCUSSION**

Purification of strep-tagged BetP from *C. glutamicum* was achieved after heterologous expression of the strepbetP gene in *E. coli*. Both the isolation and reconstitution procedures were optimized, resulting in highly active BetP-mediated betaine uptake with a $V_{max}$ of 2.3 mmol/mg of protein/min. The reconstituted carrier protein fully retained its functional properties with respect to substrate specificity and affinity toward its substrates betaine and Na$^+$. Furthermore, after homologous expression in *C. glutamicum* the N-terminal tag was shown to have no significant influence on the regulatory properties of BetP.

The main topic studied in this paper concerns the characterization of the modulating influences of osmolality and lipid surroundings on the membrane-inserted BetP protein. Thus, the mechanisms of osmosensing by an osmoregulated carrier protein are directly addressed. Analysis of the transport activity regulation by changes in the composition of the external medium demonstrated that BetP fully retained its regulatory properties in proteoliposomes. Activation because of increasing external osmolality was observed both after addition of NaCl and sorbitol, whereas an equivalent addition of the membrane-permeable solute glycerol did not lead to significant activation of BetP. The difference in stimulation by NaCl as compared with sorbitol can putatively be attributed to the difference in the action of these solutes. Whereas an increase in external sorbitol leads to an osmotic stress only (transmembrane osmolality difference), in addition to osmotic stress, NaCl induces ionic stress (change in ionic strength). Interestingly, this difference seems to be abolished at higher osmolalities. The fact that glycerol, which freely equilibrates across the proteoliposomal membrane, did not lead to a similar response of BetP indicates that a symmetric increase of the concentration of a neutral solute on both sides of the liposomal membrane does not affect betaine transport. Consequently, anhydrotic stress does not seem to play a role for reconstituted BetP (37).

In view of the results obtained previously with BetP in intact *C. glutamicum* and *E. coli* cells (5, 6), it is interesting to note that BetP in liposomes is active already at zero transmembrane osmotic gradient, although only to a low extent. This was not observed for reconstituted *E. coli* ProP in the only study available that uses a comparable approach (2). It is too preliminary, however, to speculate why and to what extent BetP may be active without the presence of an osmotic stress. However, this result illuminates the fact that at least in this respect, the situation in intact cells is not directly comparable with that of proteoliposomes. Intact cells do have an osmotic gradient across the plasma membrane because of the turgor pressure that is necessary to maintain the cell's viability and growth. This is not the case in proteoliposomes. Thus, the “zero values” are principally different in the two cases, i.e. a true zero transmembrane osmotic gradient in the case of proteoliposomes and the physiologically turgor-related transmembrane osmotic gradient in the intact cell. Further studies with extended variations of internal and external solute concentration and composition, and correlation of these data with the situation in intact cells are necessary to fully elucidate the significance of this difference.

A series of different primary signals can, in principle, be made responsible for modulating the activity of an osmoregulated membrane-inserted carrier system, irrespective of the kind of cell in which osmoregulation takes place (2, 10, 13). Although this complexity is significantly reduced in the liposomal system because of the lack of both a cytoplasm and a cell wall, it is still a question of whether this signal input is related to the change in the external compartment, the transmembrane gradient, or whether the input is via the membrane directly. We addressed this question by adding the local anesthetic tetracaine, which, when added in low concentrations, is known to change the intrinsic phase behavior of lipid membranes directly (30–32). Although not to a large extent, addition of tetracaine in concentrations below 1 mM increased transport activity mediated by the reconstituted BetP, as observed previously in intact cells (6). The optimum stimulation in the two largely different surroundings was found to be surprisingly similar, particularly in view of the fact that only the lower part of the titration experiment, i.e. the course of increasing activity due to activity stimulation, is significant. The consecutive decrease was previously shown to be related to a damaging effect of tetracaine on the membrane stability and should thus be significantly different in a simple proteoliposomal bilayer as compared with the complex ensemble of bilayer-structured cell wall and plasma membrane of *C. glutamicum*. Nevertheless, the observation of an activity modulation by addition of tetracaine indicates that at least part of the primary signal transferred to BetP comes directly from the membrane.

