Regulatory Properties and Interaction of the C- and N-Terminal Domains of BetP, an Osmoregulated Betaine Transporter from *Corynebacterium glutamicum*[†]

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Received July 14, 2008; Revised Manuscript Received September 29, 2008

ABSTRACT: The glycine betaine carrier BetP from *Corynebacterium glutamicum* responds to changes in external osmolality by regulation of its transport activity, and the C-terminal domain was previously identified to be involved in this process. Here we investigate the structural requirements of the C-terminal domain for osmoregulation as well as interacting domains that are relevant for intramolecular signal transduction in response to osmotic stress. For this purpose, we applied a proline scanning approach and amino acid replacements other than proline in selected positions. To analyze the impact of the surrounding membrane, BetP mutants were studied in both *C. glutamicum* and *Escherichia coli*, which strongly differ in their phospholipid composition. A region of ~25 amino acid residues within the C-terminal domain with a high propensity for α-helical structure was found to be essential in terms of its conformational properties for osmodependent regulation. The size of this region was larger in *E. coli* membranes than in the highly negatively charged *C. glutamicum* membranes. As a novel aspect of BetP regulation, interaction of the C-terminal domain with one of the cytoplasmic loops as well as with the N-terminal domain was shown to be involved in osmosensing and/or osmoregulation. These results support a functional model of BetP activation that involves the C-terminal domain shifting from interaction with the membrane to interaction with intramolecular domains in response to osmotic stress.

Soil bacteria like *Corynebacterium glutamicum* respond to continuous changes in their environment with homeostatic mechanisms, one of the most important being osmoregulation. *C. glutamicum* is well-equipped with systems to cope with hyperosmotic stress, with respect to both synthesis and uptake of compatible solutes. The most effective uptake system is the betaine carrier BetP. Together with ProP from *Escherichia coli* and OpuA from *Lactococcus lactis*, BetP is one of the three best-studied model systems for this kind of bacterial stress response at the level of membrane transport (*1*−*4*).

BetP, a secondary glycine betaine transporter, is energetically coupled to the cotransport of sodium. It comprises three functions, namely, transport catalysis (betaine uptake), activity regulation (adaptation of the transport activity to the actual extent of hyperosmotic stress), and stimulus sensing (perception of stress and intramolecular signal transduction) (*3, 4*). In the absence of osmotic stress, BetP is inactive but switches to the active state with an increase in external osmolality in less than 1 s (*5, 6*). The functional unit of BetP is a trimer as demonstrated by two-dimensional (2D) electron crystallography and analytical ultracentrifugation (*7*). Each subunit comprises a membrane body consisting of 12 transmembrane segments and two terminal domains, of ~55−60 amino acids each. These terminal domains, in particular, the C-terminal extension, were shown to be essential for activity regulation of BetP. In detail, at least three factors were found to contribute to the molecular mechanism of BetP activation upon osmotic stress, namely, (i) the C-terminal domain, (ii) an increase in the cytoplasmic K^+ concentration, and (iii) the properties of the surrounding bilayer surface, which is supposed to interact with the C-terminal domain (*3*). In addition, recently also the hydrophobic core of the membrane was suggested to be involved in BetP activation, at least in response to chill stress, another stimulus perceived by BetP (*8*).

The contribution of the C-terminal domain of BetP to osmosensing and osmoregulation has been investigated in previous work. The impact of truncations of this domain has been studied (*6*), and the strict dependence of its regulatory function on the specific presence of high K^+ concentrations has been demonstrated in vitro (*9*). Recently, two new features of this regulatory domain were described (*10*). By alanine scanning of the last 24 amino acids of the C-terminal domain, a decisive influence of glutamate at position 572 on the activation pattern in response to hyperosmotic stress was observed. Replacement of this amino acid with a series of others led to the loss of activation of BetP both when synthesized in the heterologous host *E. coli* and when

[†] This work was supported by Deutsche Forschungsgemeinschaft (Mo 892/2-1).

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10.1021/bi801325r CCC: $40.75
Published on Web 10/25/2008

functionally reconstituted into liposomes prepared from *E. coli* phospholipids. Surprisingly, activation by osmotic stress could be restored in some of the mutants when the recombinant forms of BetP were synthesized in *C. glutamicum* or reconstituted into liposomes harboring a high fraction of negatively charged phospholipids, thus resembling the *C. glutamicum* lipid composition. Consequently, a model was developed in which a putatively critical region centered around glutamate 572 was suggested to interact with the phospholipid membrane being responsible for a critical step in the mechanisms of BetP activation.

A number of questions, however, remained unsolved. What is the exact definition of the region within the C-terminal domain which seems to be critical for the activation process? What is the reason why this position seems to be more important than others within the C-terminal domain? Is there direct evidence for the lipid interaction of the C-terminal domain besides the discriminative functional behavior in different lipid surroundings? Are there other functionally relevant interaction partners of the C-terminal domain besides the membrane surface? Finally, what is the molecular mechanism of the functional switch of BetP in which the C-terminal domain is supposed to be involved?

We addressed these questions by two different approaches. First, a detailed molecular analysis of the impact of various amino acid positions within the C-terminal domain on the regulatory properties of BetP was carried out. This analysis was extended to the region between amino acid position 572 and the N-terminal end of the C-terminal domain. For this purpose, variants of BetP harboring amino acid replacements within the C-terminal domain were constructed, expressed in both *E. coli* and *C. glutamicum*, and functionally analyzed with respect to their sensing and regulation capacity. Second, direct methods of structural and functional interaction were applied, i.e., surface plasmon resonance spectroscopy and peptide array analysis, as well as a functional competition assay in proteoliposomes. The results of these studies clearly show that a region of ~25 amino acids within the C-terminal domain, which is not confined to or around amino acid position 572, is critically involved in the sensing and/or regulation mechanisms of BetP. Moreover, we detected at least two novel domains of BetP interacting with the C-terminal domain and being important for the activation process, namely, the N-terminal domain of BetP and cytoplasmic loop 8. These findings led to a new concept of understanding of the molecular mechanism of BetP activation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. For glycine betaine uptake measurements, *E. coli* MKH13 (*11*) was used, whereas DH5αmcr (*12*) was applied for preparative *strep-betP* expression and BL21 DE3 (Novagen, Darmstadt, Germany) for *gst* and *gst-c-betP* expression. The plasmids used for the heterologous expression of *betP* in *E. coli* are based on the pASK-IBA5 vector in which *strep-betP* is under the control of the *tet* promoter (IBA, Göttingen, Germany). pGEX-6P2 (GE Healthcare, Freiburg, Germany) and its derivative were used for expression of *gst* and *gst-c-betP*, in which *gst* is under the control of the *ptac* promotor. *E. coli* cells were grown at 37 °C in LB medium supplemented with carbenicillin (50 μg/mL). Induction of *betP* was carried out in exponentially growing cells by addition of 200 μg of anhydroxytetracycline/L of culture. The expression of *gst* and *gst-c-betP* was induced by addition of 100 μM IPTG. For homologous expression of *betP* in *C. glutamicum*, strain DHPF (*13*) was used. In this case, the *betP* genes were under the control of the *ptac* promoter of pXMJ19 (*14*). *C. glutamicum* cells were grown in Brain Heart Infusion medium (Difco, Detroit, MI) at 30 °C. Subsequently, cells were used to inoculate a fresh culture, and *betP* expression was induced with 250 μM IPTG. All constructs are summarized in Table 1S of the Supporting Information.

