Articles

The C-Terminal Domain of the Betaine Carrier BetP of *Corynebacterium glutamicum* Is Directly Involved in Sensing K^+ as an Osmotic Stimulus†

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ABSTRACT: The glycine betaine carrier BetP of *Corynebacterium glutamicum* was recently shown to function both as an osmosensor and as an osmoregulator in proteoliposomes by sensing changes in the internal K^+ concentration as a measure of hyperosmotic stress. In vivo analysis of mutants carrying deletions at the C-terminal extension of BetP indicated that this domain participates in osmostress-dependent activity regulation. To address the question, whether a putative K^+ sensor is located within the C-terminal domain, several mutants with truncations in this domain were purified and reconstituted in proteoliposomes of *Escherichia coli* phospholipids, since this in vitro system allowed variation of the K^+ concentration at the lumenal side. Truncation of 12 amino acids led to a partly deregulated BetP in terms of osmoregulation; however, K^+ sensitivity was not impaired in this mutant. The deletion of 25 amino acid residues at the C-terminal end of BetP led to both deregulation of the carrier activity, i.e., high activity independent of external osmolality, and loss of K^+-dependent transport stimulation, indicating that this region of the C-terminal domain is necessary for K^+ sensing and/or K^+-dependent carrier activation. Immunological and proteolysis analyses showed that BetP and its recombinant forms were reconstituted in a right-side-out orientation, i.e., the C-terminal domain faces the lumen of the proteoliposomes and is thus able to detect the K^+ signal at the inside. This is the first experimental demonstration of a direct connection between an osmotic stimulus, i.e., the change in internal K^+, and a putative sensor domain.

Osmotic stress is one of the most common stress situations that cells encounter in their native surroundings, affecting the cell's hydration, volume, and/or turgor pressure. A widely distributed strategy of eubacteria to overcome hyperosmotic stress is the accumulation of compatible solutes, such as glycine betaine, proline, or trehalose, by biosynthesis and/or uptake, thereby avoiding dehydration of the cytoplasm (*1*−*3*). Since uptake of compatible solutes is energetically cheaper and faster than de novo synthesis, activation of uptake systems for compatible solutes is in general the first response to hyperosmotic conditions (*4*).

Corynebacterium glutamicum, a GC-rich Gram-positive soil bacterium important for industrial amino acid production (*5*), possesses five secondary carriers for the uptake of compatible solutes (*4*, *6*−*7*, unpublished results). The activity of at least three of them, EctP, BetP, and ProP, is regulated by the external osmolality. The Na^+-dependent glycine betaine uptake system BetP, the best-studied carrier involved in osmoadaptation of *C. glutamicum* (*6*, *8*−*9*), comprises 12 transmembrane segments as well as cytoplasmically exposed terminal domains, which are important for osmostress-dependent activity regulation (*9*). The functional reconstitution of purified BetP demonstrated that this transporter harbors altogether three functions: (i) catalytic activity of glycine betaine transport, (ii) sensing of osmotic stress, and (iii) osmoregulation, i.e., adjustment of the transport rate to the extent of hypertonicity (*10*). Other compatible solute transporters that are known to be both osmosensors and osmoregulators are the secondary transporter ProP from *Escherichia coli* (*11*) and the primary (ABC) transporter OpuA from *Lactococcus lactis* (*12*).

The molecular mechanisms of activation, in particular the kind of stimulus relevant for osmosensing and the putative domains within these carriers relevant for detecting the trigger, are still under debate. Proteoliposomes, carrying purified osmosensitive carrier proteins, are well-suited for analyzing the nature of osmotic stimuli (*13*−*15*), since they offer the possibility to dissect putative triggers by varying only one of the possible parameters while keeping constant all other conditions. By using this in vitro system, we were recently able to demonstrate that the internal K^+, Rb^+, or Cs^+ concentration functions as a measure for hyperosmotic stress in the case of BetP of *C. glutamicum*, whereas changes of membrane strain, external osmolality, or other cations such as ammonium, choline, or Na^+ had no influence (*13*,

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unpublished results). The signal input site of BetP for K^+, however, remained to be elucidated. A first indication that the C-terminal extension may be involved in regulating BetP activity came from the observation that truncation of this domain led to a loss of the osmostress-dependent activation of BetP in intact *C. glutamicum* cells (*9*). This result indicates that the domain is in fact involved in K^+ sensing but does not rule out the possibility that the sensor is located elsewhere in BetP. These considerations are interesting in particular because of the fact that the C-terminal domain of ProP of *E. coli* has been implicated in osmosensing and/or osmoregulation of this secondary carrier, too (*16*). Although it has been demonstrated that the C-terminal domain is important for the regulatory properties of ProP (*16*), a direct correlation of the putative stimuli for ProP, namely, ions and macromolecular solutes in the lumenal space, has not been achieved.

This work addresses the question whether the C-terminal domain of BetP is involved in K^+ sensing, which was shown to be the relevant signal for the stimulation of this carrier protein under hyperosmotic conditions (*13*). The study of C-terminally truncated BetP mutants, carrying deletions of 12, 25, or 45 amino acid residues, was carried out in proteoliposomes, since the internal K^+ concentration is not accessible to experimental variation in intact cells. For this purpose, it was essential to prove the membrane topology of BetP as well as of its recombinant forms with respect to the location of the terminal domains when reconstituted in proteoliposomes. In this well-defined reconstituted system we were able to obtain evidence for direct involvement of the C-terminal domain of BetP in K^+ sensing or K^+-dependent activation of BetP.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. E. coli MKH13 (*17*) was used for glycine betaine uptake measurements; *E. coli* DH5αmcr (*18*) was used for all other applications. The plasmids used were pASK-IBA5 (*19*), pASK-IBA5 *betP* (*10*), pAcl1 (*13*), and pAcl CΔ12, pAcl CΔ25, and pAcl CΔ45 (this work) in which *strepbetP* is under the control of the *tet* promoter. *E. coli* cells were grown at 37 °C in LB[1] medium supplemented with carbenicillin (50 μg/mL).

