The major uptake carrier for the compatible solute glycine betaine in *Corynebacterium glutamicum* is the secondary transport system BetP. It is effectively regulated by the external osmolality both on the level of expression and of activity. BetP carries highly charged domains both at the N- and at the C terminus. We investigated the role of these extensions in the regulatory response to hyperosmotic stress. Mutants of the betP gene coding for proteins with truncated N- and C-terminal extensions were expressed in the *C. glutamicum* betP deletion strain DHP1 and were functionally characterized with respect to regulation of activity. The optimum of activation at 1.3 osmol/kg in wild type was shifted in the recombinant strains to about 2.6 osmol/kg in mutants with deletions in the N-terminal part. Deletions in the C-terminal domain resulted in a complete loss of regulation. The altered response to changes in osmolality led to severe consequences in the cellular adaption to hyperosmotic stress. Whereas in the wild type, the steady state level of glycine betaine accumulation is maintained by activity regulation of the BetP system itself, in the mutant with BetP proteins carrying truncations in the C-terminal domain, the observed steady state betaine accumulation was found to be due to a kinetic balance of unregulated glycine betaine uptake by the modified BetP and efflux via the mechanosensitive efflux channel for compatible solutes at the same time.

Bacterial membranes are readily permeable to water but constitute an effective barrier to most other solutes. An increase in the external osmolality thus leads to instant efflux of water and to a decrease in cell turgor. To avoid dehydration of the cytoplasm, bacteria have developed adaptation mechanisms to hyperosmotic stress, both long term responses, manifested during growth at high osmolality, and short term responses, which occur instantly after changes in the external osmolality (1–3). A general defense mechanism against high osmolality is the uptake and accumulation of compatible solutes like glycine betaine, proline, or ectoine. Unlike other solutes, these substances do not interfere with vital cellular functions when present at high concentration. The uptake of compatible solutes is mediated by transport systems that are regulated in dependent of the change in external osmolality.

Mechanisms and systems of osmo-adaption have been intensively studied in the Gram-negative *Escherichia coli* and *Salmonella typhimurium*. Two uptake systems for compatible solutes have been identified in these organisms, namely the binding protein-dependent transporter ProU and the secondary carrier ProP. Whereas ProU is regulated both on the level of transcription and activity (4, 5), ProP is mainly regulated on the activity level (6–8). Another well studied organism in this respect is the Gram-positive *Bacillus subtilis*. In this organism, glycine betaine is taken up via three osmo-regulated transport systems, belonging either to the ABC-type or to the class of secondary carriers (9–11). Regulation of these transport systems in various organisms was studied in detail on the genetic level; however, less information is available concerning their regulation on the level of activity (12).

*Corynebacterium glutamicum* carries several osmo-responsive uptake systems for compatible solutes. The specific, high affinity glycine betaine transporter BetP was cloned and sequenced (13). Its function and its response to osmotic changes has been analyzed in detail (14). In addition, further osmo-regulated uptake systems for compatible solutes have been identified (15). As for other bacteria, however, nothing is known so far about the mechanism of osmo-sensing and regulation on the activity level that triggers the immediate response of the carrier protein to osmotic stress.

In the present communication we report molecular studies on the mechanism of osmo-sensing by the glycine betaine uptake system BetP of *C. glutamicum*. It has been shown that BetP is effectively regulated in dependence of external osmolality. Whereas the carrier is completely inactive under isosmotic conditions, its activity increases on hyperosmotic stress in a time range of seconds or less (14). A similar regulation pattern, although shifted in its optimum, was observed after expression of betP in *E. coli* (13). We thus concluded that BetP functions not only as an osmo-regulated carrier but also as a sensor of osmotic stress. The secondary structure of BetP was predicted to carry two extensions at the cytoplasmic face, a negatively charged N-terminal and a positively charged C-terminal extension (13). The function of these domains in sensing osmotic stress was studied by construction of deletion mutants and functional characterization of the gene products.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used are described in Table I. *E. coli* strain DH5α was grown at 37 °C in Luria Bertani (LB) medium (16). *C. glutamicum* was grown in brain heart infusion medium (BHI1; Difco, Detroit, MI.) at 30 °C. General DNA Techniques—Genomic DNA was isolated from *C. glutamicum* according to Eikmanns et al. (17). Plasmid DNA was isolated using the QIAGEN plasmid kit (QIAGEN, Hilden, FRG). *E. coli* was transformed using standard methods (18). To make *C. glutamicum*
Osmo-sensing in C. glutamicum