Table 1: Sequences of Peptides Used for Competition Experiments[a]

name	sequence
N-peptide	ELAGLLENPTNLEGKLADAEEEIILEGEDTQASLNWS
L4	STFRVGRKQLLSSAFVPLIGEK
L8	ARISRGRSIREFILG
L8 *random*	LIIGAESGRSRIRFR
L10	ADSASTVMGTMSQHGQLEANKWVTA

[a] N-Termini were acetylated, and C-termini had free carboxylic groups.

Construction of Plasmids. Plasmid pAcl1 (*9*), encoding the cysteine-free C252T variant of BetP, which has been shown to be regulated like wild-type BetP (*15*), was used as a PCR template for the construction of other *betP* variants. Mutant forms of *betp* leading to BetP variants with amino acid substitutions in the C-terminal domain were constructed by site-directed mutagenesis (Stratagene, Heidelberg, Germany) as previously described (*15*). In this case, primers containing the desired mutation were used for PCR. For primer sequences and resulting pAcl1 derivatives, see Tables 1S and 2S of the Supporting Information. For the expression of *betP* in *C. glutamicum*, the *betP* gene was isolated from the respective pAcl1 derivative by the use of restriction nucleases *Xba*I and *Nae*I. The fragment was ligated into the *Xba*I and *Sma*I site of *E. coli*/*C. glutamicum* shuttle vector pXMJ19. For construction of the *gst-c-betp* fusion, the *betP* fragment encoding C-terminal BetP amino acids 540−595 was amplified using pAcl1 as a template (Table 2S) and cloned into pGEX-6P2 after *Bam*HI and *Eco*RI restriction.

Preparation of Liposomes, Purification, and Reconstitution of strep-BetP. Liposomes were prepared, and strep-BetP was purified as described previously (*16*) using 2% dodecyl maltoside for solubilization of membrane preparations. Functional reconstitution was performed as described previously (*16*). In brief, liposomes (20 mg of phospholipid/mL) from *E. coli* polar lipid extract phospholipids (Avanti Polar Lipids, Alabaster, AL) were preformed by extrusion through polycarbonate filters (400 nm pore size) and diluted 1:4 in buffer [100 mM KP_i (pH 7.5)]. After being saturated with Triton X-100, the liposomes were mixed with purified BetP protein in a lipid:protein ratio of 30:1 (w/w). BioBeads at ratios (w/w) of 5 (BioBeads:Triton X-100) and 10 (BioBeads: dodecyl maltoside) were added in four steps to remove detergent by incubation overnight. Finally, the proteoliposomes were centrifuged and washed twice with 100 mM KP_i buffer (pH 7.5) before they were frozen in liquid nitrogen and stored at −80 °C at a concentration of 60 mg of lipid/mL.

Loading of Proteoliposomes with Peptides. To load proteoliposomes with synthetic peptides (sequences in Table 1), an aliquot of BetP proteoliposomes (70 μL) was thawed

and centrifuged (353000*g* for 20 min at 20 °C). The proteoliposome pellet was resuspended in a peptide solution [333 μM peptide in 100 mM KP_i (pH 7.5)] in a total volume of 100 μL. After three cycles of freezing (liquid N_2) and thawing, the proteoliposomes were extruded 17 times through a polycarbonate filter (400 nm pore size) in a total volume of 300 μL, to yield a final peptide concentration of 100 μM inside the proteoliposomes. This corresponds to a 10-fold molar excess of peptide to BetP protein when the amount of reconstituted BetP is related to the internal proteoliposome volume. After extrusion, the proteoliposomes were washed two times in 100 mM KP_i (pH 7.5), resuspended in 70 μL of buffer, and used for uptake measurements as described here.

Transport Assays. [^{14}C]Glycine betaine was prepared as described previously (*6*). Uptake of labeled betaine in *E. coli* cells was assessed as follows. *E. coli* MKH13 expressing a particular *strep-BetP* derivative was cultivated in LB medium (50 μg/mL carbenicillin) to an OD_{600} of 0.5. Synthesis of strep-BetP mutants was initiated by addition of 200 μg/L anhydrotetracycline. The cells were harvested after 2 h, washed in 25 mM KP_i buffer (pH 7.5) supplemented with 100 mM NaCl, and resuspended in the same buffer containing 20 mM glucose. For uptake measurements, cells were assayed in 25 mM KP_i (pH 7.5) containing 10 mM glucose, 100 mM NaCl, and different KCl concentrations to adjust the external osmolality. Subsequently, cells were stirred for 3 min at 37 °C before the reaction was started via the addition of 250 μM [^{14}C]glycine betaine (specific radioactivity of 90 μCi/mmol). At given time intervals, samples were taken, filtered through glass fiber filters (APFF02500, Millipore, Schwalbach, Germany), and washed twice with 2.5 mL of 0.6 M KP_i buffer. The radioactivity on the filters was determined by liquid scintillation counting. For transport assays in *C. glutamicum* DHPF harboring the different *betP* mutants on pXMJ19, cells were grown for 10 h in BHI medium in the presence of 25 μg/mL chloramphenicol, before they were transferred into fresh medium. Expression of *betP* genes was induced by the addition of 0.25 mM IPTG, and cells were harvested after 16 h. Washing and uptake measurements were performed as described for *E. coli* MKH13 (*10*), except that filters were washed with 100 mM LiCl. Briefly, extruded proteoliposomes with 100 mM KP_i (pH 7.5) as the internal buffer were diluted 200-fold in potassium-free buffer [20 mM NaP_i (pH 7.5) and 25 mM NaCl] containing 15 μM [^{14}C]glycine betaine (3 mCi/mmol) and 1 μM valinomycin to create an outwardly directed K^+ diffusion potential. High osmolalities of the external buffer were compensated by addition of proline. After 5 and 10 s, samples were filtered rapidly through 0.45 μm nitrocellulose filters (GS; Millipore, Schwalbach, Germany) and washed immediately with 100 mM LiCl. The betaine uptake rate was calculated as described previously (*10*).

Western Blot Analysis. To control the amount of BetP variants integrated into the membrane, Western blot analysis of membrane extracts was performed as described previously (*15, 16*) by the use of BetP specific antibodies.

Purification of Gst-C-BetP Protein and Isolation of the C-Terminal BetP Domain. E. coli BL21 cells harboring the *gst* or *gst-c-betp* fusion construct on pGEX-6P2 were cultivated in LB medium. Expression of the *gst* constructs was induced by adding 0.1 mM IPTG at an OD_{600} of 0.5. After being cultivated for an additional 3 h, cells were harvested by centrifugation, washed in PBS buffer [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3), and 1 mM EDTA], and resuspended in PBS containing 1% Triton X-100, 2 μg/mL DNase, and Complete Protease Inhibitor (Roche, Mannheim, Germany, 1 pill/100 mL). The cell suspension was passed through a French press (SLM Aminco, Colora, Lorch, Germany) three times with a pressure of 1100 psi and centrifuged at 19000*g* for 20 min to remove cell debris. The GST fusion protein was purified by glutathione affinity chromatography using a 5 mL glutathione-Sepharose column. The crude extract was loaded on the column, which had been pre-equilibrated with PBS buffer. The column was washed with 8 column volumes of PBS containing 500 mM NaCl. The column buffer was then changed to 50 mM Tris-HCl (pH 8.6), 1 mM EDTA, and 150 mM NaCl. The GST fusion proteins were eluted in this buffer containing 10 mM glutathione and used both for peptide array experiments and for isolation of the C-domain from the fusion construct. For this purpose, glutathione was removed by dialysis for 18 h against buffer in 1000-fold excess (v/v) [50 mM Tris-HCl (pH 8.6), 1 mM EDTA, and 150 mM NaCl] using Spectra Por Float-A-Lyzer (MWCO 10000, Spectrum, Breda, The Netherlands). To cleave off the GST protein, the fusion protein was incubated with Prescission protease (6 units/mg of protein) (GE Healthcare) at 4 °C overnight. Prescission protease was activated by adding 1 mM dithiothreitol. To separate the C-domain peptide from the GST fusion protein and the GST-tagged protease, the cleavage product was loaded on a pre-equilibrated glutathione-Sepharose column [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 1 mM EDTA]. The isolated C-domain peptide was eluted with 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 1 mM EDTA in 1 mL fractions. The purity of the C-domain was verified by SDS−PAGE (15% gel) and subsequent silver staining (*17*). The C-domain was desalted and concentrated using a C18 reversed-phase cartridge (SepPak C18plus,Waters, Eschborn, Germany). The cartridge was activated with 5 mL of methanol, washed with 10 mL of distilled water, and loaded with the C-domain. After being washed with 20 mL of distilled water, the protein was eluted with methanol/H_2O_{dd}/acetic acid mixtures [70:30:0 MeOH:H_2O:HAc ratio (1 mL), 80:20:0 MeOH:H_2O:HAc ratio (1 mL), and 80:15:5 MeOH:H_2O:HAc ratio (2 mL)]. Solvent was evaporated (Eppendorf, Concentrator 5301) at room temperature, and the protein was resolved in buffer [50 mM Tris-HCl (pH 7.5) and 150 mM KCl].