Construction of Plasmids. Construction of pAcl1, encoding the cysteine-free BetP protein C252T, is described elsewhere (*13*). pAcl1 was used as PCR template for the construction of *betP* C252T Δ12, *betP* C252T Δ25, or *betP* C252T Δ45, leading to BetP variants with different truncations of the C-terminal domain of BetP, by use of the following mutagenic antisense primers: Δ12, 5′-[AAGCTTCTAGCGTC-GCTTTGCAGCCAGTTC]-3′; Δ25, 5′-[AAGCTTCTAGT-GAACACGACG TTCACGGGC]-3′; Δ45, 5′-[CGAAGC-TTCTAGAGATCACATCATTGC]-3′; and the sense primer, 5′-[TCTCTTGGTCACCATTCG]-3′. The resulting PCR products of approximately 0.6 kb were ligated into the vector pDrive (Qiagen, Hilden) and sequenced for control. Subsequently, the *Hin*dIII and *Bst*EII fragment was isolated and changed against the identically treated fragment of pAcl, leading to pASK-IBA5 *betP* C252T Δ12, pASK-IBA5 *betP* C252T Δ25, or pASK-IBA5 *betP* C252T Δ45.

Purification and Reconstitution of Strep-BetP. Strep-BetP was purified essentially as described (*10*). For reconstitution liposomes (5 mg of phospholipids/mL) prepared from lipids of *E. coli* polar lipid extract (Avanti Polar Lipids, Alabaster, AL) were preformed by extrusion (13 cycles) through polycarbonate filters with a pore size of 400 nm. The liposomes were titrated by stepwise addition of 20% (v/v) Triton X-100. The insertion of detergent in the liposomes was followed by measurement of the turbidity at 540 nm. Upon saturation with detergent, the liposomes were mixed with protein at a lipid-to-protein ratio of 30:1 (w/w). Following incubation of 30 min at room temperature, BioBeads at ratios (w/w) of 5 (BioBead/Triton X-100) and 10 (BioBead/dodecylmaltoside) were added to remove detergent and incubated at room temperature for 1 h under gentle agitation. Fresh BioBeads were added and incubation was continued for 1 h. Subsequently, a double amount of BioBeads was added and incubation was continued at 4 °C overnight. Finally, the above-mentioned basic amount of BioBeads was added and incubated for 1 h. Finally the proteoliposomes were centrifuged, washed twice with 100 mM KP_i, pH 7.5, frozen in liquid nitrogen and stored at −80 °C.

Transport Assays. Uptake of [^{14}C]glycine betaine in proteoliposomes was determined essentially as described (*10*). Briefly, proteoliposomes were extruded 13 times through a polycarbonate filter (400 nm pore size) in 100 mM KP_i (pH 7.5), collected by centrifugation, and resuspended in the extrusion buffer to a lipid concentration of about 60 mg of lipid/mL. An appropriate amount of proteoliposomes was diluted 200-fold into potassium-free buffer (20 mM NaP_i, pH 7.5, and 25 mM NaCl) containing 15 μM [^{14}C]glycine betaine and 1 μM valinomycin to create an outwardly directed K^+ diffusion potential. To establish hyperosmotic conditions, sorbitol was added to the external buffer. An osmotic upshift represents the difference between the measured osmolality in the respective transport assay buffer and the measured osmolality of the buffer corresponding to the internal space of the proteoliposomes. Since liposomes behave like osmometers (*14*), the volume shrinkage and concomitant change of the internal solute concentration depends linearly on the extent of the hyperosmotic shift. After several time intervals, samples were taken and filtered rapidly through 0.45 μm nitrocellulose filters (GS; Millipore, Eschborn, Germany). The filters were washed with 100 mM LiCl and the radioactivity was determined by liquid scintillation counting. In experiments in which internal solutes were varied, proteoliposomes were resuspended in the desired buffer and the internal buffer was exchanged by extrusion as described previously (*13*).

Uptake of [^{14}C]glycine betaine in cells was determined as follows: *E. coli* MKH13 expressing the genes of Strep-BetP derivatives were cultivated in LB (carbenicillin 50 μg/mL) up to an OD_{600} of 1.5. Synthesis of the single Strep-BetP mutants was initiated by addition of 200 μg/L anhydrotetracycline. After 2 h the cells were harvested, washed in 100

[1] Abbreviations: CSPD, (3-(4-methoxyspiro{1,2-dioxetan-3,2-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate, disodium salt; EDTA, ethylenediaminetetraacetic acid; LB, Luria−Bertani; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl)aminomethane.

mM Tris/Mes (pH 7.5), and resuspended in the same buffer containing 20 mM glucose. For the uptake measurements, cells were assayed in 100 mM Tris/Mes (pH 7.5) containing 10 mM glucose, 50 mM NaCl, and different sorbitol concentrations varying between 50 and 850 mM. Subsequently, cells were stirred for 3 min at 37 °C before the reaction was started by the addition of 250 μM [^{14}C]glycine betaine. At given time intervals, samples were taken, filtered through glass fiber filters (GF, Schleicher & Schuell GmbH, Dassel, Germany), and washed twice with 2.5 mL of 500 mM sucrose and 50 mM $MgCl_2$. The radioactivity on the filters was determined by liquid scintillation counting.