TABLE I
Strains and plasmids used in this study

| Strains/plasmids | Relevant genotype or characteristics | Source/reference |
|------------------|--------------------------------------|------------------|
| **Strains**      |                                      |                  |
| C. glutamicum    | wild type                            | (30)             |
| ATCC 13032       | betP deletion strain                 | (13)             |
| DHP1             |                                      |                  |
| E. coli          | mcrA, Δ(mrr-hsdRMS-mcrBC)            | (31)             |
| DH5s mcr         |                                      |                  |
| **Plasmids**     |                                      |                  |
| pUC18            | ColE1 ori                            | (32)             |
| pEKEEX2          | lacP, ptac                           | (17)             |
| pH2              | pUC18 containing the betP gene       | (13)             |
| pGT7             | pEKEEX2, betP (PCR) 1796 bp          | this study       |
| pN0              | pEKEEX2, betP (PCR) 1697 bp          | this study       |
| pN1              | pEKEEX2, betP (PCR) 1692 bp          | this study       |
| pN2              | pEKEEX2, betP (PCR) 1727 bp          | this study       |
| pN3              | pEKEEX2, betP (PCR) 1641 bp          | this study       |
| pC0              | pEKEEX2, betP (PCR) 1701 bp          | this study       |
| pC1              | pEKEEX2, betP (PCR) 1722 bp          | this study       |
| pC2              | pEKEEX2, betP (PCR) 1761 bp          | this study       |
| pC3              | pEKEEX2, betP (PCR) 1453 bp          | this study       |
| pNO0             | pEKEEX2, betP (PCR) 1515 bp          | this study       |
| pNO0C            | pEKEEX2, betP (PCR) 1536 bp          | this study       |
| pNO0C2           |                                      |                  |

TABLE II
Primers and annealing temperatures for constructing deletion mutants of betP

| Clone  | Primer 1 | Primer 2 | Annealing temperature |
|--------|----------|----------|-----------------------|
| GTG    | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-CTATCGAGCTTCCCCCGG-3′ | 64 °C |
| N0     | 5′-ATGTAGCAGTCATGGTCCCCGG-3′  | 5′-CTATCGAGCTTCCCCCGG-3′  | 64 °C |
| N1     | 5′-ATGAGGCGGAGACACCGCAG-3′  | 5′-CTATCGAGCTTCCCCCGG-3′  | 64 °C |
| N2     | 5′-ATGAGGCGGAGACACCGCAG-3′  | 5′-CTATCGAGCTTCCCCCGG-3′  | 64 °C |
| N3     | 5′-ATGAGGCGGAGACACCGCAG-3′  | 5′-CTATCGAGCTTCCCCCGG-3′  | 64 °C |
| C0     | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 62 °C |
| C1     | 5′-AAGGCGGCGGTTAGAACCGG-3′  | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 62 °C |
| C2     | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 60 °C |
| C3     | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 60 °C |
| C0N0   | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 60 °C |
| C1N0   | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 60 °C |
| C2N0   | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 5′-GTGAGTTTTATGACTACATCTGAC-3′ | 60 °C |

COMPETENT, STRAINS WERE GROWN IN LB IN THE PRESENCE OF 0.4% ISOCITRIC ACID HYDRAZIDE, 2.5% GLYCINE, AND 0.1% TWEEN 80 (19). CELLS WERE WASHED FOUR TIMES WITH 10% ISOCITRIC ACID. }

The regulation pattern was identical in the wild type strain ATCC 13032 independent of either NaCl or sorbitol (14). For a more general analysis with respect to the question of whether BetP is regulated by the external osmolality and/or by the ionic strength, we compared betaine uptake of C. glutamicum wild type strain ATCC 13032 independent of either NaCl or sorbitol for 3 min at 30 °C. For determination of the Na+ affinity, cells were washed with 50 mM morpholinepropanesulfonic acid (MOPS)-KOH buffer (pH 7.0), resuspended, and measured in the same buffer containing 10 mM glucose. The osmolality in the uptake assay was adjusted to 600 m osm/kg by the addition of solutes, e.g., NaCl or sorbitol for 3 min at 30 °C. To determine the level of activity by the external NaCl concentration (14).
Osmo-sensing in C. glutamicum