Peptide Arrays. An immobilized peptide library representing BetP (15-mer peptides, three-amino acid offset) was synthesized on activated cellulose membranes containing polyethylene glycol 600 amino spacers (ASP222, Abimed, Langenfeld, Germany) using Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] amino acid derivatives (*18, 19*). The membrane was blocked [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, TBS buffer, 3% skim milk powder, and 0.05% Tween 20] and incubated with GST (negative control) or GST-C-BetP fusion protein diluted in TBS buffer. After being washed (TBS, 0.5% skim milk powder, and 0.05% Tween 20), the membrane was incubated with anti-GST antibody, washed again, and then incubated with anti-goat horseradish peroxidase conjugate. Binding of GST-C-BetP was detected by

chemiluminescence (uptilight HRP blot chemoluminescent substrate, Uptima Interchim, Montlucon, France).

Surface Plasmon Resonance. Interaction of the isolated C-terminal BetP domain with lipid surfaces was investigated using a BIAcore 3000 biosensor instrument (Biacore, Uppsala, Sweden). Liposomes made from synthetic phospholipids [dioleoylphosphatidylenthanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylglycerol (DOPG) from Avanti Polar Lipids] were used as mixtures with the following ratios: 1:1 DOPE:DOPC, 1:1:1 DOPE:DOPC:DOPG, and 1:1:4 DOPE:DOPC:DOPG. Liposomes were prepared by mixing DOPC/DOPC liposomes with DOPG liposomes at the indicated ratios followed by extrusion through a polycarbonate filter (400 nm pore size) before being gently frozen and thawed two times. Liposomes of various compositions were then extruded 17 times (100 nm pore size) and immobilized on an L1 chip in the different flow cells. Liposome suspensions (25 μL, 0.33 mg of lipid/mL) were injected at a flow rate of 5 μL/min. The stability of the liposome surface was qualified by washing with 50 mM NaOH (5 μL, 10 μL/min). For each injection, a new liposome surface was generated. Conditions were comparable for each interaction experiment as the degree of surface coverage of the chip was closely similar for each liposome coupling. The protein or peptide of interest in 50 mM Tris (pH 7.5) and 150 mM KCl was injected at different concentrations at a flow rate of 30 μL/min until equilibrium was reached. The L1 chip was regenerated by washing the sample with 50 mM CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}.

RESULTS

Previous work on BetP in intact cells and in proteoliposomes showed that a correct conformation and/or orientation of the C-terminal domain is critical for K^+ sensing and thus for activity regulation of BetP. We were interested in quantifying the contribution of different regions within the C-terminal domain of BetP to the regulatory response in more detail. For this purpose, a set of mutants (proline walk and particular amino acid substitutions) was generated and tested (for the location of the substitutions, see Figure 4). We analyzed the region between amino acid positions 550 and 572 and focused on substitutions with an impact on the conformational constraints of the domain. As a prerequisite, it was shown that the mutant forms of BetP used, at least those changed in the C-terminal domain, were synthesized to approximately the same level in intact cells (Figure 1S of the Supporting Information).

Analysis of Proline Mutants in the C-Terminal Domain of BetP. To study the impact of conformational constraints within the C-terminal domain, amino acids in selected positions were replaced with proline, the corresponding constructs were expressed in *E. coli*, and the response of betaine uptake upon hyperosmotic stress was analyzed. As a basis for all mutant forms, BetP C252T, the Cys-less version of BetP, was used, which is regulated like wild-type BetP (*15*). Selected results of the kinetic analysis are shown in panels A and B of Figure 1. The regulatory capacity of the C-terminal domain was lost in all the BetP mutants starting from the predicted N-terminal end of this domain to amino acid position 578, indicating a strong impact of proline substitutions throughout the region tested.

To compare the regulatory capacity of these mutants, we defined the ratio of transport activity at two osmolalities, namely, 0.8 osm/kg (stimulatory) and 0.2 osm/kg (not stimulatory), as an indicative activation factor (Figure 1C). Obviously, the regulatory property of the C-terminal domain is lost upon proline substitution throughout the whole domain up to position 576/578. It is partly regained at positions closer to the C-terminal end of BetP. The original extent of regulatory capacity as measured in parental strain C252T, however, was never fully reached.

In some of the mutant strains, a small but reproducible increase in activity was observed when the external osmolality was shifted from 0.2 to 0.4 osm/kg (Figure 1A,B). This could indicate that a significant increase in transport activity may occur below the lowest external osmolality tested (0.2 osm/kg). If true, the change in the activation pattern would be interpreted not as a loss of regulation but rather as a shift of the optimum of activation to lower values. Transport at such low osmolalities cannot be tested under the experimental conditions described for panels A and B of Figure 1, since the range of osmotic variation when starting from a particular osmotic condition is limited. Consequently, we changed the experimental setup to investigate the region of lower osmolality (Figure 1D). The results, which are provided for a few selected BetP mutants in comparison to the parental strain, clearly show that there is no hidden range of potential activation under conditions that include a low external osmolality.

Analysis of Replacements with Amino Acids Other Than Proline. We studied the impact of replacements with amino acids other than proline by introducing amino acids which significantly differ from the native amino acids at this position but are not supposed to severely affect the α-helix propensity. In a previous work, replacements were analyzed in a particular region at the very end of the C-terminal domain, between amino acid positions 571 and 595 using alanine scanning (*10*). Here, we chose a number of charged amino acids (e.g., E555, R568, and R576) which were replaced with neutral amino acids or with amino acids with opposite charge. Alternatively, charged amino acids were introduced (A564). The results shown in Figure 2 demonstrate that elimination, conversion, and introduction of charges affect the regulatory capacity of the C-terminal domain in a manner similar to that observed for proline replacements. This, however, holds true for only a core region of the sensitive domain. At the borders of this region, i.e., near the N-terminal (Y550A) or the C-terminal part (R576A), the regulatory capacity was less strongly impaired, in contrast to effects of proline substitutions.