Production of Antibodies against BetP. For the detection of BetP in Western blot analyses, antibodies raised either against BetP or against the C-terminal extension of BetP were generated. In the former case BetP was purified as described above and 1 mg of pure BetP was used for the production of antibodies in rabbit. In the latter case a peptide covering the last 55 amino acid residues of BetP was purified. For that purpose at first the desired DNA fragment encoding the sequence of the last 55 amino acids of BetP was amplified by means of PCR with 5′-[CAT ATG GTC AAG GAC TTA AGC]-3′and 5′-[CCC GGG TCG ACG CTT CCC CGC]-3′ as sense and antisense primers, respectively. The purified PCR fragment was cloned into the *Sma*I site of pUC18 by use of the Sure Clone ligation kit (Pharmacia, Freiburg, Germany), the accuracy of the inserted DNA sequence was confirmed by sequencing, and subsequently, the fragment was restricted with *Nco*I and *Sma*I and ligated into the similarly treated vector pTYB2 (NEB, Bad Schwalbach, Germany), creating a 3′ gene fusion with the intein/chitin binding domain. The resulting plasmid pTYB2-C-EX was transformed with *E. coli* strain ER2566 (NEB, Bad Schwalbach, Germany) as host. Expression of the fusion protein was performed as follows: The strain ER2566/pTYB2-C-EX was incubated overnight in 100 mL of LB (50 μg/mL carbenicillin) on a rotary shaker. This culture was used as inoculum for a 500 mL LB culture. Starting from an optical density of $OD_{600} = 0.1$, cells were grown at 25 °C until an optical density of $OD_{600} = 0.5$ was reached, and then expression of the fusion gene was induced by the addition of 50 μM IPTG. The cells were incubated at room temperature overnight before they were harvested and washed with 0.9% saline. A crude extract was prepared by two passages of the cells in a French press in 20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, and 0.1 mM EDTA in the presence of the protease inhibitor Complete (Roche, Mannheim, Germany) and 1 μg/mL DNase. The crude extract was centrifuged to get rid of cell debris. The resulting supernatant was used for affinity chromatography with the Impact T7 one-step protein purification system (NEB, Bad Schwalbach, Germany) according to the manufacturer's protocol. In the first step the fusion protein was specifically bound to the chitin beads before it was induced to undergo an intein-mediated self-cleavage, liberating the C-terminal domain of BetP. An SDS−15% polyacrylamide gel showed that the elution fractions contained one protein band with an apparent molecular mass of approximately 6.5 kDa, which corresponds well with the calculated molecular mass of 6.8 kDa. The fractions containing the C-extensions of several purifications were pooled and concentrated and desalted by means of solid-phase extraction on a Sep Pak Plus C18 cartridge from Waters. After activation of the cartridge with 5 mL of methanol and washing with 10 mL of water, the C-extension of BetP was bound. The elution profile was as follows: 1.5 mL of 70:30 methanol/water, 1.5 mL of 80:20 methanol/water, and 2 mL of 80:5:5 methanol/water/1 M CH_3COOH. The C-extension was completely found in the last elution fraction. After lyophilization, the protein was dissolved in water to a concentration of 1 mg/mL. For the production of antibodies, in guinea pigs, 1 mg of protein was used.

Depending on whether antibodies raised against BetP or antibodies raised against the C-terminal extension of BetP were used in the immunoblot analysis, either alkaline phosphatase-coupled anti-rabbit IgG (Sigma, Deisenhofen, Germany) or alkaline phosphatase-coupled anti-guinea pig IgG (Sigma, Deisenhofen, Germany) was used as second antibody.

Orientation of Strep-BetP in Proteoliposomes. Orientation of StrepBetP in the bilayer membrane of the proteoliposomes was determined by defining the accessibility of the C-terminal extension from the internal or external side of the liposomes with polyclonal antibodies raised against the intact protein or the C-terminal domain, respectively. The detection of epitopes accessible from the outside of the proteoliposomes was carried out as follows: Preformed proteoliposomes were diluted to 50 ng of BetP/μL and incubated for 90 min with one the of two different antibodies or the corresponding preimmune sera (1:1000), respectively. The proteoliposomes were washed four times with 10 mM TBS (pH 7.5) and subsequently, the BetP/antibody complex was separated by SDS−PAGE (7.5%), with β-mercaptoethanol omitted from the sample preparation, and blotted onto a PVDF Western blotting membrane (Roth, Germany). Antibodies bound to BetP before in the binding assay were detected with a secondary antibody conjugated with alkaline phosphatase by use of CSPD (Roche; Mannheim, Germany). For immunodetection of intraliposomal protein domains, proteoliposomes were prepared as mentioned above and antibodies raised against the intact protein or the C-terminal domain were added (1:100). The antibodies were allowed to enter the lumen of the proteoliposomes by two freeze−thaw cycles. Subsequently, the proteoliposomes were extruded again, washed once, and incubated for 90 min at 30 °C to allow binding of antibodies. The complete procedure was repeated twice and the amount of antibodies bound to BetP was detected as mentioned above. As control for unspecific binding of the antibodies to the lipid bilayer, the whole procedure was carried out with protein-free liposomes, too.

Site-Specific Proteolysis. The accessibility of the N-terminal domain of BetP was measured both in the solubilized and in the reconstituted state in proteoliposomes. Both preparations were diluted in 10 mM TBS, pH 8.0, to 0.25 mg of BetP/mL. Aminopeptidase from *Aeromonas proteolytica* and aminopeptidase I from *Streptomyces griseus* (Sigma Aldrich, Deisenhofen, Germany) were added at a BetP/protease ratio of 100:1 (w/w). Proteolysis was carried out at 30 °C. At given time intervals the reaction was stopped by adding the protease inhibitor Complete (Roche, Mannheim, Germany). The proteins were subjected to SDS−PAGE (12%) and either were stained with Coomassie Blue or a Western blot analysis was carried out as described previously (*10*). Cleavage of N-terminal domain was fol-

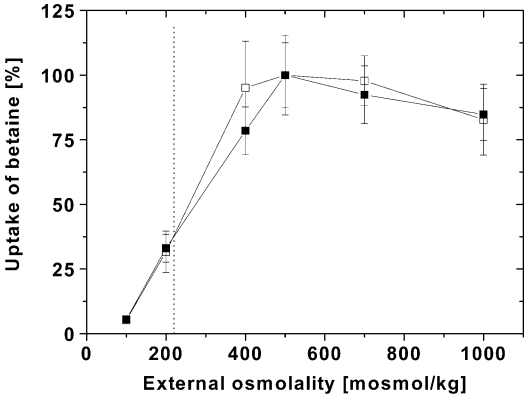

FIGURE 1: Activity regulation of wild-type BetP and BetP C252T in liposomes and dependence on external osmolality. Osmolality of the internal buffer (100 mM KP_i, pH 7.5) was 220 mosmol/kg; that of the basic external buffer (20 mM NaP_i, pH 7.5, and 25 mM NaCl) was 100 mosmol/kg. The external osmolality was increased by adding sorbitol up to 1000 mosmol/kg, thus creating an osmotic gradient, which consequently led to liposome shrinkage due to water efflux. Uptake of glycine betaine by BetP (□) and BetP C252T (■) is shown. The dashed line represents iso-osmolality between internal and external buffer. The transport rate at an osmolality of 500 mosmol/kg was set to 100%. The absolute values at 100% activity were 874 nmol/(min·mg of dry wt) for wild-type BetP and 1198 nmol/(min·mg of dry wt) for BetP C252T.

lowed by use of the streptavidin−alkaline phosphatase conjugate (IBA, Göttingen, Germany) or the antibodies raised against BetP.