Glycine betaine uptake activity was measured independent of the external osmolality (A) and of added tetracaine (B). C. glutamicum cells were grown overnight in BH complex medium. Uptake was started by the addition of 100 μl labeled glycine betaine. A, the osmolality of the uptake assay was increased either by the addition of NaCl (solid circles) or by the addition of sorbitol (open circles) in the presence of 50 mM NaCl. The value for uptake at 1,350 mosmol/kg external osmolality was set to 100%, and the absolute values at 1,350 mosmol/kg were 105 nmol min⁻¹mg of dry weight⁻¹ for NaCl and 38 nmol min⁻¹mg of dry weight⁻¹ for sorbitol stimulation. As a control, proline uptake by the PutP system of C. glutamicum independent of external NaCl is shown (stars). The absolute value at 1,380 mosmol/kg was 9.6 nmol min⁻¹mg of dry weight⁻¹. B, glycine betaine uptake of C. glutamicum wild type (solid symbols) and C. glutamicum DHP1 (ΔbetP) (open symbols) was measured. The osmolality of the uptake assay was adjusted with NaCl to a final value of 400 mosmol/kg (circles), 600 mosmol/kg (squares), or 1,000 mosmol/kg (triangles).

For a more fundamental approach, we investigated whether the change in the physical state of the membrane (mechanostress) is the direct signal for carrier activation. We applied a method known from biophysical studies on membranes, i.e., the addition of local anesthetics, to influence the membrane tension in a defined manner (21–25). For this purpose, the influence of tetracaine on betaine uptake in the wild type strain C. glutamicum ATCC 13032 as well as in the betP deletion strain DHP1 was determined (Fig. 1B). The osmolality was adjusted with NaCl to a final value of 400, 650, or 1,000 mosmol/kg, respectively, and tetracaine was varied from 0 to 1.25 mM. Whereas at 400 mosmol/kg the uptake activity of the wild type reached its optimum between 0.5 and 0.75 mM added tetracaine, no increase in activity was observed in strain DHP1. At higher osmolalities (650 and 1,000 mosmol/kg) the tetracaine-induced increase in uptake activity of the wild type was less pronounced and reached optimal values between 0.75 and 1 mM tetracaine. The results of Fig. 1B demonstrate that BetP can be stimulated by the addition of tetracaine alone and this stimulation is related to the activation of the carrier protein by hyperosmotic stress.

Construction of N- and C-terminal Deletion Mutants of BetP—From the nucleotide sequence of the betP gene, a protein of 595 amino acids was derived that shares a high degree of identity to two other osmotically regulated bacterial carriers, OpuD of B. subtilis and BetT of E. coli (9, 26). The secondary structure prediction (PHDhtmtop program; EMBL, Heidelberg, FRG) of these three proteins revealed a common structure of 12 transmembrane segments as well as a common hydrophilic C-terminal extension. In contrast to the other two carriers, BetP possesses an additional hydrophilic N-terminal domain, which is longer than the C-terminal one. Interestingly, the two hydrophilic domains, which are predicted to face the cytoplasm, are both highly charged; however, with different polarity. Whereas 15 negatively charged and only two positively charged residues are located within the 62 amino acids of the N-terminal part, 21 positively charged and 8 negatively charged amino acid residues are located within the 55 amino acid residues of the C-terminal domain (Fig. 2). We investigated the role of these two extensions in osm-sensing and regulation by constructing mutant genes of betP coding for proteins with deletions in one or both extensions. The two domains were truncated stepwise, resulting in four mutants of each domain. In the case of a full deletion of the extensions (C0 and N0) only two and three amino acid residues, respectively, were left in front of the first putative transmembrane segment or at the end of the last segment. In addition, three double mutants lacking the N-terminal and part or all of the C-terminal domain were constructed (C0N0, C1N0, C2N0) (Fig. 2).