Regulatory Behavior of Mutant Strains in the Host C. glutamicum. In earlier investigations, an influence of the surrounding membrane on BetP regulation was observed (*10, 16*). Thus, the impact of selected amino acid replacements within the C-terminal domain was studied after expression of the *betP* constructs in the original host, *C. glutamicum* (Figure 3). The difference in the regulatory properties of BetP when it was expressed in *E. coli* and *C. glutamicum* is likely to be caused by the different phospholipid compositions of the plasma membrane of the two organisms. The amino acid range within the C-terminal domain of BetP, in which amino acid replacements led to the loss of the regulatory properties when they were analyzed in *C. glutamicum*, turned out to

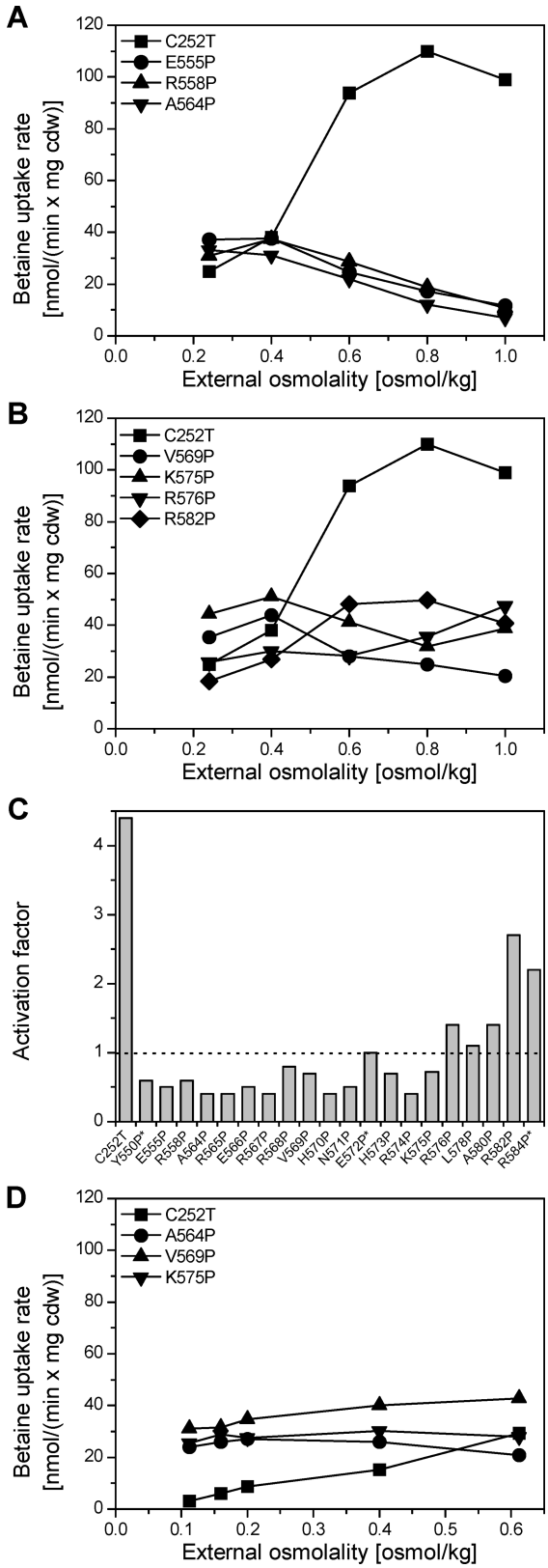

FIGURE 1: Activity regulation of BetP variants with C-terminal proline substitutions in *E. coli* MKH13 cells. The osmolality of the external buffer (25 mM KP_i and 100 mM NaCl) was adjusted by the addition of KCl. (A and B) Regulation of betaine uptake as a function of the external osmolality for various proline mutant strains. (C) Activation factors for all proline mutants in the C-terminal domain of BetP after an osmotic upshift from 0.2 to 0.8 osm/kg. Mutants marked with asterisks were taken from ref *10*. (D) Regulation of BetP variants at low osmolality. The osmolality of the external buffer (15 mM KP_i and 50 mM NaCl) was adjusted by the addition of KCl. The corresponding absolute values were 29.3, 27.1, 42.7, and 30.0 nmol of betaine min^{-1} (mg of cdw)$^{-1}$ for C252T, A564P, V469P, and K575P, respectively.

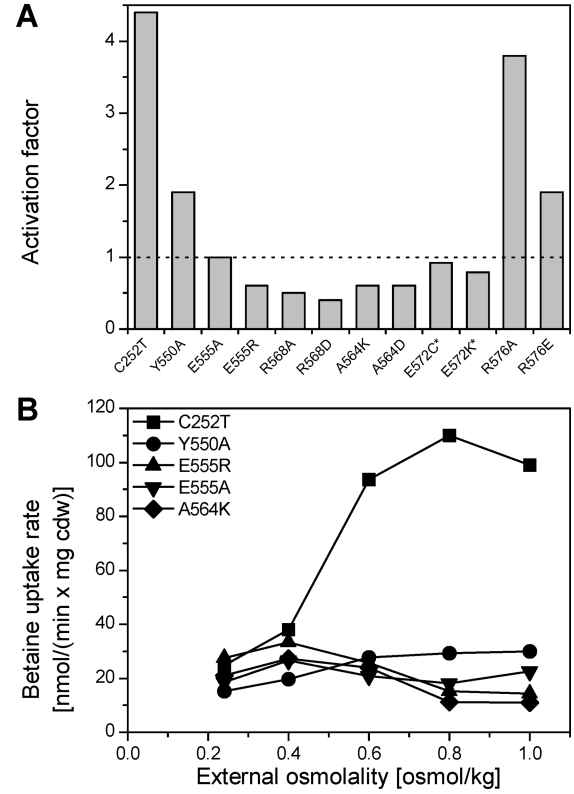

FIGURE 2: Activity regulation of BetP variants with C-terminal substitutions with amino acids other than proline in *E. coli* MKH13 cells. The osmolality of the external buffer (25 mM KP_i and 100 mM NaCl) was adjusted by the addition of KCl. (A) Regulation of betaine uptake as a function of the external osmolality for selected non-proline mutant strains. (B) Activation factors for all non-proline mutants in the C-terminal domain of BetP after an osmotic upshift from 0.2 to 0.8 osm/kg. Mutants marked with asterisks were taken from ref *10*.

be different from the pattern detected in the *E. coli* background. Whereas the observed loss of regulatory properties is highly similar for substitutions in the core region of the C-terminal domain, this is not the case for amino acid substitutions at the border of this region, e.g., in variants Y550A, E555A, K575P, and R576P. Intermediate cases were observed, too. The mutant E555A is not activated in *E. coli* but is regulated in *C. glutamicum*, whereas activation was not observed when an opposite charge was introduced (E555R). An interesting difference was found for the two proline replacements, K575P and R576P, which both fully lack activation in *E. coli* but are clearly stimulated when embedded in the *C. glutamicum* membrane, however, with a changed activation profile.