RESULTS

Previous studies in intact cells indicated that the N- and in particular the C-terminal domain of BetP might be directly involved in osmosensing and/or osmoregulation (*9*). To define the role of the C-terminal domain in detail, a series of C-terminally truncated BetP mutants was constructed. All mutants, carrying the StrepTagII at their N-terminal ends, were purified and reconstituted into liposomes. These recombinant forms of BetP were based on BetP C252T, a Cys-less version of BetP, to be able to introduce cysteines at strategic places in future experiments. For this reason, it was necessary to compare the functional properties of the Cys-less version with that of wild-type BetP.

Wild-type StrepBetP and StrepBetP C252T were reconstituted into *E. coli* lipids and their transport activity was determined in response to increasing osmotic stress (Figure 1). If an external osmolality below 220 mosmol/kg was used, proteoliposomes were exposed to a mild osmotic downshock, creating an osmotic gradient of Δ120 mosmol/kg or less, which caused a dilution of the internal solutes due to the influx of water. This should not result in leakage of solutes, since White et al. (*20*) showed recently that the integrity of liposomes from *E. coli* polar lipid extract was not impaired by osmotic downshocks of relatively low extent (cf. also Figure 3, where the same downshock conditions were applied). The dependence of activity regulation of BetP C252T on the external osmolality was found to be identical to that of wild-type BetP. Although the specific uptake rate of StrepBetP C252T was slightly higher than that of wild-type StrepBetP, the maximum of activity was observed at an external osmolality of around 500 mosmol/kg in both cases, which is similar to the result obtained in *E. coli* cells

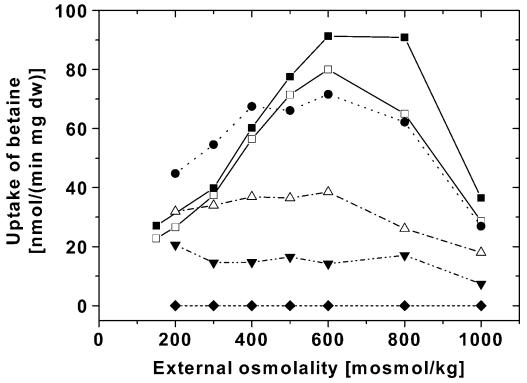

FIGURE 2: Uptake of glycine betaine by plasmid-encoded C-terminally truncated BetP mutants in *E. coli* MKH13 and dependence on external osmolality. The external buffer basically contained 100 mM Tris/Mes, pH 7.5, and 50 mM NaCl. Higher osmolalities were adjusted by addition of sorbitol. Uptake of glycine betaine was started by addition of 250 μM [^{14}C]glycine betaine. The uptake activity of the following BetP derivatives is shown: BetP wild type (□), BetP C252T (■), BetP Δ12 (●), BetP Δ25 (△), BetP Δ45 (▼), and the empty vector pASK-IBA5 (◆).

(cf. Figure 2). Thus, the Cys-less variant of BetP was suited for the intended study.

Osmoregulation of Plasmid-Encoded C-Terminally Truncated BetP Variants in E. coli MKH13. We first characterized the C-terminally truncated BetP variants in *E. coli* MKH13, a strain that is deficient in transport of compatible solutes (*17*); consequently, glycine betaine uptake is solely provided by plasmid-encoded BetP in this strain. In these experiments, we tested whether the BetP mutants used were still active with an N-terminally fused StrepTagII, which is essential for protein purification. In addition, by varying the osmolality in the uptake assay, we were able to analyze whether the regulation pattern of some of these mutants (BetP Δ12 and BetP Δ25), which had been characterized in intact cells of *C. glutamicum* before (*9*), is in fact similar in the heterologous host *E. coli*.

In Figure 2, glycine betaine uptake in *E. coli* MKH13 containing StrepBetP, StrepBetP C252T, or C-terminally truncated forms of it is shown at varying osmolalities. Besides MKH13 carrying the empty plasmid, all strains were active in uptake, thus, the StrepTagII is not inhibitory. Glycine betaine uptake activity of the wild type, BetP C252T, and BetP Δ12 increased in dependence on the imposed osmotic stress, reaching closely similar maximal uptake rates around 600 mosmol/kg; thereby the activity of BetP Δ12 was already elevated at low external osmolalities. In contrast, the mutant proteins BetP Δ25 and BetP Δ45 showed a maximum transport activity at very low osmolalities; thereby the maximum transport rates reached values of approximately 35% and 20% of the fully activated state of the wild-type protein, respectively. To exclude the possibility that these mutants only appear to be independent of the external osmolality because of a lower expression level in the heterologous host, we investigated the relative BetP amounts in the cytoplasmic membrane by means of Western blot analysis (Figure 5A). BetP C252T and the truncated BetP variants were found to be present in comparable amounts. We thus conclude that BetP Δ25 and BetP Δ45 were indeed deregulated with respect to osmotic stress.

When compared to results previously obtained in *C. glutamicum* for wild-type BetP, the activation optimum

Table 1: Kinetic Constants of BetP, BetP C252T, and the C-Terminally Truncated Cysteine-Free Variants BetP Δ12, BetP Δ25, and BetP Δ45 in Proteoliposomes

	glycine betaine		NaCl
protein	K_m (μM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	K_m (mM)
BetP wild type	3.0 ± 0.3	1693 ± 44	38.1 ± 2
BetP C252T	3.8 ± 0.6	3222 ± 136	44 ± 2
BetP Δ12	5.1 ± 0.9	3360 ± 206	44.2 ± 3
BetP Δ25	4.0 ± 0.7	3288 ± 192	147 ± 16
BetP Δ45	1.9 ± 0.5	470 ± 27	130 ± 19

seems to be shifted to a lower osmolality in *E. coli*. This finding may be attributed to a different membrane lipid composition of the two species (*10*) and/or to a different internal K^+ concentration (*13*). Nevertheless, the extent of deregulation observed in BetP Δ25 was found to be identical in *E. coli* and in *C. glutamicum* cells.