Kinetic Discrimination of BetP-mediated Glycine Betaine Uptake of C. glutamicum DHP1—The C. glutamicum betP deletion mutant DHP1 showed a reduced betaine uptake rate of about 10% that of the wild type, which is due to at least one additional osmo-regulated uptake system (15). In contrast to specific uptake via BetP, betaine uptake by the additional transport system(s) was competitively inhibited by ectoine or proline. To discriminate the activity of the mutant BetP proteins in C. glutamicum DHP1, we tested betaine uptake of strain DHP1/pGTG carrying the wild type betP gene and of strain DHP1/pEKEKX2 (vector without insert) in the absence and presence of ectoine (Fig. 3). Betaine uptake of the betP deletion strain DHP1/pEKEKX2 was completely abolished by the addition of ectoine in 100-fold excess. In contrast, strain DHP1/pGTG showed high betaine uptake in the presence of ectoine, the activity of which depended on the external osmolality exactly as in C. glutamicum wild type. Thus, the betaine uptake observed in the presence of a 100-fold excess of ectoine represents the activity of BetP only. These conditions were used in all further measurements.

Glycine Betaine Uptake Activity of betP Mutants in C. glutamicum Strain DHP1—Fig. 4 shows betaine uptake of the various plasmid-encoded BetP mutant carrier proteins in the presence of unlabeled ectoine in comparison to strain DHP1/pGTG. All BetP proteins with deletions in the N-terminal extension were active; however, the regulation pattern was altered. Whereas the wild type reached its maximum activity at
600 mM NaCl (corresponding to 1.35 osmol/kg), the optimal activation of the proteins with N-terminal deletions was shifted to higher values. All mutant proteins behaved similarly and reached their maximum of activity at osmolalities 2-fold higher than that of the wild type. Fig. 4 summarizes the uptake rates of proteins with truncated C-terminal part. Strain DHP1/pC0 completely lacking the extension was not active at all. The activity of all other C-terminal deletion mutants increased with increasing NaCl, similar to the wild type. However, the optimum activity was shifted to lower NaCl concentrations of about 200 mM. Furthermore, uptake activity of strains DHP1/pC1 and DHP1/pC2 did not decrease as strongly as the wild type activity at higher NaCl concentrations but remained nearly constant between 200 and 1,500 mM NaCl. For further analysis, double mutants were constructed with deletions in both, the C- and the N-terminal domains. As expected, strain DHP1/pC0N0, i.e. the mutant protein with deletion of both extensions, was not active, as was strain DHP1/pC0 (Fig. 4C). The double mutants DHP1/pC1N0 and DHP1/pC2N0 were active in transport and behaved similar to the corresponding C-terminal deletion mutants DHP1/pC1 and DHP1/pC2.

Na⁺ Dependence of Glycine Betaine Uptake of the betP Mutants—Glycine betaine uptake via the BetP system is coupled to symport of two Na⁺ ions (14). As a consequence, Na⁺ acts on betaine transport both as cosubstrate and as osmo-stimulants. To discriminate between the two effects, we measured betaine uptake at a constant NaCl concentration of 50 mM and varied the external osmolality with sorbitol. 50 mM NaCl does not stimulate uptake in the wild type and is not sufficient to fully stimulate uptake in the respective BetP mutants. Fig. 5 shows the uptake activities of strains DHP1/pC1, DHP1/pC2, and DHP1/pC1N0 in comparison to strain DHP1/pGTG. Whereas the wild type carrier was stimulated by sorbitol similar as observed for NaCl, the mutant strains did not respond to the change in osmolality. High sorbitol concentrations led to a decrease of glycine betaine uptake in strains DHP1/pC1 and DHP1/pC1N0, whereas uptake of strain DHP1/pC2 remained constant. Consequently, the data of Fig. 4B must be re-interpreted. Stimulation of uptake activity of the proteins C1 and C2 at low NaCl concentrations between 10 and 200 mM must in fact be caused by the increasing concentration of Na⁺ ions but not by the increase in osmolality.