Definition of Conformationally Sensitive Regions within the C-Terminal Domain of BetP. The strong effect of proline substitutions within the C-terminal domain of BetP was correlated with the secondary structure prediction for this part of the protein (Figure 4C). Proline insertions within the region of the C-terminal domain which is obviously critical for osmoregulation and/or -sensing lead in fact to local disorder and a significant reduction of predicted α-helical structure around the position of replacement. This could not be shown for the region directly adjacent to the last transmembrane domain of BetP (positions 548−553), for which a β-sheet structure is predicted. The strong impact of proline substitutions in this region was independent of the

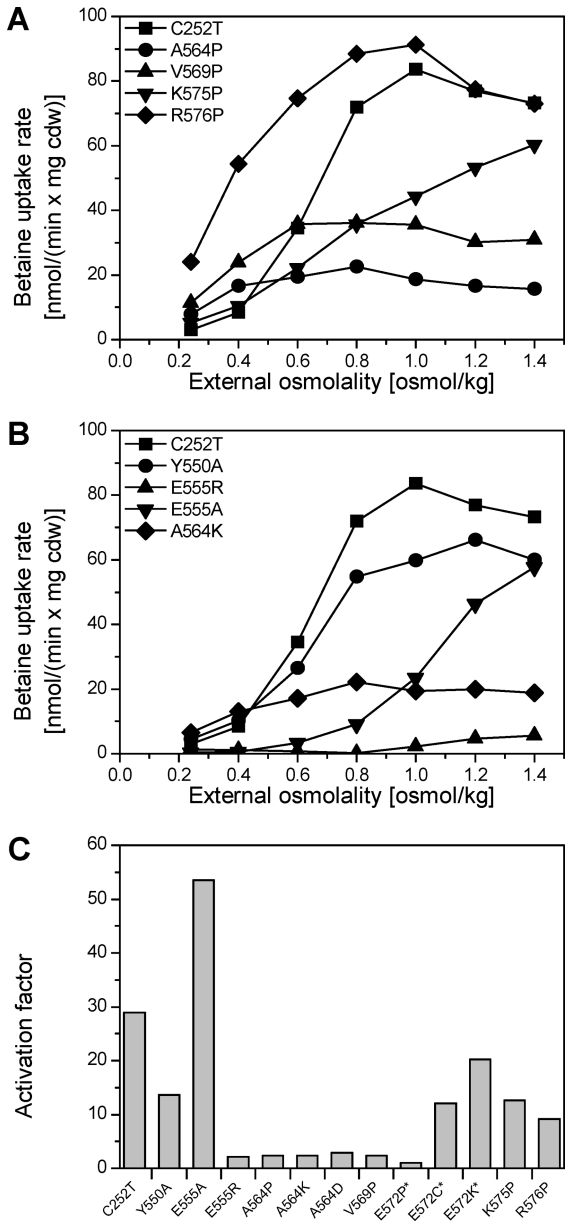

FIGURE 3: Activity regulation of BetP variants with C-terminal amino acid replacements in *C. glutamicum* DHPF cells. The osmolality of the external buffer (25 mM KP_i and 100 mM NaCl) was adjusted by the addition of KCl. (A) Regulation of betaine uptake as a function of the external osmolality for proline mutant strains. (B) Regulation of betaine uptake as a function of external osmolality for non-proline mutant strains. (C) Activation factors for all mutants in the C-terminal domain of BetP after an osmotic upshift from 0.2 to 1.0 osm/kg. Mutants marked with asterisks were taken from ref *10*.

lipid surrounding. Replacements with amino acids other than proline, which led to changes in the charge distribution but, according to structure prediction, do not have a major impact on the α-helical region, affect the regulatory properties of the C-terminal domain differently. They strongly influence the functional properties of BetP when located in the sensitive core region (positions 555−572), but their impact seems to be lipid-dependent in the border regions of the sensitive part of the domain.

Interaction of the C-Terminal Domain with Other Domains of BetP. We observed that the functional properties of the C-terminal domain of BetP during osmoresponsive activation critically depend on conformational constraints. This may be interpreted as a specific interaction of the domain with other parts of BetP. To study this question in detail, we applied two different experimental strategies.

In the first approach, we used peptide arrays, in which synthetic 15mer peptides, covering the whole sequence of BetP, were synthesized on cellulose membranes. Every individual peptide was shifted by three amino acids compared to the previous peptide on the array, leading to 195 peptides altogether (Table 3S of the Supporting Information). To this peptide array was added GST-coupled, heterologously synthesized C-terminal BetP peptide, and binding of this domain to the peptide array was monitored by antibody detection (Figure 5). In control experiments, it was proven that GST does not interact per se with the array peptides (not shown). Only binding patterns covering more than three consecutive spots (i.e., more than six to nine amino acids) and thus corresponding to a linear binding motif of a reasonable extent were regarded as significant. Interaction of the C-terminal domain peptide was observed with both the C-terminal domain itself and the N-terminal domain, as well as with some cytoplasmically oriented loops, in particular loops 4 and 8, and, to some extent, loop 2 (for loop location, see Figure 4A). Interestingly, a high K^+ concentration in the incubation buffer did not alter binding of the C-terminal domain (not shown). This might indicate that the observed interactions, although specific, are not directly related to the K^+ sensitivity of BetP and its terminal domain or to conformational changes related to interaction with K^+ ions.

With a second and independent strategy, we tested the interaction of the C-terminal domain with possible interacting domains by a functional approach. For this purpose, putatively interacting peptides were included during vesicle preparation into the interior of proteoliposomes harboring reconstituted BetP. It was shown previously that BetP is oriented in the proteoliposomes predominantly in a right-side-out direction (*9*); i.e., both the C-terminal domain and putative interacting domains of BetP should be accessible from the inside (Figure 6). If an interaction of the C-terminal domain with other parts of BetP at the cytoplasmic side would be functionally significant for osmotic activation, a possible competition upon addition of these interacting domains in excess should influence BetP activation. As a matter of fact, osmodependent activation was strongly affected in the presence of both the loop 8 peptide and the N-terminal domain. In comparison to that of the parental version of BetP, the activation profiles were not significantly changed upon addition of loop 4 and loop 10 peptides. The latter did not exhibit interaction in the peptide array either. As a critical control, we investigated the effect of a peptide with the same amino acid composition as loop 8, however with a randomized sequence. In contrast to the original loop 8 peptide, the randomized peptide had no significant influence on BetP activation.

Lipid Dependence of N-Terminally Truncated Mutants of BetP. In view of the results concerning the possible significance of the N-terminal segment as an interacting domain, we reconsidered its impact on BetP activation. In contrast to the C-terminal domain, a possible contribution of the N-terminal domain to regulation has been much less well studied. We found previously that various truncations of this domain led to a shift of the optimum of activation to higher values of osmotic stress; i.e., a decreased sensitivity