Kinetic Properties of BetP and Mutant Forms in Liposomes. Table 1 summarizes the kinetic parameters of BetP C252T and of the truncated mutants measured in proteoliposomes. Liposomes were preloaded with 100 mM KP_i (pH 7.5) and were diluted into a buffer with a fixed external osmolality of 600 mosmol/kg containing 20 mM Tris/Mes (pH 7.5) and varying betaine (1−30 μM) or Na^+ contents (2.5−150 mM) and sorbitol concentrations. The K_m for glycine betaine was found to be in the range of 2−5 μM for all proteins tested, whereas the K_m for Na^+ differed among the various BetP mutants. The affinity for the cosubstrate Na^+ was determined by measuring glycine betaine uptake dependence on the external Na^+ concentration (*8*). To account for the fact that the apparent Na^+ affinity may be influenced by a varying share of charged phospholipids in the *E. coli* lipid extracts, the K_m values for Na^+ had been determined upon reconstitution into several liposome preparations from different *E. coli* lipid extracts. We found a similar K_m for Na^+ for the wild type (38 mM), the Cys-less mutant BetP C252T (44 mM), and BetP Δ12 (44 mM). Deletion of the last 25 or 45 amino acid residues, however, led to a decrease of the Na^+ affinity to 147 and 130 mM, respectively. BetP C252T and its derivatives had a 2-fold higher V_{max} as compared to the wild type, except the mutant lacking the last 45 amino acids, which showed a reduced V_{max}.

In experiments where the external osmolality was varied, an additional source of potential misinterpretation had to be considered, namely, the possibility that the K_m for betaine or Na^+ may depend on the osmolality applied in different experiments. A change of the substrate affinity dependent on osmotic shifts has in fact been shown for the compatible solute carrier ProP of *E. coli*, where the K_m for proline was increased approximately 8-fold with increasing external osmolalities (*14*). We found, however, that the K_m of BetP C252T for betaine and Na^+ did neither changed to a statistically significant extent nor systematically varied with the extent of the applied stress within the range of osmolality used in these experiments (results not shown). Thus, we can exclude that any changes in the transport rates at low or high external osmolalities were caused by a change in the substrate affinity of BetP.

Osmoregulatory Properties of BetP and Its Truncated Variants in Proteoliposomes. In contrast to studies in intact cells, the flexibility and accessibility of the proteoliposomal system allows us to investigate the osmosensing and osmoregulation mechanism of truncated BetP variants by analyzing different putative stimuli separately. For this purpose, the different BetP mutant forms were purified individually and reconstituted in proteoliposomes (Figure 3). Liposomes were prepared at an osmolality of 220 mosmol/kg and subjected to osmotic shifts by varying the external osmolality between 100 and 1000 mosmol/kg by the addition of sorbitol. Since liposomes behave like osmometers (*14*, *20*) the resulting volume swelling/shrinkage depends linearly on the extent of the osmotic down- or upshift. This in turn leads to a corresponding change of the internal solute concentration and in particular to a change in the lumenal K^+ concentration, which was previously shown to be responsible for activation of BetP. In the external buffer, the concentration of the cosubstrate Na^+ was kept constant at 61 mM, except for the truncated variant BetP Δ45, where it was raised to 116 mM because of the low Na^+ affinity and decreased V_{max} of this mutant. The fact that we necessarily had to cover also low osmolality conditions in these experiments restricted the external Na^+ concentrations to values at or even below the K_m value.

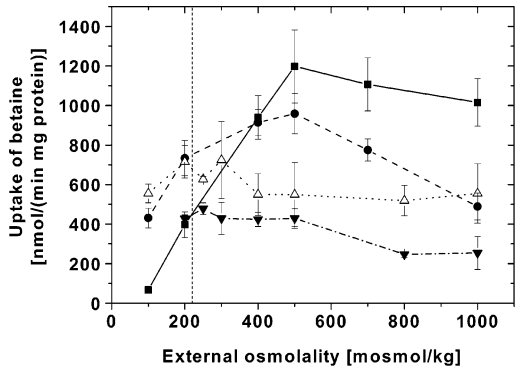

FIGURE 3: Activity regulation of BetP and the C-terminally truncated BetP derivatives in liposomes and dependence on external osmolality. All proteoliposomes used were prepared in buffer (100 mM KP_i, pH 7.5) with an osmolality of 220 mosmol/kg. The external buffer basically contained 20 mM NaP_i, pH 7.5, and 25 mM NaCl (total [Na^+] approximately 60 mM) and had a basic osmolality of 100 mosmol/kg, except for BetP Δ45, where the external buffer contained 20 mM NaP_i, pH 7.5, and 80 mM NaCl (total [Na^+] approximately 120 mM). The external osmolality was increased by adding sorbitol up to 1000 mosmol/kg, thus creating an osmotic gradient that led to liposome shrinkage due to water efflux and consequently to an increase in internal solute (and K^+) concentration. The uptake of glycine betaine by BetP C252T (■), BetP Δ12 (●), BetP Δ25 (△), and BetP Δ45 (▼) is shown. The dashed line represents iso-osmolality between internal and external buffer. The experiment indicated by squares is identical to that shown in Figure 1.

All mutants were found to be functionally active after reconstitution (Figure 3). As a control, the activity pattern of BetP C252T was included in this experiment. As shown by in vivo measurements (cf. Figure 2), truncation of 45 or 25 amino acids rendered the protein permanently active, i.e., these mutants were deregulated in terms of osmoregulation. The fact that BetP Δ25 is nearly fully active already at low external osmolalities and reached 60% of the activity of the fully activated BetP C252T indicates that BetP Δ25 is constitutively active rather than unable to become activated. The regulation pattern of BetP Δ12 was more pronounced in proteoliposomes as compared to the in vivo measurements.

