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Stimulus analysis of BetP activation under in vivo conditions

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The secondary active, Na^+ coupled glycine betaine carrier BetP from *Corynebacterium glutamicum* BetP was shown to harbor two different functions, transport catalysis (betaine uptake) and stimulus sensing, as well as activity regulation in response to hyperosmotic stress. By analysis in a reconstituted system, the rise in the cytoplasmic K^+ concentration was identified as a primary stimulus for BetP activation. We have now studied regulation of BetP in vivo by independent variation of both the cytoplasmic K^+ concentration and the transmembrane osmotic gradient. The rise in internal K^+ was found to be necessary but not sufficient for BetP activation in cells. In addition hyperosmotic stress is required for full transport activity in cells, but not in proteoliposomes. This second stimulus of BetP could be mimicked in cells by the addition of the amphiphile tetracaine which hints to a relationship of this type of stimulus to a change in membrane properties. Determination of the molecular activity of BetP in both cells and proteoliposomes provided experimental evidence that in proteoliposomes BetP exists in a prestimulated condition and reaches full activity already in response to the K^+ stimulus.

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1. Introduction

Osmotic stress is a common type of environmental stress, in particular for soil bacteria like the grampositive organism *Corynebacterium glutamicum*. Cells counteract hyperosmotic stress by accumulation of compatible solutes provided either by synthesis or uptake, the latter being in general preferred for reasons of speed and energy economy. The glycine betaine carrier BetP from *C. glutamicum* is, together with ProP from *Eschericha coli* and OpuA from *Lactococcus lactis*, the best-studied osmoregulated uptake system both in terms of function and structure [1–3]. Different mechanisms of transport and osmostress-dependent regulations have been identified for these three model systems of osmoregulated solute uptake [4].

BetP is a secondary active carrier and a member of the BCCT family of transporters. It comprises 595 amino acid residues, 12 transmembrane segments, and two long terminal domains exposed to the cytoplasm [5]. Glycine betaine uptake is driven by cotransport with Na^+ in a 1:2 stoichiometry [6]. Regulation on the level of both transcription [7] and activity [6,7] has been studied in detail, the latter mainly by functional analysis upon reconstitution into proteoliposomes [8]. In the absence of osmotic stress in intact cells, BetP has a very low activity. It becomes instantly activated in response to a rise of the external osmolality. As the primary stimulus for activation of BetP, the increase in cytoplasmic K^+ as a consequence of high external osmolality was identified based on experiments in proteoliposomes [9]. BetP switches to the active state when cytoplasmic K^+ exceeds a threshold concentration of around 100 mM. Activation was shown to be specific for K^+, Rb^+, and Cs^+, whereas Na^+ or NH_4^+ were not effective [9,10]. Both terminal domains are involved in regulation of BetP, and the C-terminal domain with a size of around 54 amino acid residues was recognized as being required for K^+ sensing and subsequent transport regulation [5,11,12]. Consequently, BetP was identified as a transport system which harbors two independent functions, transport catalysis, on the one hand, and sensing as well as activity regulation, on the other [8].

In the last years, the 2D and the 3D structure of BetP were elucidated to high resolution [3,13]. BetP turned out to be a homotrimer in the membrane and the C-terminal, regulatory domain was found to establish, in addition to direct interaction sites at the periplasmic face of the membrane embedded parts of BetP, a special contact between adjacent protomers in the trimer specifically interacting with cytoplasmic loops of the neighboring protomer [3]. Crystallization of BetP under different conditions and analysis of recombinant forms of BetP provided a basis for the detailed elucidation of fundamental aspects of this transporter with respect to transport catalysis, substrate specificity, substrate/co-substrate coupling, phospholipid interaction, and domain function [14–17].

Although K^+ was identified as an important stimulus for activation of BetP in the reconstituted system, this concept was never rigorously proven under in vivo conditions due to an obvious experimental restriction of the analysis in intact cells. Bacteria in general require high internal K^+ concentrations for proper metabolic function. It is thus rather difficult to freely manipulate the internal concentration of this ion in the bacterial cytoplasm in particular in the low millimolar range [18]. We have recently unraveled that K^+ homeostasis is surprisingly simple in *C. glutamicum* involving only one single transport system, namely the CglK channel [19]. In connection to this finding it turned out that *C. glutamicum* has an exceptional tolerance to low cytoplasmic K^+

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concentrations [19]. This favorable experimental situation enabled us to study the K^+ dependence of BetP also under in vivo conditions. In the present analysis we confirmed cytoplasmic K^+ as a relevant stimulus required for BetP activation, however, it turned out that this stimulus is not sufficient for full activation of BetP embedded in the plasma membrane of intact cells. Under in vivo conditions, a second type of stimulus was identified as a prerequisite for full stimulation of BetP in response to osmotic challenge.

2. Materials and methods

2.1. Materials

Specific chemicals were L-α-lysophosphatidylcholine from egg yolk (Sigma-Aldrich), tetracaine hydrochloride (Sigma-Aldrich), *E. coli* polar lipid extract (Avanti), *strep*MAB Classic (IBA), and anti-mouse IgG (whole molecule)-alkaline phosphatase antibody produced in goat (Sigma-Aldrich). All other chemicals were of analytical grade.

2.2. Bacterial strains, plasmids, and growth conditions

For glycine betaine uptake measurements, *E. coli* MKH13 [20] was used, whereas *E. coli* DH5αmcr [21] was applied for preparative expression of *strep-betPC252T*. The plasmids used for expression of *betP* in *E. coli* are based on the pASK-IBA5 vector [22] in which *strep-betPC252T* is under the control of the *tet* promoter (IBA) [9]. *E. coli* cells were grown at 37 °C in LB medium supplemented with carbenicillin (100 mg/l). Induction of *betP* was carried out in exponentially growing cells by the addition of 0.2 mg of anhydroxytetracycline/l of culture. For expression of *strep-betP C252T*in *C. glutamicum* strain DHPF [23] was used. This strain was also used for glycine betaine import measurements. In this case, the *betP* was under the control of the *ptac* promoter of pXMJ19 [24]. *C. glutamicum* cells were grown in Brain Heart Infusion medium (Difco) at 30 °C. Subsequently, cells were used to inoculate a fresh culture, and *betP* expression was induced with 50 μM IPTG. For K^+-limited cultivation of *C. glutamicum* MMI minimal medium [12] was used, in which K^+ salts were replaced by the corresponding Na^+ salts. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}).