It could be argued that the initial stimulation by 50 mM NaCl is not due to the availability of Na⁺ ions specifically but to the change in ionic strength. We thus measured uptake activity of the C-terminal mutants at increasing NaCl concentration (10–150 mM) and adjusted the ionic strength by KCl to a constant salt concentration of 1,200 mM. Whereas the wild type and the C3 mutant reached their full activity below 50 mM NaCl, the activity of the C1 mutant increased up to 100 mM NaCl (data not shown). These results indicate that neither the increase in
osmolality nor in ionic strength is responsible for uptake stimulation in these mutants. Consequently, the Na\textsuperscript{+} affinity of the C1 mutant must be altered as compared with the wild type. To determine the Na\textsuperscript{+} affinity of the mutants we adjusted the osmolality of the assays by sorbitol to a constant value of 600 mosmol/kg and varied NaCl in the range of 1–150 mM (Fig. 6). The Na\textsuperscript{+} dependence of strain DHP1/pC3 did not differ significantly from the wild type, whereas the affinity toward Na\textsuperscript{+} was decreased in strains DHP1/pC1 and DHP1/pC2, corroborating the results of Fig. 5. The dependence of uptake activity on Na\textsuperscript{+} was found to be sigmoidal in shape. However, when the results of all four strains are analyzed according to the Hill equation, irrespective of the significantly changed affinity for Na\textsuperscript{+}, the best fit for the value of (Hill)\textsuperscript{n} actually lies between 2.2 and 2.35 in all four cases (plot not shown).

For a further characterization of the mutant proteins, the \(K_m\) values for glycine betaine were determined also (Table III). Except for strains DHP1/pC1 and DHP1/pC1N0, \(K_m\) was not altered as compared with the wild type. Strains DHP1/pC1 and DHP1/pC1N0 showed a 6- to 10-fold lowered substrate affinity; however, the uptake rate of these strains was quite low, thereby rendering the discrimination against additional betaine uptake systems by use of the competitor ectoine problematic. The \(V_{\text{max}}\) values of uptake of the other strains varied from 35 to 61 nmol min\(^{-1}\) (mg of dry weight\(^{-1}\)). Despite various attempts, we did not succeed in obtaining a specific antiserum against BetP. Thus, we could not decide whether the differences in the maximum rates resulted from a different degree of expression or whether they were caused by the mutations directly. Consequently, in contrast to the \(K_m\) values, the observed maximum rates are not a meaningful characterization of the different mutant strains.

Regulation of BetP Mutant Proteins under Conditions of

FIG. 4. Glycine betaine uptake in various mutant strains independent of the external NaCl concentration. Glycine betaine uptake was measured in strain DHP1/pGTG (wild type) (solid symbols) and in strains of DHP1 carrying different mutated betP alleles on a plasmid (open symbols). Cells were grown overnight in BHI complex medium containing 0.2 mM IPTG and 20 mg/liter kanamycin. Uptake was started by the addition of 100 \(\mu\)M labeled glycine betaine and 10 mM unlabeled ectoine. Maximum uptake at optimum conditions of external osmolality was set to 100% as explained in the legend to Fig. 1 in all cases. For absolute values, see data in Table III. A, uptake activity of strains DHP1/pN0 (squares), DHP1/pN1 (rhomboids), DHP1/pN2 (circles), and DHP1/pN3 (triangles). B, uptake activity of strains DHP1/pC0 (dotted line, hexagons), DHP1/pC1, DHP1/pC2, and DHP1/pC3 (broken line, stars, triangles, and squares, respectively). C, uptake activity of strains DHP1/pC0N0 (dotted line, hexagons), DHP1/pC1N0, and DHP1/pC2N0 (broken lines, squares, and circles, respectively).
Kinetic Equilibrium—So far we have used initial uptake rates to characterize the various mutant carrier proteins. To test whether the regulation under steady state conditions is changed in the mutant proteins, betaine uptake was analyzed under equilibrium conditions by measuring the internal [14C]glycine betaine for 2.5 h after a hyperosmotic shift in strain DHP1/pC2 as compared with the wild type (Fig. 7, A and B). Both strains accumulated betaine until a steady state was reached at about 1 h, whereafter internal betaine remained virtually constant (130 mM for DHP1/pGTG and 210 mM for DHP1/pC2). The observed steady state can be explained by at least three different models. (i) The activity of BetP is switched off when the internal osmolality matches that of the medium, (ii) BetP remains active, and the observed constant concentration of betaine is the sum of uptake and efflux via BetP, (iii) BetP remains active, and the observed constant internal label is the sum of the uptake activity of BetP and the efflux via another system.