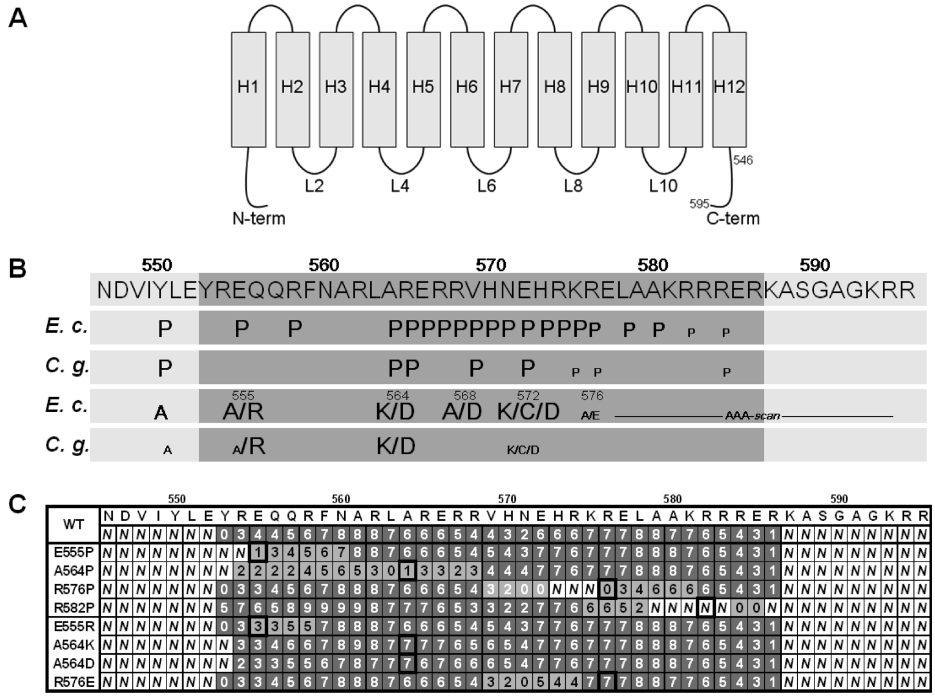

FIGURE 4: (A) Topological scheme of BetP, indicating internal loops and terminal domains. For the C-terminal domain, the flanking amino acid positions are indicated. (B) Impact of various mutations in the C-terminal region of BetP in different membrane surroundings on BetP function. The effects of various amino acid replacements are shown as four lines, separately proline and non-proline substitutions for both *E. coli* (*E. c.*) and *C. glutamicum* (*C. g.*). Large letters indicate a strong effect on BetP regulation (cf. Figures 2−5), whereas small letters represent a minor or no influence. Selected mutants (Y550P, all E572 mutants, R584P, and AAA scan) were taken from ref *10*. (C) Predicted influence of selected mutations on the secondary structure of the C-terminal domain. For secondary structure prediction, 3D-PSSM was used. Likelihood of a predicted α-helical structure in each position: 0 = low and 9 = high (with shaded background and white letters). Predictions of secondary structure elements other than α-helices (coiled or *β*-sheet structures) are indicated by *N*. Light gray boxes indicate decreased α-helix propensity resulting from the respective amino acid replacements. The positions of amino acid replacements are highlighted with bold boxes.

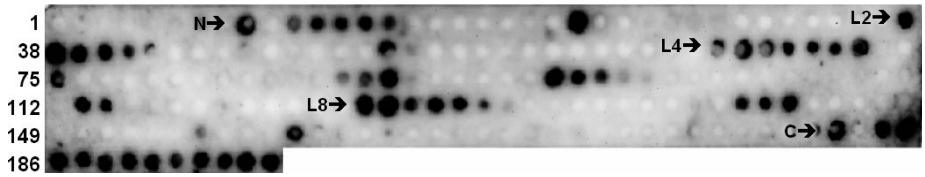

FIGURE 5: Interaction of the C-terminal domain of BetP with other parts of the BetP protein monitored by peptide array analysis. The cellulose membrane with the BetP peptide library (15mer oligopeptides with an offset of three amino acids from spot to spot synthesized in parallel) was incubated with the C-terminal BetP domain fused to GST. Interacting peptides were detected by GST-specific antibodies using chemiluminescence. The 195 peptides used are listed in Figure 2S of the Supporting Information.

to hyperosmotic stress was observed as well an a decrease in maximum velocity (*6*). We have now extended our analysis to the influence of the lipid surrounding on the contribution of the N-terminal region to regulation (Figure 7). Upon partial (C252T Δ29) and full deletion of the N-terminal domain (C252T Δ53), a decreased sensitivity of BetP, i.e., a shift of the optimum of osmotic activation to higher values of osmotic stress, was observed when the recombinant forms of *betP* were expressed in *C. glutamicum* (Figure 7B). This result is comparable to previous observations (*6*), which were, however, made with wild-type BetP instead of the C252T variant as the parental protein and using different *betP* expression systems.

Surprisingly, osmoregulation of these mutant forms was lost upon expression in *E. coli* (C252T Δ29), and the full N-terminal truncation of BetP (C252T Δ52) was not active in the heterologous host. It has to be taken into account, however, that the expression or, alternatively, the stability of these constructs was in general rather low (cf. Figure 1S of the Supporting Information), which in part may explain the observed low activity of the C252T Δ29 mutant, but not the complete lack of activity for the fully truncated mutant. The observation of a different impact of N-terminal truncations of BetP on its regulatory properties when expressed in the two different host organisms argues for the significance of the surrounding membrane for a regulatory contribution of the N-terminal domain of BetP. This result cannot be due to a direct interaction between the N-terminal domain and the lipid surface, since the experiments were carried out using the mutant in which the N-terminal domain is completely missing. Consequently, an indirect effect via another type of interaction, e.g., with the C-terminal domain, has to be taken into consideration.

Interaction of the C- and N-Terminal Domains with the Lipid Surface. Interaction of the regulatory, C-terminal domain of BetP with the surrounding membrane has been deduced from two kinds of experiments. Variation of the charge of the surrounding lipids had an impact on the sensitivity of BetP to osmotic stress, which led to a change in the set point of osmotic activation. This has been studied

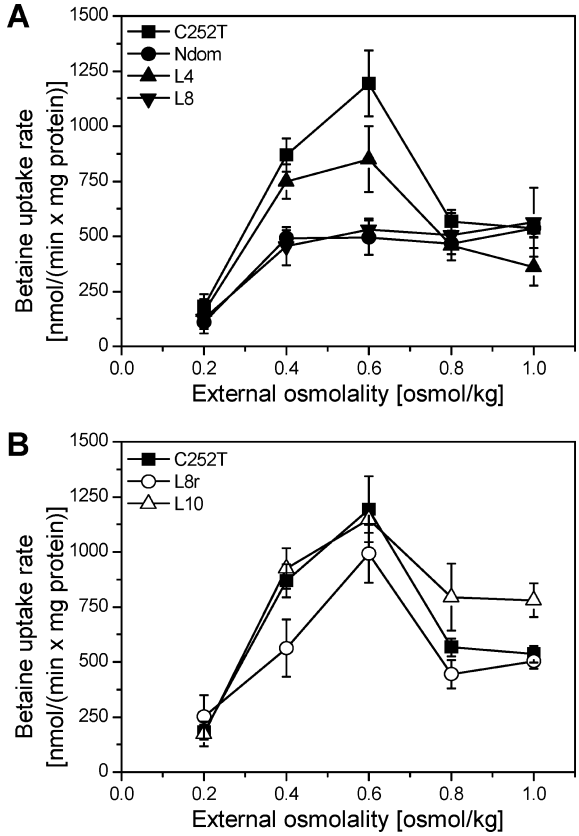

FIGURE 6: Functional competition by peptides with BetP C252T reconstituted in *E. coli* lipid proteoliposomes. Betaine uptake as a function of increasing external osmolality was measured. The osmolality of the external buffer [20 mM NaP_i (pH 7.5) and 25 mM NaCl] was adjusted via addition of proline. The proteoliposomes were loaded with various peptides corresponding to selected parts of BetP (see the text) (L4, loop 4 peptide; L8, loop 8 peptide; L8r, randomized loop 8 peptide; L10, loop 10 peptide; Ndom, N-terminal peptide; for sequences, see Table 1). An ~10-fold molar excess of peptide compared to the concentration of reconstituted BetP C252T when related to the internal liposome volume was used.