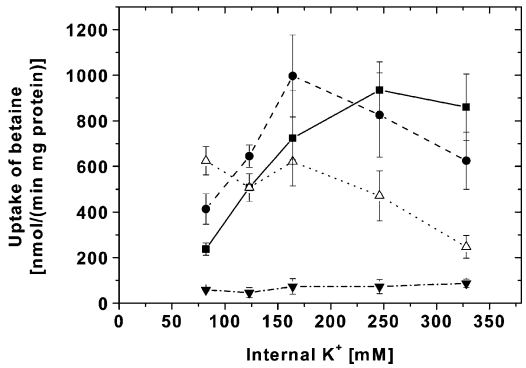

FIGURE 4: Activity regulation of BetP C252T and its C-terminally truncated BetP derivatives and dependence on the liposomal K^+ concentration under iso-osmotic conditions. The internal buffer of the proteoliposomes contained 45.6, 68.4, 91.2, 136.8, 182.4, or 228 mM KP_i, pH 7.5, corresponding to internal K^+ concentrations of 82, 123, 164, 246, 328, and 410 mM. The external buffer basically contained 20 mM NaP_i, pH 7.5, and 25 mM NaCl (100 mosmol/kg). Iso-osmolality between liposomal interior and exterior was adjusted by addition of sorbitol to the external buffer. Data for BetP C252T (■), BetP Δ12 (●), BetP Δ25 (△), and BetP Δ45 (▼) are shown.

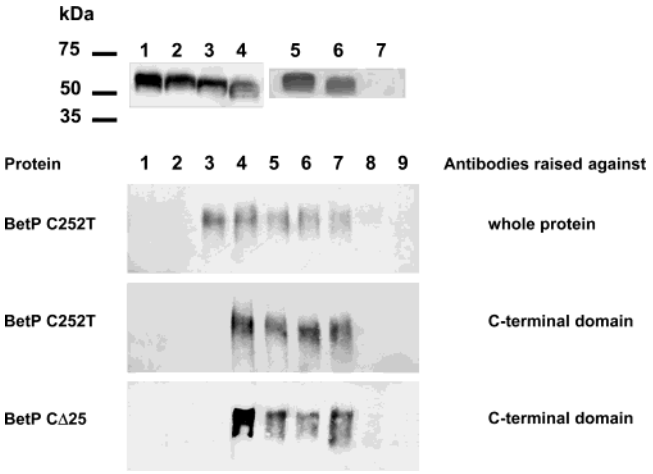

FIGURE 5: Specificity of BetP antibodies and orientation of BetP C252T and BetP Δ25 in proteoliposomes. (A) Specificity of antibodies raised either against the intact carrier protein or against the C-terminal extension of BetP. Proteins of the membrane fraction were separated in an SDS−polyacrylamide gel and blotted. The immune detection of BetP was performed with antibodies raised against BetP (lanes 1−4) or against the last 55 amino acids of BetP (lanes 5−7): BetP C252T (lanes 1 and 5); BetP Δ12 (lane 2); BetP Δ25 (lanes 3 and 6); BetP Δ45 (lanes 4 and 7). (B) (Proteo)liposomes were incubated with the indicated antibodies either directly, to detect the external accessibility of the C-terminal domain (lanes 1−3), or after the antibodies were enclosed within the (proteo)liposomes (lanes 4−9), to determine accessibility of BetP domains from the lumenal side. After incubation, the antibody/BetP complex was separated in an SDS−polyacrylamide gel and blotted onto a PVDF membrane. Antibodies, which were bound to BetP before in the binding assay, were detected by Western blot analysis. Shown are reaction of the preimmune serum with proteoliposomes (lane 1), binding of antibodies to BetP-free liposomes (lane 2) or to proteoliposomes (lane 3), and binding of enclosed antibodies to proteoliposomes (lanes 4−6) or BetP-free liposomes (lanes 7−9) after two, four, or six freeze−thaw cycles (for details see Experimental Procedures).

In both systems BetP Δ12 was still able to respond to osmotic stress to a significant extent, which was detectable in particular in the experiments in which a very low external osmolality was applied. This mutant, however, reached already 45% of its maximum activity at an external osmolality of 100 mosmol/kg, where the wild-type protein was found to be almost inactive. For this reason we refer to this mutant as being partially deregulated.

Chemosensing by BetP and Its C-Terminally Truncated Derivatives in Proteoliposomes. Osmostress-dependent BetP activation was recently interpreted in a proteoliposomal system in terms of a chemosensory event in response to an increase in the lumenal K^+ concentration (*13*). In the experiment described above (Figure 3), the external osmolality was elevated by the addition of sorbitol, leading to water efflux and consequently to an increase of the internal K^+ concentration, which results in activation of wild-type BetP. To investigate the influence of K^+ on the activity regulation of mutant BetP variants in more detail and to be able to dissect the effect of internal K^+ from other parameters that are changed upon a hyperosmotic shift, for example, liposomal shrinkage or external osmolality, the experimental setup was changed. To avoid water fluxes and any other volume-related changes, glycine betaine uptake by the mutant carrier proteins was determined under iso-osmotic conditions in a series of proteoliposome preparations differing exclusively in their internal K^+ concentration (Figure 4). BetP C252T and BetP Δ12 were found to be activated solely by an increase in the liposomal K^+ concentration, whereas the activity of BetP Δ25 and BetP Δ45 could not be stimulated by K^+, i.e., they were active in glycine betaine transport independent of the internal K^+ concentration. Although the activity of BetP Δ45 was very low in this kind of experiment in comparison to BetP C252T, it has to be emphasized that the mutant was not inactive. Taken together, the results indicate that BetP C252T and the partly deregulated mutant BetP Δ12 were able to sense changes in internal K^+ concentration, whereas BetP Δ25 and BetP Δ45 were not. It has to be pointed out that this hypothesis is essentially based on the assumption that BetP and its recombinant forms when reconstituted in proteoliposomes have, at least to a major part, a right-side-out orientation, with their N- and C-terminal extensions facing the lumen of the liposomes.