2.3. Reconstitution of strep-BetP, and variation of liposome properties

Strep-BetP C252T was purified and functionally reconstituted in liposomes as described previously [8] using 2% dodecyl maltoside for solubilization of membrane preparations. To alter the protein/lipid ratio of proteoliposomes they were fused with liposomes from *E. coli* polar lipid extract. For this purpose, BetP proteoliposomes were mixed with an appropriate amount of liposomes, filled with 0.1 M KP_i (pH 7.5) to a volume of 0.7 ml, and extruded 17 times through a polycarbonate membrane (Nucleopore) with a pore size of 0.4 μm. The volume of the proteoliposome suspension was then adjusted to 1 ml and exposed to two cycles of shock freezing in liquid nitrogen and thawing at room temperature. Subsequently the proteoliposomes were extruded through a polycarbonate filter again, centrifuged at 350,000 *g* at 20 °C for 20 min and resuspended in 0.1 M KP_i (pH 7.5). The final lipid concentration in the proteoliposome suspension was approximately 60 mg/ml. These vesicles were used for the transport assays. To exchange the internal medium of proteoliposomes an aliquot was thawed, centrifuged, and the supernatant was removed. The sediment was resuspended in1 ml of the new buffer and exposed to one freeze–thaw cycle. Subsequently the vesicles were extruded 17 times through a polycarbonate membrane, sedimented by centrifugation and resuspended in 1 ml buffer. The vesicles were frozen and thawed again, extruded, centrifuged, and finally resuspended in buffer to the final lipid concentration of approximately 60 mg/ml.

2.4. Transport assays

[^{14}C]-glycine betaine was synthesized and uptake of labeled betaine in *E. coli* and in *C. glutamicum* cells was measured as described previously [5,12]. For steady state accumulation of betaine, the external osmolality was 1.5 osmol/kg, the concentration of [^{14}C]-glycine betaine 0.5 mM. In these assays, glass fiber filters were washed with 0.8 M KP_i buffer (pH 7.5). For transport assays in K^+-depleted *C. glutamicum* DHPF the cells were first cultivated in BHI medium for 8 h and washed twice with 50 ml saline (0.9% NaCl). The cells were then transferred into K^+-free MMI medium with 50 μM IPTG (initial $OD_{600}$of 1). After 16 h the cells were harvested, washed twice in 25 mM NaP_i buffer with 0.1 MNaCl (pH 8, 0.24 osmol/kg), and resuspended in the same buffer containing 20 mM glucose. For uptake measurements, cells were assayed in 25 mM NaP_i with 0.1 M NaCl (pH 8, 0.24 osmol/kg) containing 10 mM glucose and different sorbitol concentrations to adjust the external osmolality. The cells were incubated for 10 min at 30 °C in buffer with or without 2.5 mM KCl. The uptake was started by the addition of 0.25 mM [^{14}C]-glycine betaine. If betaine import was measured in the presence of lyso-phosphatidylcholine (LPC) or tetracaine (TC) the cells were incubated for 10 min with 10 μM LPC or for 3 min with 0.8 mM TC, respectively, prior to the addition of glycine betaine. The OD_{600} of cell suspensions containing amphipathic compounds was 1.5–1.7.

Transport assays in BetP-proteoliposomes were performed as described previously [8] except that the osmolality of the external buffer was altered using sorbitol, and for the filtration of vesicles 0.45 μm nitrocellulose filters (GS, Millipore) were used. The betaine uptake rate was calculated as described previously [25]. Amphipathic compounds were added directly to proteoliposomes without preincubation. BetP activity was measured using the standard protocol [8] except that the external buffer was supplemented with different concentrations of LPC or TC prior to the addition of proteoliposomes. The lipid concentration of proteoliposome suspensions containing amphipathic compounds was approx. 0.3 g/l.

2.5. Determination of the size of proteoliposomes using multiangle dynamic light scattering (MDLS)

For the determination of the size of the proteoliposomes using dynamic light scattering, the vesicles were suspended in 0.1 M KPi buffer (pH 7.5) which was prefiltered through a 0.2 μm cellulose acetate membrane filter (Filtropur S 0.2, Sarstedt). For the measurement a light scattering photometer (ALV, D-63225 Langen) was used equipped with a fiber optic detection system in combination with an ALV-5000 multiple-tau digital correlator. The light source was an argon ion laser Koheras Model 165LGS (Sacher Lasertechnik GmbH, D-35037, Marburg) operating at 10 mW. The scattered light intensities from the sample were measured in a range of scattering angles between 30° and 150° in 10° intervals. Each measurement was performed in triplicate. The temperature was 25 °C, the refractive index and the viscosity of water were used. The intensity-intensity auto-correlation function $G^{(2)}(\tau)$ measured by the correlator has been analyzed following the procedure given in [26] using the program CONTIN [27] to obtain the decay rates Γ ($\Gamma = 1/\tau_c$; τ_c relaxation time) and the corresponding distribution functions $A(\Gamma)$.