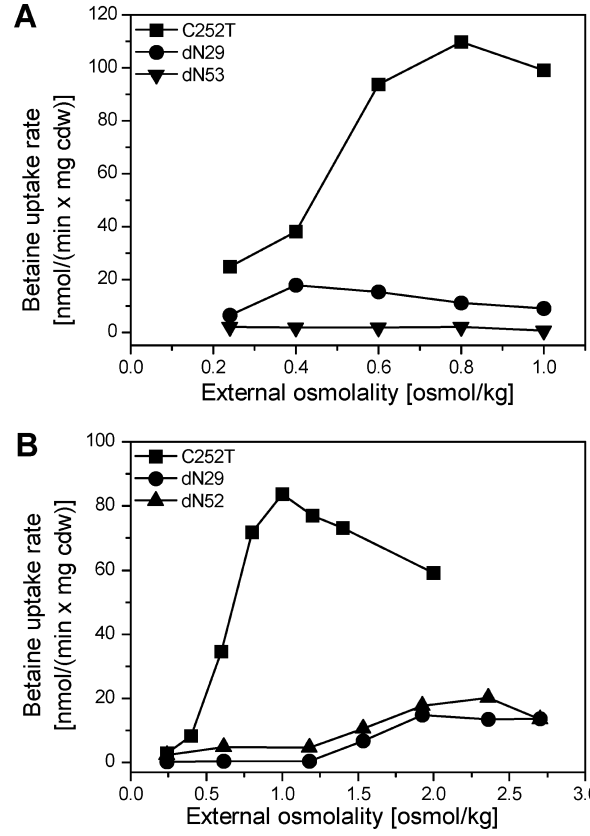

FIGURE 7: Impact of truncations of the N-terminal region of BetP on osmodependent regulation. Activity regulation of N-terminally truncated *betP* variants expressed in *E. coli* MKH13 (A) and *C. glutamicum* DHPF cells (B). The osmolality of the external buffer (25 mM KP_i and 100 mM NaCl) was adjusted by the addition of KCl (dN29 and dN53 BetP C252T truncated by 29 and 52 or 53 amino acids, respectively, at the N-terminal end).

in different lipid surroundings both in vitro (*10, 16*) and in vivo (ref *10* and this work). In view of the results presented in this work supporting the idea of multiple interactions of the C-terminal and possibly also the N-terminal domain with other partners, we have now directly assessed the interaction of the isolated terminal domains with lipid surfaces. For this purpose, we used surface plasmon resonance spectroscopy by applying bilayer surfaces of different compositions and assessed binding of the isolated C-terminal domain peptide. To mimic the impact of surface charge density on binding of the C-terminal domain, we varied the content of the anionic phosphatidylglycerol (DOPG) in the background of an equimolar mixture of the zwitterionic phospholipids phosphatidylcholine (DOPC) and phosphatidylethanolamine (DOPE). We measured an increasing binding affinity of the C-terminal domain peptide at an increasing content of negatively charged phospholipids (Figure 8). From these data, K_D values for binding were fitted by steady-state affinity analysis using BIA-Evaluation. This analysis led to the following values for K_D: 8.0 μM for 1:1 DOPE/DOPC, 5.2 μM for 1:1:1 DOPE/DOPC/DOPG, and 3.3 μM for 1:1:4 DOPE/DOPC/DOPG mixtures. In contrast to the C-terminal domain, the peptide covering the N-terminal part of the

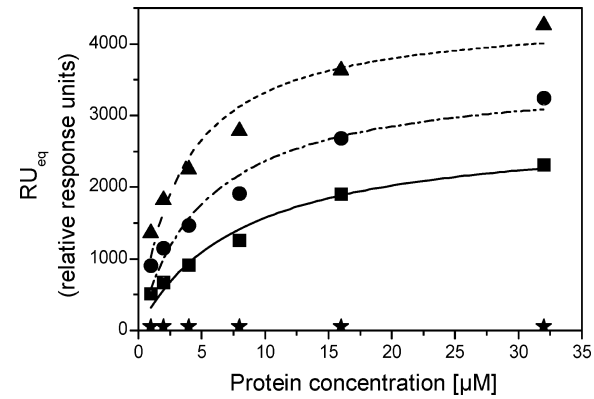

FIGURE 8: Interaction of the terminal domains of BetP with lipid surfaces monitored by surface plasmon resonance spectroscopy. RU values in the equilibrium of the interaction were used for steady-state affinity analysis with BIA-Evaluation. Buffer conditions included 50 mM Tris (pH 7.5) and 150 mM KCl. Interaction of the C-domain with the following lipid mixtures is shown: 1:1 DOPE/DOPC (■), 1:1:1 DOPE/DOPC/DOPG (●), and 1:1:4 DOPE/DOPC/DOPG (▲). The N-peptide did not interact with the tested lipid surfaces (★).

N-terminal domain did not bind to any of these surfaces to a measurable extent.

Consequently, we found a lipid-dependent change in the osmosensing properties and in the regulatory response of BetP variants truncated in the N-terminal domain but did not observe binding of this domain to lipid surfaces. An explanation for this result could be a possible interaction of

the N-terminal domain with its C-terminal counterpart, which was observed in the peptide array experiments. The latter has been proven to bind to lipid membranes; thus, a lipid dependence of the contribution of the N-terminal domain to BetP regulation may be mimicked. When we assessed binding of the C-terminal domain to lipid surfaces in the presence of an excess of the N-terminal domain, we in fact found a reduction in the level of binding of the C-terminal peptide to lipid bilayers. Addition of an excess of an unspecific peptide (loop 10 peptide) did not significantly reduce the level of binding of the C-terminal domain. This result again indicates a mutual interaction of the two terminal domains (data not shown).

DISCUSSION

BetP from *C. glutamicum* is an interesting object for studying intramolecular signal transduction in a protein that combines catalytic, regulatory and sensory properties. It is, besides ProP from *E. coli* (*20*) and OpuA from *L. lactis* (*21*), one of the model systems for this purpose (*9, 16*). The significance of terminal domains of these proteins, in particular the C-terminal domain of BetP, in sensing and regulation has previously been documented (*2*−*4, 6, 10, 15, 22*). The molecular mechanism of how regulatory domains in these model systems are functioning, however, is not known for any of these transporters. We are interested in elucidating how structural properties of the terminal domains of BetP can be correlated to the regulatory mechanism with which the activity of this secondary betaine transporter is controlled.

In a previous study using alanine scanning of the C-terminal domain of BetP, we identified a particular amino acid position (E572) that is involved in sensing and/or regulation of BetP. Interestingly, this property was influenced by the type of lipid surrounding in which BetP was embedded (*10*). This result was interpreted as indicating an interaction of the C-terminal domain with the membrane surface. We therefore carried out a detailed analysis of the functional significance of different regions within the complete C-terminal domain comprising approximately 50−55 amino acid residues.

It had been shown that the functional consequence of replacements at amino acid position 572 of BetP depended on the nature of the amino acid replacing the original glutamate, proline being the only amino acid which led to a loss of regulatory properties under all circumstances. Structure prediction of the C-terminal domain reveals a high probability of an α-helical structure throughout the largest part of the domain, excluding only the beginning (close to the last transmembrane segment) and the end (the terminal 15−20 amino acid residues) of this domain. In this work, we thus replaced a number of amino acids with proline at selected positions, focusing on the predicted α-helical region and on the direct neighborhood of residue E572. It turned out that the loss of sensory and/or regulatory properties after amino acid replacement with proline is not restricted to position 572 or to its close neighborhood but is effective throughout the major part of the predicted helical structure except the C-terminal part. The strong effect of proline substitutions agrees well with their predicted effect on helix stability. It is interesting to note that a weakening of the local helix probability holds true also for amino acid replacements at positions higher than 575, where only a weak effect on regulatory properties was observed. This argues for the part of the C-terminal domain comprising amino acids 576−595 not being critical for sensing and/or regulation.

When amino acids other than proline were used, the position of the replacement became more important. Amino acid substitutions at the border of the α-helical region had a weaker influence, even if a change in charge was involved. When these mutants were analyzed in *C. glutamicum* membranes bearing a high extent of negative surface charges, the part of the C-terminal domain in which a replacement led to a loss of regulatory properties was more restricted as compared to *E. coli*. This held true with respect to both sides of the predicted helical region, and for both proline and non-proline replacements. This result indicates that a high negative surface charge of the surrounding membrane stabilizes the functionally active conformation of the C-terminal domain of BetP.