Recently we have shown that *C. glutamicum* is able to release compatible solutes via stretch-activated (mechanosensitive) channels which are tightly regulated under hyposmotic conditions (outside low) (27). We did not find any indication for a betaine efflux carrier, as reported for *Lactobacillus plantarum* (28). To discriminate between the three different possibilities, we added unlabeled betaine in 33-fold excess to cells 1.5 h after the addition of labeled betaine, i.e. after the steady state of betaine accumulation had been reached (Fig. 7). The addition of 50 mM unlabeled substrate did not lead to a major decrease of internal label in strain DHP1/pGTG. The low efflux of glycine betaine thus indicates that both the reverse reaction of BetP and the activity of a putative efflux channel must be very low, i.e. model (i) held true in this case. In contrast, the addition of unlabeled betaine to strain DHP1/pC2 resulted in a rapid decrease of internal label. Consequently, the observed steady state must be the sum of both uptake and efflux, i.e. models (ii) or (iii) are valid. Thus, either the mutant BetP is functionally changed, being now active in a (futile) exchange under iso-osmotic conditions, or the mutant protein catalyzes uptake of unlabeled substrate, and the label is released from the cytosol via the efflux channel. For a discrimination, unlabeled substrate was added after 5 min, i.e. when betaine was still taken up at the maximum rate. As expected, the internal label remained constant in strain DHP1/pGTG, indicating again that no efflux occurred (Fig. 7A). The uptake kinetics seems to stops at the point of addition because of the low specific label of external betaine after the addition of unlabeled substrate. In strain DHP1/pC2 no efflux of label occurred within the first 30 min.
min after addition of unlabeled substrate (Fig. 7B). Interestingly, the onset of the efflux occurred at the same time, when the cells without betaine addition were approaching the steady state. On the basis of these results models (ii) and (iii) can be discriminated. Since no initial efflux was observed after the addition of unlabeled betaine to cells that carry the mutant protein and were actively taking up betaine, the mutant carrier also does not catalyze counter-exchange under these conditions, identical to the wild type protein. Consequently, the rapid decay of internal label on the addition of unlabeled substrate after 1.5 h is not due to exchange activity of the modified BetP but represents the sum of uptake activity of BetP and efflux activity via the channel.

**DISCUSSION**

Osmotic stress is a major challenge in the habitat of a soil bacterium like *C. glutamicum*. Consequently, this organism is not only equipped with a number of transport systems responding to osmotic stress, but these systems are also efficiently regulated, both on the level of expression and, most important for an instant stress response, on the activity level. We have previously described that the glycine betaine carrier BetP, the major osmo-reactive uptake system in *C. glutamicum*, effectively responds to osmotic changes within a time range of seconds or less (14). We were interested in the mechanism of how the BetP protein senses the osmotic stress. The data presented here indicate that both the negatively charged N- and the positively charged C-terminal extension of BetP are involved in sensing and/or transducing osmotic changes to the domain of the protein responsible for the translocation of the substrate glycine betaine.

The usual way to exert osmotic stress to a bacterial cell is the addition of salts, e.g. NaCl. In the case of BetP being a Na+-coupled secondary system (14), the addition of NaCl changes not only the osmolality but also the ionic strength as well as the concentration of the coupling ion. To discriminate between these mechanisms, we extended our previous studies of NaCl-dependent regulation of BetP to the uncharged solute sorbitol and to the membrane-active amphipathic local anesthetic tetracaine. Tetracaine is known to change the physical state of the membrane (21–24), which is the structural basis of osmo-stress (mechanostress) on the level of membrane structure. In a similar study on osmo-sensing by the KdpD protein, a sensor kinase of the *E. coli* Kdp system, local anesthetics were found to modulate the activity of the membrane-inserted protein (25).

Both the addition of sorbitol and of local anesthetics led to an activation of the BetP protein, similar to that evoked by the addition of NaCl. This indicates that in fact membrane/protein interaction is the basis of the transduction of osmo-stress to the carrier protein. Interestingly, additional transport systems involved in osmo-regulation in *C. glutamicum* do not seem to respond to addition of tetracaine (Fig. 1B), which indicates again that the effect of the local anesthetic is not at all simply an unspecific membrane action.