Domain-Specific Antibodies. One possibility to determine the orientation of the C-terminal extension of BetP when integrated into proteoliposomes would be the use of domain-specific antibodies. For this purpose, polyclonal antibodies raised either against BetP or against the C-terminal extension of BetP were generated. The specificity of the two different antibodies was tested. The expression of genes encoding the different C-terminally truncated forms of BetP was induced with anhydrotetracycline in different MKH13 strains as described under Experimental Procedures. Cells were harvested by centrifugation, disrupted by use of glass beads, and fractionated into cytoplasm and membrane fraction by ultracentrifugation. Aliquots (50 μg) of the membrane fractions were separated on SDS−12.5% polyacrylamide gels and blotted onto PVDF membranes. The immune detection of BetP was performed either with antibodies raised against BetP (Figure 5A, left panel) or with antibodies raised against the last 55 amino acids of BetP, corresponding to the hydrophilic C-terminal extension (Figure 5A, right panel). As expected, the former antibodies were able to detect all forms of BetP, whereas the latter only detected wild-type BetP C252T and BetP Δ25 but failed to detect BetP Δ45, confirming thus its specificity for the C-terminal extension.

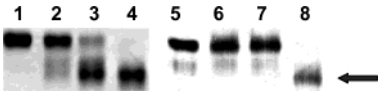

FIGURE 6: Orientation of Bet C252T in liposomes. Solubilized protein (lanes 1−4) or proteoliposomes (lanes 5−8) were incubated with aminopeptidases for 0, 0.5, 4, and 12 h (lanes 1−4) or 0, 4, and 12 h (lanes 5−7). Only after solubilization of the proteoliposomes with Triton X-100 was the C-terminal domain of reconstituted BetP accessible to proteolytic digestion (lane 8). After proteolysis, the samples were separated by SDS−PAGE and stained with Coomassie brilliant blue. The specificity of the aminopeptidases, in terms of BetP digestion at the N-terminal end, was confirmed in a parallel experiment (not shown). Whereas all protein bands were recognized by polyclonal antibodies raised against the C-terminal extension of BetP, partially digested BetP (indicated by an arrow) was not detected by the streptavidin−alkaline phosphatase conjugate, because the Strep-TagII at the N-terminus had been degraded (data not shown).

Additionally, this shows that the remaining 30 amino acids residues of the C-terminal domain of BetP Δ25 still contained enough epitopes necessary for the immune reaction.

Orientation of BetP C252T and BetP Δ25 in Proteoliposomes. Whereas the cytoplasmic orientation of the N-and C-terminal domains had been determined in intact cells (*13*), the topology of reconstituted BetP in proteoliposomes was not known. Polyclonal antibodies described above were used to determine whether this domain is accessible from the internal and/or external side of the liposome membrane. Binding of antibodies of the C-extension-specific antibodies could only be observed after their enclosure into the proteoliposomal lumen by freezing and thawing (Figure 5B, middle panel, lanes 4−6) but not if intact proteoliposomes were incubated (Figure 5B, middle panel, lane 3), indicating that the C-terminal domain is only accessible from the lumenal side. To distinguish between a specific binding of antibodies to BetP and mere enclosure in the lumen, the amounts of antibodies in proteoliposomes and liposomes were diluted by several freeze−thaw cycles, thereby allowing the enclosed antibody to equilibrate with the external medium. After the sixth cycle no antibodies could be detected in protein-free liposomes, whereas in BetP-containing proteoliposomes the amount of bound antibodies remained almost unchanged (Figure 5B, middle panel, lanes 4−9). The pattern of antibody binding was nearly identical if the mutant BetP Δ25 was used (Figure 5B, bottom panel). As a control for the binding conditions, similar experiments were carried out with polyclonal antibodies raised against the intact protein. As expected, in this case BetP C252T was detected by the antibodies at both sides of the membrane as shown in Figure 5B (top panel).

To verify this observation in an independent experiment, proteolysis studies were carried out by incubating either solubilized or reconstituted BetP C252T for up to 12 h with aminopeptidases. In contrast to the solubilized protein, the N-terminal domain of reconstituted BetP was only accessible to digestion after solubilization of the proteoliposomes with detergent (Figure 6), indicating that also the N-terminal extension is exclusively facing the lumen of the liposomes. To exclude the possibility that during the six freeze−thaw cycles applied above either the activity or the orientation of BetP changed, we carried out additional control experiments. These revealed that the maximum activity of BetP decreased only by 14% after six freeze−thaw cycles and showed furthermore that the N-terminal domain was still only accessible to digestion by the aminopeptidase after solubilization of the proteoliposimes with detergent (data not shown). Taken together, these results argue for a right-side-out orientation of BetP C252T and BetP Δ25 in the liposomal membrane, which is an essential prerequisite for the C-terminal extension to be able to directly detect the stimulus indicating hyperosmotic stress, i.e., the increase of K^+ concentration at the inside of the proteoliposomes.

DISCUSSION

Three uptake systems for compatible solutes, namely, the ABC system OpuA from *L. lactis* and the secondary carriers ProP from *E. coli* and BetP from *C. glutamicum*, fulfill the criteria of being both osmosensors and osmoregulators, since they retained the ability to detect hyperosmotic conditions and to respond to the extent of a hyperosmotic shift in proteoliposomes after purification and reconstitution (*10*−*12*). The underlying molecular mechanisms of how osmotic stress is actually sensed by these systems are a matter of current research (*21*). In all three cases, changes of internal conditions seem to be used as a measure for hyperosmotic stress. In detail, either a change of the carrier/membrane interaction induced by increasing internal ionic strength (OpuA, *15*), concentration of cytoplasmic ions and osmolytes (ProP, *22*), or the internal potassium concentration (BetP, *13*) were made responsible for triggering activation of the individual carrier proteins. In none of these examples, however, has a direct correlation of the identified stimuli and particular subunits or domains of these carriers been achieved so far. This, however, is a prerequisite for a molecular understanding of the sensing and/or activation process related to the response to osmotic stress.