2.6. Western blot analysis

The amount of BetP variants integrated into the membrane was determined by Western blot analysis of membrane extracts. Cells were suspended in buffer (0.1 M NaCl, 50 mM NaPi pH 8) and disrupted with glass beads in a cell homogenizer Precellys 24 (Bertin Technologies). Four disruption cycles (45 s at 6500 rpm) were performed. The cell homogenate was supplemented with N-lauroylsarcosine (0.5 % w/w) and incubated for 1 min at 40 °C. The unsolubilized particles were removed by centrifugation (30 s, 15,000 *g*), the supernatant was

diluted 1:2 with buffer (0.1 M NaCl, 50 mM NaPi pH 8) and spotted on nitrocellulose membranes, which were then dried for 20 min at room temperature. The membrane was incubated for 1 h in TBS (0.9% NaCl, 50 mM Tris pH 7.5) with 3% skim milk powder. Treatment with BetP-specific and anti-strep-antibodies was performed as described previously [8,11]. The samples were stained with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine/nitro-blue tetrazolium chloride, and the membrane was washed three times with TBS buffer. The stained membranes were transferred into reaction buffer (1 g/l para-nitrophenyl phosphate, 0.5 mM $MgCl_2$, 10 mM diethanolamine pH 9.5), and incubated at room temperature for 3 h. Subsequently the para-nitrophenol formed was quantified by measuring the absorption at 406 nm. For calibration purified cys-less Strep-BetP mixed with cell homogenate of *E. coli* MKH13 was used.

2.7. Determination of the average mass of phospholipids

E. coli polar lipid extract dissolved in chloroform was dried in a reagent tube and incubated for 1 h with 0.3 ml perchloric acid at 180 °C. The samples were cooled down to ~50 °C, 1 ml H_2O was added and the solution was incubated for 10 min. The sample was supplied with 0.8 ml of 0.75% ammonium molybdate (w/w) and 2.5% ascorbic acid solution (w/w) and incubated for 15 min at 110 °C. The sample was cooled down and the absorption at 810 nm was measured. For calibration 1–12 nmol KH_2PO_4 was used. To determine the dry mass of phospholipids 0.4 ml of *E. coli* polar lipid extract was slowly spotted on glass fiber filters, which were weighted before. After completely evaporating the solvent chloroform the filters were weighted again and the mass of phospholipids was calculated. The measured concentration of lipids was identical to that indicated by the manufacturer (25 mg/ml).

2.8. K^+ uptake measurements in C. glutamicum

K^+ uptake was quantified by monitoring its intracellular concentration by atom emission spectrometry using a flame photometer ELEX 6361 (Eppendorf). Bacteria were cultivated as described above for K^+-depletion, the cells were harvested, washed in 25 mM NaP_i buffer with 0.1 M NaCl (pH 8, 0.24 osmol/kg), and resuspended in the same buffer containing 20 mM glucose. For uptake measurements, cells were assayed in 25 mM NaP_i (pH 8) with 0.1 M NaCl containing 10 mM glucose and different KCl concentrations (70 μM–2.5 mM). Optionally the osmolality of the external buffer was increased up to 1 osmol/kg by the addition of sorbitol. Prior to use, the cells were stirred in buffer for 10 min at 30 °C. For measurement of intracellular K^+ the cell suspension was centrifuged for 30 s at 20,000 *g* at 30 °C, the supernatant was thoroughly removed by suction, and the bacterial sediment was resuspended in distilled H_2O. The cells were disrupted by 45 min sonication at 80 °C (Sonorex Super Plus 10P Digital, Bandelin electronic). After removal of cell debris by centrifugation, the K^+ concentration in the supernatant was measured. The cytoplasmic volume was calculated using the conversion factors determined previously for similar conditions. At the external osmolality of 0.24 osmol/kg 1 mg cell dry mass corresponds to 1.6 μl and at 1 osmol/kg to 1.4 μl of cytoplasmic volume [28].

3. Results

3.1. Stimulus analysis in C. glutamicum

High K^+ concentration at the cytoplasmic side of BetP was identified under in vitro conditions in proteoliposomes as the primary stimulus for BetP related to hyperosmotic stress and no alternative stimulus was detected [9]. In this work, we set out to experimentally discriminate this specific stimulus from a general stimulus related to hyperosmotic stress under in vivo conditions in intact cells of *C. glutamicum*. Knowing the threshold of the previously identified K^+ stimulus of BetP [9], and taking advantage of the unique tolerance of *C. glutamicum* to low internal K^+ [19], we carried out a differentiated stimulus analysis starting with K^+-depleted cells at low osmolality.

For this purpose we added to these cells either (i) external K^+ in the absence of osmotic stress resulting in an increase of cytoplasmic K^+ (K^+ stimulus) due to K^+ uptake by the CglK transporter [19], (ii) sorbitol leading to hyperosmotic stress (osmotic stimulus) in the absence of external K^+, or (iii) both compounds or stimuli, respectively, together (Table 1A, lines 1–4). Sorbitol was used in these experiments as an osmolyte, since high NaCl concentrations would increase the driving force for BetP [8], and sorbitol is not effectively metabolized by *C. glutamicum* [29]. Although all internal solutes including K^+ passively increase upon hyperosmotic stress, the resulting cytoplasmic K^+ concentration is far below the threshold of activation. Increase of internal K^+ stimulated betaine uptake, similar to that observed in proteoliposomes [9,11]. Osmotic stress in the absence of sufficiently high internal K^+ did not stimulate BetP to a significant extent. Unexpectedly, and different to the results obtained in proteoliposomes, full activity was only achieved if both stimuli are applied together. The value measured for full activity is very similar to that measured in cells under hyperosmotic stress when grown in minimal medium supplemented with K^+, which was determined to be 46.3 ± 5.5 μmol · (mg dw · min)$^{-1}$.

In previous experiments in proteoliposomes full stimulation of BetP was interpreted to be caused solely by the rise in internal K^+ [9,11]. As shown here, effective activation of BetP in intact cells requires the presence of at least two independent stimuli. The pattern described in Table 1A argues for BetP being in a pre-stimulated condition when solely osmotic stress is present in the absence of sufficient K^+. Full stimulation is observed when, in addition to the osmotic pre-stimulus, cytoplasmic K^+ exceeds the threshold for activation.

3.2. Characterization of the 2nd stimulus in intact cells

Osmotic challenge may give rise to a large number of potential stimuli acting on membrane-embedded proteins [30], making it difficult to define the correct physical nature of the relevant stimulus under particular conditions. Since the second stimulus differing from the previously identified K^+ stimulus was observed only in intact cells and not in proteoliposomes, we assumed that this stimulus is related to properties of the cell envelope and/or the cell membrane, which is strikingly different in the two systems used. For this reason, we tested the impact of altered membrane properties on BetP.