The charged N- and C-terminal extensions of BetP are an obvious target for studies on osmotic regulation, especially in view of the hypothesis on ProP function in *E. coli*, which shows a comparable C-terminal extension (12). All four different deletion mutants in the N-terminal region of BetP were active in transport. Furthermore, even the carrier protein with a completely missing N-terminal extension did not show a change in the binding affinity toward glycine betaine. On the other hand, the pattern of osmo-regulation of these recombinant proteins on the level of activity was altered. We observed an optimum of stimulation at a 2-fold higher external osmolality, i.e. at a NaCl concentration where the wild type protein is already largely inhibited.

The consequences of changes in the C-terminal region were completely different. Truncation of only 12 amino acid residues (C3 mutant) led to activity of the carrier at low external osmolality, i.e. in a range where the wild type protein is inactive. Deletion of 23 and 32 residues in the C2 and C1 mutants, respectively, resulted in proteins that were not only active at low osmolality but remained active over a broad range of osmolality. The full extent of the change in regulation properties of BetP proteins with truncated C-terminal extensions became obvious after a detailed functional analysis. Proteins with a significantly shortened C-terminal extension (C2 and C1 mutants) had completely lost regulation by osmotic stress both at high and low osmolality. The initial rise in activity, which seemed to be similar to the wild type, was found to be solely the consequence of a changed affinity for the co-substrate Na+.

In the presence of a low basic concentration of Na+, we demonstrated by the use of sorbitol instead of NaCl that these mutant BetP proteins are in fact not sensitive to osmo-stress anymore. The dominance of the C-terminal extension for this regulation was corroborated by the construction of double mutants with deletions both in the N- and in the C-terminal region. The double mutants turned out to be functionally identical to the respective proteins with altered C-terminal region only.

In summary, the following pattern of consequences of truncating terminal regions of BetP emerges. Deletions in the N-terminal extension cause a shift to higher values of the optimum of osmotic stimulation. A truncation in the C-terminal region by 12 amino acids (C3) lowers the optimum of activation. Larger deletions of 23 (C2) or 32 amino acids (C1) lead to a complete loss of the response to osmotic stress as well as to a significant decrease in the Na+ affinity. In addition, deletion of 32 amino acids (C1) results in a decrease of the affinity toward glycine betaine. After truncation of the whole C-terminal extension, no transport activity was detected anymore.

These findings to some extent resemble a hypothesis that was put forward for the *E. coli* ProP protein (12). The *E. coli* proline/betaine transporter ProP also functions both as osmo-sensor and osmo-regulator and carries a C-terminal extension of about 50 amino acids, which was supposed to be involved in osmo-sensing. Furthermore, a related hypothesis on the location of sensory and regulatory domains in a membrane protein involved in the response to osmotic stress was suggested for the KdpD sensor kinase in *E. coli*. In this case, however, transmembrane segments were made responsible for the putative sensory domain (29).

The results on the BetP protein provide the first step for a molecular analysis of membrane-protein interactions involved in osmo-sensing by a bacterial carrier system. Moreover, our results demonstrate that the altered regulation of the mutant proteins has severe consequences for the response of the cell to osmostress. *C. glutamicum* carrying the C2 mutant with the truncated C-terminal region reached a significantly higher level of steady state accumulation of betaine. This indicates that the basis for the kinetic steady state in wild type cells and in cells with the mutant BetP carrier are different. A kinetic analysis of the situation revealed that this is in fact the case. By measuring exchange of labeled (internal) and unlabeled (external) betaine in the wild type and in mutant strains, we showed that the steady state in the wild type is based on a down-regulation of BetP activity due to the fact that the stimulus, i.e. the osmotic stress, is abolished after a high internal concentration of compatible solute is reached (14). In contrast, in the mutant strain carrying the BetP protein that is insensitive to hyperosmotic stress, the observed steady state was found to be the result of an unchanged uptake activity counter-balanced by glycine betaine efflux via a different pathway.
independent from BetP. Consequently, in this case, a futile cycle is created. The efflux pathway, being a security valve under these conditions, most likely is the mechanosensitive efflux channel that we recently described in C. glutamicum (27).

Acknowledgment— We thank E. Galinski for a gift of ectoine, J. Wood for stimulating exchange of information, and H. Sahm for continuous support and helpful discussions.