In previous studies we have shown that an increasing lumenal K^+ (Rb^+ or Cs^+) concentration is able to trigger the activation of BetP when reconstituted in *E. coli* phospholipids, and this stimulus could be discriminated from others like membrane strain, vesicle shrinkage, change in osmolarity, change in external ion conditions, or a change of other lumenal cations (*13* and unpublished results). Furthermore, results obtained in intact cells indicated that truncations at the N- and the C-terminal domains of BetP led to an alteration in its sensory and/or regulatory function (*9*). In this study we used proteoliposomes in which C-terminally truncated BetP variants were reconstituted in order to experimentally correlate these two observations, i.e., to prove the assumption that the K^+-sensitive domain of BetP is located in the C-terminal extension of the carrier.

The characterization of several BetP mutants in proteoliposomes led in fact to the identification of the protein domain responsible for, or at least critically involved in, chemosensing of K^+ by the glycine betaine carrier. A segment of amino acids between positions 571 and 583 of BetP, which has a total number of 595 residues, seems to be critically involved in the K^+-dependent activation upon hyperosmotic stress, since the truncation of 25 amino acids was sufficient to rendered BetP insensitive to changes of the internal K^+ concentration, resulting in a constitutively active transporter. A truncation of 12 amino acids resulted in a carrier that is only partially regulated, showing around half

the activity of a fully stimulated BetP already at low osmolalities, but this mutant form is still able to sense K^+. Truncation of 45 amino acids and thus virtually of the full C-terminal domain, on the other hand, led to a BetP variant that is similar to BetP Δ25 concerning its insensitivity to hyperosmotic stress and K^+. Although the activity of this mutant was relatively low in comparison to the other BetP variants, BetP Δ45 was not inactive. Our results cannot completely rule out the possibility that an intact C-terminal domain is essential only for the catalytic activity of betaine transport, rather than for K^+ sensing, since it is not easy to clearly decide whether a mutant, like BetP Δ45, is constitutively active or unable to become efficiently activated. Two observations are in favor of our hypothesis that the C-terminal domain of BetP is indeed involved in the K^+-sensing process. (i) The extent of deregulation, i.e., the loss of osmostress-dependent activation and/or K^+ sensitivity, seems to be related to the size of the truncation. For the other scenario, suggesting that the C-terminal domain is important for the transport reaction only, both osmoregulation and K^+ sensitivity should be lost concomitantly in BetP Δ12. (ii) The constitutively active BetP Δ25 mutant, which reached 60% of the activity of BetP C252T in the proteoliposome system, cannot be interpreted as unable to be activated, which should be the case if this domain is essential for the transport reaction. Therefore, we conclude that the C-terminal extension is either directly involved in K^+ sensing or its interaction with a K^+ sensor within BetP is necessary for the observed regulation.

For a correct interpretation of these results, it is absolutely essential to know in which orientation BetP is embedded in the proteoliposomes, or in other words, on which side of the membrane the putative osmosensor, the C-terminal domain, is located. In cells we recently could show that BetP has an even number of transmembrane segments, whereby the N- and C-terminal domains face the cytoplasm (*13*). With antibodies in hand recognizing only the C-terminal domain of BetP and by the use of aminopeptidases detecting the N-terminal domain of BetP, it was now possible to determine the topology of the N- and C-terminal domains with respect to the liposomal membrane, too. Both an immunological and a proteolytic method showed that BetP is unidirectionally integrated in a right-side-out orientation, i.e., the N- and C-terminal extensions are facing the lumen of the liposomes. The residual 30 amino acids of the C-terminal extension in BetP Δ25 still allowed antibody binding. Consequently, by use of antibodies raised against the C-terminal domain, it could be shown that the K^+-insensitive mutant BetP Δ25 has the same orientation as BetP C252T. Excluding the possibility that the truncated C-terminal domain of BetP Δ25 may be mislocated further strengthens the hypothesis that the K^+ stimulus is sensed by the last 25 amino acid residues of this domain in the wild-type protein.

Recently, we showed that BetP activation is specific for K^+ (or closely similar ions such as Rb^+ or Cs^+); neither NH_4^+, choline (*13*), nor even Na^+ (unpublished results) was able to activate BetP. The discrimination between different cations argues for a K^+ binding site in which the C-terminal domain of BetP should be involved. On the other hand, however, in proteoliposomes, half-maximal activation of BetP was found at an internal K^+ concentration of 220 mM (*13*), which appears to be rather high for a specific binding site. Since the primary sequence of the C-terminal domain does not include a motif known to be involved in K^+ binding/recognition, it remains to be elucidated whether (i) additional regions within BetP cooperate with the C-terminal domain in K^+ sensing, (ii) oligomers of BetP are the active entity for recognizing K^+, or (iii) the membrane may be involved in creating K^+ specificity of the sensing process.

As mentioned above, three uptake systems for compatible solutes, namely, OpuA from *L. lactis*, ProP from *E. coli*, and BetP from *C. glutamicum*, have been studied to a significant extent with respect to their properties of being osmosensors and osmoregulators as well as with respect to the kind of stimuli relevant for signaling hyperosmotic stress. In the case of OpuA, it is not yet known which domain within the ABC transporter is involved in perceiving osmotic stimuli, whereas in the case of the secondary carriers ProP and BetP, their C-terminal domains seem to be implicated in osmosensing (*9*, *16*, this work). Despite this similarity, the underlying sensing mechanisms seem to be quite different in BetP and ProP, not only in terms of the kind of stimuli that are detected (see above) but also in terms of secondary structures of the domains involved. We do not have evidence yet for a particular secondary structure of the C-terminal domain of BetP, and in isolated form, it seems to adopt a random coil structure (unpublished results). The C-terminal extension of ProP, on the other hand, is predicted to form an antiparallel α-helical coiled-coil, which was in fact shown to influence the regulation properties of ProP in vivo (*16*). The orientation of ProP has not been experimentally proven in proteoliposomes so far, and it is thus not unequivocally clear whether this domain of ProP or mutant forms of it are located inside the proteoliposomes and therefore able to detect internal changes in ionic conditions. Taken together, these results on BetP activation by hyperosmotic shifts in proteoliposomes represent the first direct experimental correlation of the osmotic stimulus, K^+ (or ions in general), and a particular domain involved in signal perception.

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