Direct approaches to alter the membrane lipid composition of *C. glutamicum* by fatty acid supplementation of mutants in which both fatty acid synthetase complexes (FasA and FasB) were deleted [31], or by the addition of lyso-phospholipids to *C. glutamicum* cells [32,33] were not successful. Finally, we altered the properties of the plasma membrane by direct addition of local anesthetics as membrane active compounds. This experimental approach has already successfully been applied in previous studies of osmoregulated transport systems, namely BetP and OpuA [5,23,34,35]. When using local anesthetics, it has to be taken into consideration that these compounds, besides being membrane active, may also act directly on the target protein and, in addition, may lead to membrane damage [36,37]. The latter effect would compromise the electrochemical potential of the plasma membrane, which, in turn, would affect BetP activity by decreasing its driving force. To rule this out, we have quantified the steady state betaine accumulation by BetP in cells in dependence of added tetracaine (Fig. S1A). Steady state betaine accumulation is in thermodynamic equilibrium with the driving force and is thus a direct and relevant measure of the electrochemical Na^+ potential at the plasma membrane. It turned out that the energetic situation of the cells is not significantly changed up to concentrations of 1.0 mM tetracaine when added to cells at around 1 g cdm/l. Another prerequisite for the application of tetracaine was the optimization of the concentration to be applied (Fig. S1B). In all further experiments

Table 1
Stimulus analysis of BetP in intact *C. glutamicum* cells.

A. K^+ depleted *C. glutamicum* cells

Osmolality (osmol/kg)	External K^+ (mM)	Tetracaine (mM)	Betaine uptake rate (nmol · (mg dw · min)$^{-1}$)
0.24	None	None	2.4 ± 1.2
0.24	2.5	None	10.5 ± 3.3
1.0	None	None	3.3 ± 0.9
1.0	2.5	None	39.0 ± 7.5
0.24	None	0.8	22.4 ± 5.4
0.24	2.5	0.8	33.1 ± 4.9
1.0	None	0.8	10.2 ± 6.0
1.0	2.5	0.8	37.0 ± 6.6

B. Proteoliposomes

Luminal K^+ (mM)	Tetracaine (mM)	Betaine uptake rate (nmol · (mg protein · min)$^{-1}$)
70	None	213 ± 52
≥280	None	871 ± 269
70	0.2	306 ± 135
≥280	0.2	799 ± 162

Betaine uptake rates in K^+ depleted *C. glutamicum* cells were measured in media with different osmolality (sorbitol was used for increasing the medium osmolality) and upon addition of KCl (K^+ stimulus), tetracaine, or both reagents. For determination of betaine uptake rates in proteoliposomes, vesicles prepared in the presence of 25 mM NaP_i/0.1 M NaCl at two different internal K^+ concentrations were used, and tetracaine was added were indicated. The values are means of at least 3 experiments.

with intact cells, we used 0.8 mM tetracaine at the abovementioned cell density.

We added tetracaine to *C. glutamicum* cells under conditions of both low and high internal K^+, and both in the absence and presence of osmotic stress, respectively (Table 1A). Tetracaine did not further stimulate betaine uptake when BetP was already fully activated (Table 1A, lines 4 and 8), whereas strong stimulation was observed when BetP was only partially active (lines 1, 5, and 2, 6). This is clearly different from the effect of K^+, which strongly activated BetP in the presence of an osmotic stimulus (lines 3, 4), whereas it was less effective at low osmolality (lines 1, 2). Consequently, the tetracaine stimulus seems to be more closely related to the osmotic than to the K^+ stimulus.

3.3. State of BetP regulation in proteoliposomes: pattern of activation

We also analyzed tetracaine for activation of BetP in proteoliposomes, thereby extending previous studies [5,8,23] (Table 1B, Fig. 1). Similar to that observed for lyso-phospholipids (see above), tetracaine did not significantly stimulate reconstituted BetP under these conditions, neither in the presence of high internal K^+, nor at K^+ below the threshold of activation. In the latter case, a minor and not significant effect was observed (Table 1, lines 1, 3). In these experiments the internal K^+ concentration at non-stimulatory conditions was higher than in the corresponding experiments using intact cells. The reason is the requirement of an appropriate driving force for betaine uptake in proteoliposomes by generating a K^+-diffusion potential upon addition of valinomycin, which precludes very low internal K^+ concentrations to be used.

Taken together, we identified two different stimuli for BetP in intact cells but only a single one (internal K^+) in proteoliposomes. This raises the question why the second stimulus is not observed in vitro, and, consequently, what the true activation state of BetP in the reconstituted system is. In the presence of high internal K^+ reconstituted BetP is thus either, similar to cells, in a partially activated state, or it has already achieved, in difference to cells, the fully activated state. The latter interpretation means that reconstituted BetP, in the absence of stimulatory K^+ concentrations, has to be already in a pre-activated form, equaling BetP in cells in the presence of low K^+ and osmotic stress. In

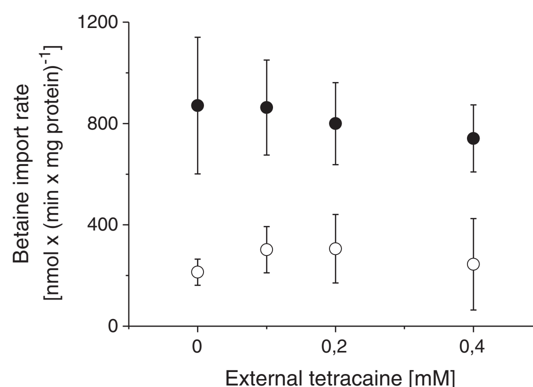

Fig. 1. Betaine uptake by BetP in proteoliposomes: response to tetracaine addition. Proteoliposomes with low (70 mM) and high internal K^+ concentration (≥280 mM), respectively, were used. The values are means from at least three experiments using independent preparations of proteoliposomes.

experiments, osmotic stress may be replaced by tetracaine, since the action of this amphiphile on BetP seems to resemble osmotic stress.