Taken together, the core part of the C-terminal domain, i.e., between amino acid positions 555 and 572, is highly sensitive to amino acid replacement in any membrane surrounding. It is thus assumed that this core part needs to be in a strictly defined conformation presumably optimized for functional interaction with other domains of BetP, e.g., cytoplasmic loops. This region is actually not located around the previously identified amino acid position 572. On the contrary, glutamate 572 turned out to be at the border of this region, which also explains why the impact on BetP regulation of replacement of E572 with different amino acids depended on the lipid surrounding. By accident, the C-terminal boundary of this critical region is exactly the location where truncations of the C-terminal domain were engineered by which the participation of this domain in the sensing and/or regulation mechanism was identified (*6*).

Effects of lipid on osmodependent regulation have been observed in other model systems, too. Besides BetP, ProP of *E. coli* and OpuA of *L. lactis* respond to an increased fraction of negatively charged phospholipids with an increased extent of osmotic stress necessary for carrier activation (*23, 24*). In the case of OpuA, the response to hyperosmotic stress was altered when part of the C-terminal CBS domain was truncated (*25*). The C-terminal domain of ProP, on the other hand, seems to interact specifically with cardiolipin in the membrane, which affects the cellular localization of the transporter (*26*).

Regulation of activity by a C-terminal motif with a defined conformation was also observed for ProP in *E. coli.* In this case, the terminal domain was shown to adopt an antiparallel coiled-coil structure leading to dimerization of the ProP protein (*27*−*29*). Although BetP was shown to oligomerize, too, the trimeric form of BetP as observed in 2D crystals cannot be caused by C-terminal interactions, since a mutant form lacking 45 amino acids from the C-terminal domain was used for 2D crystallization of the trimer (*30*).

In this work, we provide the first experimental evidence of a possible regulatory interaction between the terminal domains of BetP and other parts of the transporter. Previous findings already indicated a contribution of the N-terminal domain to BetP regulation (*6*). Here we show not only that truncations of the N-terminal domain lead to a shift in sensitivity toward osmotic stress but also that the surrounding

lipids affect the impact of N-terminal truncations on the regulatory properties of BetP. Since this was also observed in mutants in which the N-terminal domain was fully removed, this result was interpreted to be indirect in nature. Consequently, our results may indicate that the regulatory influence of the N-terminal domain is not based on direct peptide−membrane interaction, but rather on protein−protein interaction with the opposing C-terminal domain, which in turn may interact directly with the membrane. Obviously, further studies using specific amino acid replacements within the N-terminal domain of BetP have to be carried out to elucidate the significance of this domain and its putative interaction with the C-terminal domain for BetP regulation.

This putative domain interaction was corroborated by two independent techniques. We used an in vitro assay monitoring binding of the C-terminal domain to a peptide array derived from the total BetP sequence. The results of this experiment not only indicate a mutual interaction of the two terminal domains of BetP but also hint at a possible interaction of C-terminal domains from different BetP monomers within the functional trimer. The latter results thus argue for a particular geometric organization of the three terminal domains within the BetP trimer. This indicates a functional cross talk between individual BetP monomers via their C-terminal domains; however, the correct interpretation of this finding will become clear only when more direct structural information about BetP is available. In particular, these results also provide for the first time experimental evidence of a specific interaction of the regulatory C-terminal domain with cytoplasmic loops of BetP. This observation cannot be due to unspecific interaction, since (i) external loops as well as transmembrane segments of BetP were not recognized and (ii) not all cytoplasmic loops were recognized either.

This result was further investigated by applying a direct competition assay, in which domains putatively interacting with the C-terminal domain were added to the internal space of proteoliposomes carrying reconstituted BetP in a functionally active state. The osmodependent activation of reconstituted BetP was found to be strongly affected when either a peptide identical to the terminal part of the N-terminal domain or a loop 8 peptide was added. A peptide derived from loop 4 had only a minor effect or no effect at all; consequently, loop 4 does not seem to have a major impact on regulation. The specificity of the competition assay was proven by two results. First, loop 10, which had proven not to interact in the peptide array experiment, also did not affect BetP activation, and second, a loop 8 peptide with randomized sequence failed to inhibit in contrast to the original loop 8 peptide.

The results of this work seem to be important for a mechanistic understanding of the osmodependent activation of BetP. First, a conformationally critical region of the C-terminal domain was identified that is pivotal for the correct function of this domain for BetP activation. Second, the dependence of activation on the surrounding membrane was confirmed at a more detailed level. Moreover, at least two new domains of BetP interacting with the C-terminal domain have been identified, the N-terminal domain, and in particular its N-terminal region, as well as loop 8 of the membrane body of BetP. Since BetP is a functional trimer (*7*), our results do not discriminate between the interaction

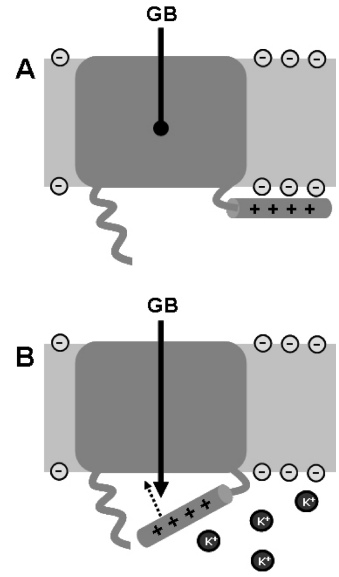

FIGURE 9: Functional model of BetP switching between the active and inactive state as a function of the location of the C-terminal domain and its interaction partners.

of domains of the same or of different subunits within the BetP oligomer. Furthermore, the question of whether interaction signals with loop 4 (and possibly loop 2), as observed in the peptide array experiment, may in fact have functional significance must be resolved in future experiments.

It is of particular interest that loop 8 seems to be the most important interaction partner of the C-terminal domain. BetP belongs to the BCCT family of transporters, and these carriers are characterized by a conserved motif stretching from the eighth transmembrane segment into exactly cytoplasmic loop 8. This conserved motif, the loop part of which contains a cluster of arginine residues, was, although without experimental evidence, suggested to be involved in binding of the positively charged substrates of this type of transporter (*31*).

These results now provide the basis for a more elaborate model for the activation process of BetP. Since an increased level of binding of the C-terminal domain to the membrane surface in the presence of negatively charged lipids makes BetP activation increasingly difficult, the membrane-bound state of the C-terminal domain can be regarded as the inactive state. The interaction with the membrane surface, obviously an important step in the process of BetP (in)activation, critically depends on the charge composition of the bilayer membrane. This concept is in line with previous observations of a shift in the activation optimum to higher values of osmolality with an increase in membrane surface charge density (*15*). Since a competitive inhibition of the interaction of the C-terminal domain with other putative interacting domains (loop 8, N-terminal domain) led to inhibition of BetP activation, the protein-bound or protein-interacting state of the C-terminal domain is suggested to be the active state of BetP. These results can be combined in a putative model describing the functional switch of BetP between the active and inactive state depending on the location of the C-terminal domain (Figure 9). It is obvious that this model is not able to describe all aspects of BetP activation, since it also has been shown that the composition of the hydrophobic part of the surrounding membrane and/or its physical state seems

to influence the activity state of BetP (*8, 32*). Molecular switches depending on electrostatic interactions have been proposed also for other proteins related to osmosensing and regulation, and a basic model has been derived on the basis of this idea (*33*). This holds not only for OpuA and ProP, as already mentioned above (*2*), but also for the sensor kinase KdpD of *E. coli* (*34, 35*).

ACKNOWLEDGMENT

We thank Dr. C. Ziegler for providing N-terminally truncated mutants of BetP (see Table 1S).

SUPPORTING INFORMATION AVAILABLE

Plasmids used in this study (Table 1S), oligonucleotides used for site-directed mutagenesis (Table 2S), peptides synthesized on the peptide array described in Figure 5 (Table 3S), and the expression level of the constructs used in this study (Figure 1S). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801325R