REFERENCES

1. Yantzey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) Science 217, 1214–1222
2. Csonka, L. N. (1989) Microbiol. Rev. 53, 121–147
3. Csonka, L. N., and Hansen, A. D. (1991) Annu. Rev. Microbiol. 45, 569–606
4. Cairney, J., Booth, I. R., and Higgins, C. F. (1985) J. Bacteriol. 164, 1224–1232
5. Lucht, J. M., and Bremer, E. (1994) FEMS Microbiol. Rev. 14, 3–20
6. Cairney, J., Booth, I. R., and Higgins, C. F. (1985) J. Bacteriol. 164, 1218–1223
7. Grothe, S., Krogsrud, R. L., McClellan, D. J., Milner, J. L., and Wood, J. M. (1986) J. Bacteriol. 166, 253–259
8. Milner, J. M., Grothe, S., and Wood, J. M. (1988) J. Biol. Chem. 263, 14900–14905
9. Kappes, R., Kempf, B., and Bremer, E. (1996) J. Bacteriol. 178, 5071–5079
10. Kempf, B., and Bremer, E. (1995) J. Biol. Chem. 270, 16710–16715
11. Lin, Y., and Hansen, J. N. (1995) J. Bacteriol. 177, 6874–6880
12. Culham, E. D., Lasby, B., Marangoni, A. G., Milner, J. L., Steer, B. A., van Nues, R. W., and Wood, J. M. (1995) J. Mol. Biol. 229, 268–276
13. Peter, H., Burkovski, A., and Kramer, R. (1996) J. Bacteriol. 178, 5229–5234
14. Farwick, M., Siewe, R. M., and Kramer, R. (1995) J. Bacteriol. 177, 4690–4695
15. Peter, H., Iader, A., Burkovski, A., Lambert, C., and Kramer, R. (1997) Arch. Microbiol. 168, 143–151
16. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Eikmanns, B. J., Thum-Schmitz, N., Eggeling, L., Lüdtke, K. U., and Sahm, H. (1994) Microbiology 140, 1817–1828
18. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2172–2175
19. Liebl, W., Bayerl, A., Schein, B., Stillner, U., and Schleifer, K. (1989) FEMS Microbiol. Lett. 65, 299–304
20. Landsfield, B., and Strom, A. R. (1986) J. Bacteriol. 165, 849–855
21. Bottner, M., and Winter, R. (1993) Biophys. J. 65, 2041–2046
22. Hornby A. P., and Cullis, P. R. (1981) Biochim. Biophys. Acta 647, 285–292
23. Lambert, C., Erdmann, A., Eikmanns, M., and Kramer, R. (1995) Appl. Environ. Microbiol. 61, 4334–4342
24. Shimouza, T., Shibata, A., and Terada, H. (1992) Biochim. Biophys. Acta 1104, 261–268
25. Sugiiwa, A., Hirokawa, K., Nakashima, K., and Miruno, T. (1994) Mol. Microbiol. 14, 929–938
26. Lamark, T., Kaassen, I., Eshoo, M. W., Falkenberg, P., Mc Dougall, J., and Strom, A. R. (1991) Mol. Microbiol. 5, 1049–1064
27. Ruffert, S., Lambert, C., Peter, H., Wendisch, V. F., and Kramer, R. (1997) Eur. J. Biochem. 247, 572–580
28. Glaasker, E., Konings, W. N., and Poolman, B. (1996) J. Biol. Chem. 271, 10060–10065
29. Zimmann, P., Puppe, W., and Altenedorf, K. (1995) J. Biol. Chem. 270, 28282–28288
30. Abe, S., Takayama, K., and Kinoshita, S. (1987) J. Gen. Microbiol. 13, 279–301
31. Grant, S. G. N., Jessee, J., Bloom, F. R., and Hanahan, D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4645–4649
32. Vieira, J., and Messing, J. (1982) Gene 19, 259–268
Osmo-sensing by N- and C-terminal Extensions of the Glycine Betaine Uptake
System BetP of *Corynebacterium glutamicum*
Heidi Peter, Andreas Burkovski and Reinhard Krämer

*J. Biol. Chem.* 1998, 273:2567-2574.
doi: 10.1074/jbc.273.5.2567

Access the most updated version of this article at [http://www.jbc.org/content/273/5/2567](http://www.jbc.org/content/273/5/2567)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/273/5/2567.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 31 references, 17 of which can be accessed free at [http://www.jbc.org/content/273/5/2567.full.html#ref-list-1](http://www.jbc.org/content/273/5/2567.full.html#ref-list-1)