For a decision between these two alternatives, we compared the activation pattern of BetP in intact cells by the three different stimuli applied, K^+, osmotic stress, and tetracaine, with the pattern observed in proteoliposomes (Fig. 2). Obviously, the activation patterns in cells and proteoliposomes only match if we assume BetP in proteoliposomes to be in a pre-activated state, similar to the partially activated state of

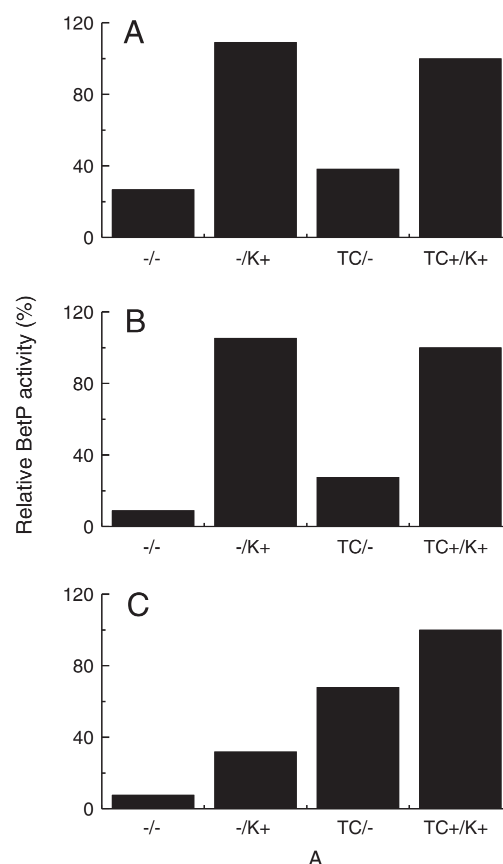

Fig. 2. Pattern of activation of BetP in *C. glutamicum* cells and proteoliposomes by different types of stimuli. Relative betaine uptake rates in proteoliposomes (panel A) and in *C. glutamicum* (panels B and C) at different states of activation due to the presence of tetracaine (0.3 and 0.8 mM in proteoliposomes and cells, respectively), internal K^+, and varying osmolality (panel B: 0.24 osmol/kg, panel C: 1.0 osmol/kg). The values were taken from Table 1 and normalized to the uptake rate at full stimulation (simultaneous presence of all three stimuli).

BetP in cells which is observed at high osmolality in the absence of stimulatory K^+ concentrations. This result indicates the presence of the second type of stimulus in proteoliposomes already under basic conditions, independent from the K^+ stimulus. In Fig. 2, a difference between the two systems under non-stimulating conditions is observed, since the basic activity in proteoliposomes is somewhat higher. This is due to the fact that the K^+ concentration in proteoliposomes under the basic conditions applied was higher than in cells because of experimental requirements (see above).

3.4. State of BetP regulation in proteoliposomes: molecular activity

The validity of this approach analyzing and comparing the activation pattern of BetP in both cells and proteoliposomes is limited by the fact that the mechanistic nature of the tetracaine stimulus is not properly defined [38]. For this reason, we applied a second, completely independent strategy to decide upon the functional state of reconstituted BetP. A basic approach for characterizing a particular enzyme or transporter under different conditions is to determine its molecular activity (turnover number). Determination of this value in both experimental systems used should allow defining whether K^+ stimulation in proteoliposomes results in full or in partial activation of BetP, respectively. The molecular activity of transporters has only rarely been determined in detail because of technical difficulties. For this purpose three values have to be measured both in cells and in proteoliposomes, (a) the specific transport activity of BetP, (b) the concentration of BetP, and (c) the share of functionally active BetP proteins. The first value is easy to determine as shown previously [6,8]. Accurate quantification of the second value is more difficult. For intact cells, we used quantitative Western blotting calibrated with purified BetP as a standard. The content of purified BetP in proteoliposomes can be measured directly. The third parameter is undoubtedly important and has, to our knowledge, not been rigorously taken into consideration in previous measurements of related in vitro systems.

For the analysis of molecular activity in intact cells, we heterologously expressed *betP* in *E. coli*MKH13 which lacks all carriers for compatible solutes. The reason for choosing *E. coli* as a host was based on the fact that we also used *E. coli* phospholipids for reconstitution. It makes perfect sense to keep the lipid surrounding constant when comparing the molecular activity of a transporter in different systems. It was essential to determine the share of active carrier, since *betP* was overexpressed. We quantified both amount and specific activity of BetP synthesized in *E. coli* for different time periods after induction and calculated its molecular activity at different times of expression or synthesis (Table 2). It turned out that up to about 1 h of expression, the molecular activity remains constant at a value of around 3300 ± 350 min^{-1}. A slight overestimation of this value has to be taken into account in view of the fact that we may have lost some protein during extraction. We carried out the same experiment using *C. glutamicum* instead of *E. coli* and obtained a molecular activity of 2560 ± 260 min^{-1} which is rather similar to the value found in *E. coli*. This value was, however, not as reliable as that from *E. coli*, since quantitative protein extraction from *C. glutamicum* membranes proved to be not as reliable and reproducible as in *E. coli*.

The corresponding experiment in proteoliposomes turned out to be much more difficult. Both determination of BetP activity and content is straightforward, since purified BetP was used for reconstitution (Fig. S2). The challenge was to determine the share of active BetP after reconstitution. We have developed a way to quantify the number of active BetP molecules via determination of the number of transport-active proteoliposomes under conditions of limiting amounts of reconstituted BetP protein. At very low protein to lipid mass ratios (PLR) only a small share of liposomes will incorporate BetP and each of these vesicles will contain only one BetP trimer. Only those vesicles harboring an active transporter will accumulate betaine. Titrating increasing amounts of BetP into the liposomes at a constant lipid concentration will result in increasing numbers of active vesicles and thus in an increasing amount of accumulated betaine. Consequently, under these conditions this amount will be proportional to PLR. Once every vesicle carries at least one active BetP trimer no further increase in the total amount of accumulated betaine upon further incorporation of BetP will be observed. This particular PLR is referred to as the saturation PLR (PLR_{sat}). If PLR is increased further, the same steady state value will be reached faster. As a control, we confirmed that the functional properties of reconstituted BetP indicated by the initial betaine uptake rate remained unchanged throughout the range of this titration (Fig. 3A).