When the regulatory properties of *C. glutamicum* BetP were studied in the heterologous host *E. coli*, a shift in the optimum of stimulation by the increase of external osmolality was observed from 1.3 osmol/kg (*C. glutamicum*) down to 0.5 osmol/kg (*E. coli*) (7). This observation can be attributed either to the different membrane surroundings of BetP in the two different organisms or to the difference in turgor pressure between *E. coli* and *C. glutamicum*. The liposomal system offers the possibility to test at least the second alternative. Because it was, unfortunately, not possible to prepare stable liposomes from lipid extracts of *C. glutamicum*, presumably due to the mycolic acid contaminants from the bilayer-containing *C. glutamicum* cell wall (35, 36), we used pure phosphatidyl glycerol, which represents 87% of phospholipids of the *C. glutamicum* plasma membrane, to mimic the conditions in *C. glutamicum*. The analysis of osmotic stimulation of BetP in proteoliposomes prepared from different mixtures of phosphatidyl glycerol and *E. coli* lipids, which mainly consist of phosphatidyl ethanolamine, revealed a similar difference in the optimum of stimulation as previously found when comparing BetP inserted in intact *E. coli* and *C. glutamicum* cells (7). More conclusively, this shift in the observed optimum was linearly related to the increase in the content of phosphatidyl glycerol in the *E. coli* lipids. This indicates that the observed alteration in BetP function was not due to the simple presence of phosphatidyl glycerol but to its share in the bilayer membrane. This argument is strengthened by the fact that *E. coli* lipids naturally contain 15% phosphatidyl glycerol and 5% diphasphatidyl glycerol. Consequently, the regulatory pattern of BetP seems to be, at least to a significant extent, directly related to properties of the surrounding membranes. These properties are obviously not local effects, e.g. direct interaction of a particular phospholipid species with the inserted protein, rather global effects related to the state of the surrounding membrane must be assumed.

In principle, two models may be put forward to explain these results. The difference may be either related to the difference of the two lipids in (surface) charge or to their difference in bilayer-forming capacity. Phosphatidyl ethanolamine, the major *E. coli* lipid, is a typical non-bilayer lipid (38), whereas phosphatidyl glycerol is well known for its bilayer forming...
capacity (39). The result, that the optimum of stimulation is independent of whether the neutral sorbitol or the ionic NaCl is used in high concentrations, which, in the latter case would lead to a marked decrease of the surface potential in the direct neighborhood of BetP, weakens the argument of the surface charge being the major reason for the observed shift in activation. Furthermore, the significant content of negatively charged lipids in the E. coli lipid extract (20% negatively charged phospholipids; see above) argues against this explanation.

Taken together, the analysis of factors influencing osmoregulated betaine uptake catalyzed by membrane-inserted BetP in proteoliposomes led to the following conclusions. BetP alone is fully sufficient for the regulatory response in transport activity independent of whether the neutral sorbitol or the ionic NaCl is used in high concentrations, which, in the latter case would lead to a marked decrease of the surface potential in the direct membrane bilayer. Such a shift in activation capacity has experimentally been related to the mechanism of osmosensing. It should be taken into account that the different membrane changes caused by osmotic and ionic stress at or near the turgor pressure. However, in view of the fact that effective regulation of BetP proved to be preserved in the liposomal system, turgor does not seem a good candidate for regulating BetP in intact cells.

A series of widely different parameters have theoretically been made responsible as osmosensor stimuli, including influences originating from the composition and physical state of the cell wall or the plasma membrane, respectively, as well as from direct interactions with external solutes or internal compounds (solutes and macromolecules). Although included in this summary of hypothetical effectors, to our knowledge this is the first time that the phospholipid composition of the membrane has experimentally been related to the mechanism of osmosensing. It should be taken into account that the different putative regulatory parameters tested in this study seem to act on different levels. We have no indication so far that BetP could be activated by a particular lipid surrounding itself; in contrast, the lipid membrane seems to basically determine the possible range of activation for BetP in case it becomes activated. On the other hand, the event triggering the activation seems to be directly related, at least to a significant extent, to membrane changes caused by osmotic and ionic stress at or across the membrane bilayer.

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