For experimental reasons, BetP was not successively titrated into liposomes, but a stock amount of proteoliposomes with a high PLR of 33.3 μg/mg was generated and the PLR value was then step by step decreased upon fusing proteoliposomes with increasing amounts of liposomes lacking BetP protein. Accumulation of [^{14}C]-betaine was then measured in vesicles at different PLR values (Fig. 3B). To determine

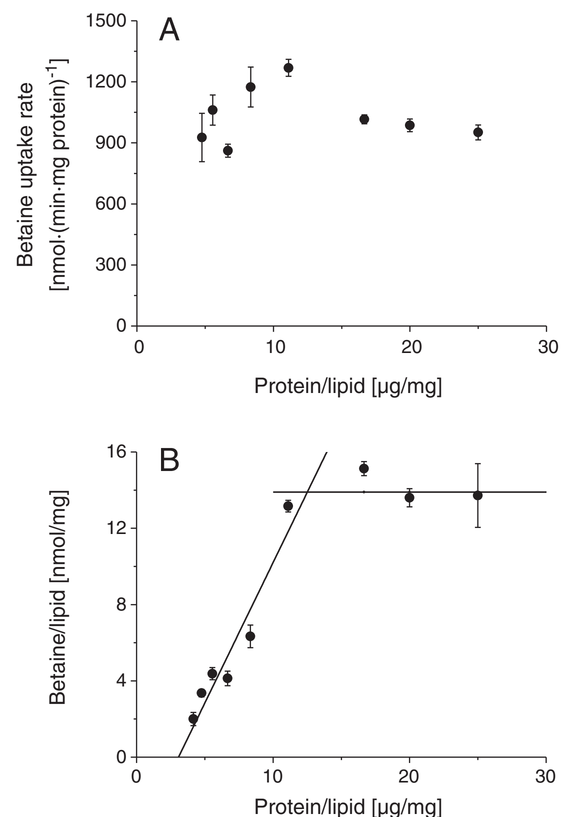

Fig. 3. Betaine accumulation in proteoliposomes: titration of BetP content. A. Dependence of betaine uptake on BetP content of proteoliposomes. The content of BetP protein was determined after reconstitution using the method [51]. The uptake of betaine was normalized to the content of BetP and plotted in dependence of the protein/lipid (w/w) ratio of the proteoliposomes used. B. The amount of betaine accumulated in proteoliposomes after 3 min of incubation with 15 μM of labeled betaine in different proteoliposome preparations reconstituted with increasing amounts of purified BetP is shown. The sample volume was 0.4 ml and the lipid concentration between 0.24 and 0.30 mg/ml. The amount of betaine accumulated in proteoliposomes was normalized to the lipid concentration. All values are means of at least three experiments with standard deviation.

Table 2
Molecular activity of BetP upon different expression times in *E. coli*.

Expression [min]	Betaine uptake [nmol·(min·mg dw)$^{-1}$]	BetP content [mg/g dw]	Molecular activity of BetP [min^{-1}]
15	118.0 ± 1.0	2.19 ± 0.23	3486 ± 325
30	143.6 ± 10.9	2.85 ± 0.32	3264 ± 378
60	118.0 ± 2.1	2.31 ± 0.25	3234 ± 310

All values are means of at least three measurements with standard deviation.

PLR_{sat}, the region with a linear slope was extrapolated as well as the region with a constant value of accumulation resulting in a value of 12.5 ± 0.6 μg/mg for PLR_{sat} equaling a m/m ratio of 1:80 ± 4.

For calculation of the molecular activity the number of BetP trimer complexes per proteoliposome is required. The size of the proteoliposomes used was determined by dynamic light scattering (Fig. S3). The mean radius of the vesicles used in these experiments was 143 ± 15 nm resulting in a surface area of 257,000 nm^2/vesicle. A single phospholipid covers an interface area of 0.6–0.7 nm^2 as determined for PG, PE, or PC [39–41], an average value of 0.65 nm^2 was used. A bilayer liposome of 143 nm radius thus contains about 790,000 lipid molecules. We measured the lipid (dry mass) and phosphate content of the phospholipids used in this study and determined an average phospholipid mass of 816 D, a single liposome thus results in approx. 645 MD. Based on the mass of 192,627 D for a BetP trimer, a proteoliposome solution containing one BetP trimer per vesicle leads to a PLR of 0.30 μg/mg. Consequently, only 1 out of 42 BetP trimers, or 2.4% of total BetP molecules reconstituted in proteoliposomes were found to be active. The transport activity of BetP in proteoliposomes was determined under standard activation conditions (0.6 osmol/kg, PLR 16.7) to1,133 ± 277 nmol · $(min \cdot mg\ protein)^{-1}$, which leads to a turnover number of 73 ± 14 min^{-1}. It is important to note, that the measured value of the molecular activity of BetP depended to some extent on the batch (quality) of *E. coli* phospholipids used for reconstitution. For this experiment, the same batch of *E. coli* lipids as in the reconstitution titration (see above) was used. Given that 2.4% of BetP molecules are active, the corrected molecular activity results to 3055 ± 745 min^{-1}, which is closely similar to the value obtained for fully stimulated BetP in *E. coli* cells, in particular in view of the fact that the value obtained in *E. coli* may be slightly overestimated (see above).

The value obtained for the molecular activity of BetP in proteoliposomes is the result of a relatively complicated set of measurements and assumptions, consequently, the impact of possible errors in this calculation has to be considered. The calculated molecular activity of BetP is linearly dependent on variations in the experimentally determined values of PLV_{sat} and the lipid mass, as well as in the calculated lipid surface. It is dependent by a power of two on variations in the vesicle radius, since BetP distributes into the vesicle surface.

4. Discussion

A detailed mechanistic understanding of stimulus perception and signal transduction has not been achieved for any osmoregulated transport system studied so far [42]. A first and fundamental step in this direction is a biochemical definition of the physical stimuli acting on the protein under study. Because of accessibility and simplicity of the experimental system, an elaborate stimulus analysis for BetP has previously been carried out in proteoliposomes only and led to identification of the internal K^+ concentration as the sole detectable stimulus for reconstituted BetP [9,23]. In the present work, we challenged these results by an investigation under in vivo conditions. For this purpose, the recently achieved characterization of K^+ uptake and homeostasis in *C. glutamicum* was instrumental [19]. As unique properties, *C. glutamicum* was found to be equipped with a single, passive K^+ uptake system only, the CglK channel, and to tolerate very low internal K^+ concentrations. This allowed for dissecting the K^+ stimulus, established in proteoliposomes, from other putative stimuli of BetP. As a surprise, it turned out under in vivo conditions that K^+ is not the only stimulus and is not sufficient to fully activate BetP. As a further component of activation, the increase in external osmolality was detected, independent from the influence of cytoplasmic K^+.

We used tetracaine as an alternative method to stimulate BetP independent of the previously established K^+ stimulus. Since the true mechanism of action of tetracaine is not known [38], its relevance for mimicking membrane stress is not proven. There are, however, arguments in favor of this conception. Tetracaine is a membrane-active compound and alters the physical state of the membrane [43]. Its pattern of BetP activation overlaps much closer with the osmotic than the K^+ stimulus. By re-arranging data from Table 1A it becomes obvious that the three types of stimulating factors applied here, i.e. K^+, osmotic stress, and tetracaine, respectively, are not independent from each other (Table 3). When cells in the presence of two of the three factors tested were additionally exposed to the third factor, strong activation was only observed when K^+ was the third stimulus, indicating that the osmotic and tetracaine stimuli are overlapping in their relevance for activating BetP and possibly also in their physicochemical nature with respect to changing BetP's membrane surrounding.

Since we detected at least two different kinds of stimuli in cells but only a single one in proteoliposomes, the true state of activity of BetP in the reconstituted system was questionable. The most simple answer, assigning the K^+-activated state in proteoliposomes to the partially activated state in cells upon the presence of the K^+ stimulus only, turned out not to be true. Here, we provide a number of experimental arguments in favor of the fact that reconstituted BetP upon K^+ stimulation alone is already in the fully active state. (a) The molecular activity of BetP in proteoliposomes in the presence of a sufficiently high internal K^+ concentration is closely similar to that of BetP in cells upon full activation. (b) The pattern of activation of BetP in proteoliposomes upon stimulation by either K^+, tetracaine, or both effectors, respectively, matches with the activation pattern of intact cells upon full stimulation, i.e. in the presence osmotic stress. (c) In obvious difference to the situation in intact cells, tetracaine is not able to activate BetP in proteoliposomes beyond the level of activation by K^+. (d) In accordance with argument c, the K^+ stimulus is not efficient in the absence of the second, osmotic stimulus in intact cells. (e) Lyso-phospholipids as membrane-active compounds are able to activate BetP in intact cells but not in proteoliposomes, in which BetP is assumed to be in a pre-activated state. (f) Activation of BetP by chill, which has been interpreted to be related to the physical state of the surrounding membrane, has not been observed in proteoliposomes [44].

There are some caveats concerning these arguments. We will discuss in particular the two most important arguments (a) and (b). The first comment concerning argument (a) refers to the surprisingly low share of active BetP in the reconstituted system. Notably, the first detailed analysis of this kind applied to a reconstituted transporter, namely the ATP/ADP carrier in mitochondria, has led to a low share of intact protein of about 8% after reconstitution in proteoliposomes, too [45]. We would like to point out, that, to our knowledge, the present analysis has not been performed before to such detail for any other transport protein for which no tightly bound ligand is available, which favored the analysis in the case of the mitochondrial nucleotide transporter. Consequently, we assume that isolation and purification, and in particular reconstitution including repeated extrusion steps may lead to inactivation and aggregation of a large share of the carrier protein.

Another objection concerning argument (a) refers to the driving force of betaine uptake, which, if significantly different in the two

Table 3
Pattern of activation of BetP in intact *C. glutamicum* cells.

Betaine uptake rate upon pre-activation with stimuli		Maximal uptake rate upon additional stimulation with	
Osmo + tetrac	10.2 ± 6.2	K^+	37.0 ± 6.6
Tetrac + K^+	33.1 ± 4.9	Osmo	37.0 ± 6.6
Osmo + K^+	39.0 ± 7.5	Tetrac	37.0 ± 6.6

The three stimuli used are: shift to hyperosmotic conditions ('osmo', shift from 0.24 osmol/kg to 1.0 osmol/kg), tetracaine addition ('tetrac', addition of 0.8 mM tetracaine), and K^+ addition (K^+, addition of 2.5 mM external K^+, leading to a strong increase in the cytoplasmic K^+ concentration). The values are generated based on the results listed in Table 1. Rates are given in nmol·$(min \cdot mg\ dw)^{-1}$.

systems used, would lead to differences in the calculated molecular activity. In view of this complication, however, we deliberately set the conditions for initial betaine uptake in proteoliposomes to roughly similar driving forces with respect to the electrochemical Na^+ potential across the plasma membrane of cells and the liposomal membrane, respectively. Also with respect to arguments concerning a putatively different membrane surrounding, we have chosen two systems in which the membrane lipid composition is identical (*E. coli* cells and liposomes from *E. coli* lipids).

Possible comments concerning the validity of argument (b) deal with the action of tetracaine on BetP activity. We have experimentally ruled out possible artifacts due to unspecific membrane damage caused by tetracaine. It is true that the precise way of action is not known for this reagent, which, however, applies for all other known stimuli, e.g. K^+, as well. Consequently, for the aim of the present work it is solely relevant that BetP is in fact activated, since only patterns of activation and not mechanistic models were used for the decision. We would like to mention previous results, however, which indicate that tetracaine, in addition to the mode of action discussed here, may exert other types of influences. It has been shown that tetracaine is able to stimulate a C-terminal truncated version of BetP, which is insensitive to osmotic stress [46].

The finding of BetP being already partially activated in proteoliposomes under basic conditions in the absence of any detectable stress is not in conflict with our previous work [9,11]. The present analysis in intact cells fully confirms K^+ as an important factor for activation of BetP. On the other hand, it demonstrates that the situation in cells is, concerning activation by hyperosmotic stress, more complicated than in proteoliposomes. These considerations lead to the question about the physical nature of the second stimulus, which seems to be constantly effective in proteoliposomes. Although we cannot properly answer this question at the moment, there are at least some indications for the nature of this stimulus. Based on the observation that it is not detectable in lipid vesicles but effective in cells, it should be related to physical differences between the two systems. At least four major aspects have to be considered: (a) the composition of the internal space, cytoplasm or lumen, (b) the membrane lipid composition, (c) the protein content and composition of the membrane, (d) the presence or absence of a cell wall, and finally (e) a slightly different conformation of BetP upon integration into proteoliposomes as compared to insertion into the plasma membrane of the cell.

Aspect (b) is not relevant, since we have chosen two systems with identical lipid composition. The fact that the so far unknown second stimulus was found to be effective in proteoliposomes in the absence of any unknown compounds in the luminal space strongly argues for aspect (a) not being relevant, too. Although the protein composition in the membranes of the two systems, in vivo and in vitro, is different (aspect c), the result that BetP is fully functional when inserted in a membrane from a heterologous system, e.g. *E. coli*, argues against at least a specific protein-protein interaction as a relevant contribution. The fact that the membrane protein density is dramatically different in native membranes and in proteoliposomes, respectively (also aspect c), has to be taken into account. It is easy to imagine that a significantly changed protein density of the phospholipid membrane may alter its physical properties. This factor, however, is difficult to study experimentally, since proteoliposomes become permeable at high concentrations of inserted proteins. Furthermore, the closely related aspect (e) has to be considered here. Due to the significantly different surrounding it is conceivable that the membrane-inserted BetP may adopt a slightly different conformation. Experimental evidence has been provided that the C-terminal domain of BetP, which is critically involved in activity regulation, does in fact interact with the membrane, which was relevant for the suggested switch model for BetP activation [12,25]. If the regulatory C-terminal domain would adopt an altered conformation in proteoliposomes, a changed impact of the putative membrane-related stimulus is conceivable. Finally, there is an obvious difference concerning the impact of membrane morphology (aspect d), since the effect of osmotic stress on membrane shape, i.e. the physical strain related to shape changes of a vesicular structure, is not relevant in liposomes. Lipid vesicles immediately adapt their shape to hyperosmotic conditions by shrinking to erythrocyte-like structures [35,47]. As a consequence, turgor does not exist in liposomes, whereas it may be a crucial factor for membrane proteins in intact cells responding to changes in osmotic stress and, concomitantly, to changes in physical properties of the membrane [1]. Consequently, also this aspect seems to be truly relevant.

There are a number of further experimental observations pointing to a direct significance of the membrane or the cell wall, respectively, mediating the so far unknown stimulus. First, when studying BetP activation by chill stress, we realized that the extent of stimulation was directly correlated to the fatty acid composition of the membrane lipids [44]. Second, a direct action of tetracaine on the BetP protein independent from the membrane is very unlikely, since we had already previously demonstrated that activation occurs by various compounds of different chemical nature and charges [23]. We thus conclude that the second stimulus is probably related to alterations in the properties of the plasma membrane, e.g. its intrinsic pressure, and/or the cell wall of cells.

Taken together, two different stimuli seem to be integrated in the response of BetP to osmotic stress, the first acting indirectly via the osmoresponsive increase of the cytoplasmic K^+ concentration and the second acting directly via the membrane. Whereas the first stimulus had been elucidated in detail in the reconstituted system previously, the second, membrane-related stimulus is a novel aspect of understanding osmosensing by and osmoregulation of BetP and possibly similar transporters. It has to be shown in further experiments, whether this new concept of two independent stimuli is relevant for other osmoregulated systems, too.

It is interesting to note that the presence of two different stimuli being effective for BetP activation seems to be highly relevant in terms of the physiologic response to hyperosmotic stress in *C. glutamicum*. BetP is characterized by unique regulatory properties not only concerning the onset of activation [5,6], but also with respect to downregulation of its activity in response to osmotic adaptation [28]. When osmotic compensation by BetP-mediated accumulation of high betaine concentrations in the cytoplasm is achieved, BetP was found to be downregulated to a basic level of activity even in the presence of high internal K^+ concentrations clearly above the threshold of activation. This observation, which could not be explained, is very likely to reflect the situation described here. Both stimuli are effective during the onset of BetP activation. The second stimulus, related to membrane stress only, will probably cease upon osmotic adaptation. As demonstrated here, the remaining K^+-dependent stimulus when exclusively present is only weakly effective. The remaining basic BetP activity during osmotic adaptation will then be counterbalanced by the compensatory action of mechanosensitive channels, as we have experimentally demonstrated [48].

This work furthermore contributes another facet to the old question whether reconstitution in proteoliposomes is an appropriate tool for analyzing transport systems, which was under discussion since the time of the first successful functional reconstitutions of purified carrier proteins [49,50]. On the one hand, the elaborate stimulus analysis discriminating the predominant factor, the K^+ stimulus, from a large number of principally possible parameters was exclusively possible by simplifying the experimentally highly complex situation in a reconstituted system [9]. The present work strongly indicates, on the other hand, that returning to the in vivo situation is essential to validate results obtained in proteoliposomes. By this investigation, the previous identification in the reconstituted system of K^+ being a relevant stimulus was fully confirmed. It was not confirmed, however, that K^+ is the only stimulus relevant for BetP. The true mechanistic nature of both the K^+ stimulus as well as the second, membrane-related stimulus identified under in vivo conditions in intact cells will be a challenge for future investigations.

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Appendix A. Supplementary data

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