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Biochimica et Biophysica Acta 1667 (2004) 229–240

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Activity regulation of the betaine transporter BetP of *Corynebacterium glutamicum* in response to osmotic compensation

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Received 19 August 2004; received in revised form 18 October 2004; accepted 29 October 2004
Available online 11 November 2004

Abstract

As a response to hyperosmotic stress bacterial cells accumulate compatible solutes by synthesis or by uptake. Beside the instant activation of uptake systems after an osmotic upshift, transport systems show also a second, equally important type of regulation. In order to adapt the pool size of compatible solutes in the cytoplasm to the actual extent of osmotic stress, cells down-regulate solute uptake when the initial osmotic stress is compensated. Here we describe the role of the betaine transporter BetP, the major uptake carrier for compatible solutes in *Corynebacterium glutamicum*, in this adaptation process. For this purpose, *betP* was expressed in cells (*C. glutamicum* and *Escherichia coli*), which lack all known uptake systems for compatible solutes. Betaine uptake mediated by BetP as well as by a truncated form of BetP, which is deregulated in its response to hyperosmotic stress, was dissected into the individual substrate fluxes of unidirectional uptake, unidirectional efflux and net uptake. We determined a strong decrease of unidirectional betaine uptake by BetP in the adaptation phase. The observed decrease in net uptake was thus mainly due to a decrease of V_{max} of BetP and not a consequence of the presence of separate efflux system(s). These results indicate that adaptation of BetP to osmotic compensation is different from activation by osmotic stress and also different from previously described adaptation mechanisms in other organisms. Cytoplasmic K^+, which was shown to be responsible for activation of BetP upon osmotic stress, as well as a number of other factors was ruled out as triggers for the adaptation process. Our results thus indicate the presence of a second type of signal input in the adaptive regulation of osmoregulated carrier proteins.
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Keywords: Transport; Osmoregulation; *Corynebacterium*; Betaine; Uptake

Maintenance of cytoplasmic water activity and cell turgor is essential for growth and viability in prokaryotic and eukaryotic cells. Changes in environmental osmolality lead to water flux into or out of the cell, consequently, cells have developed a variety of mechanisms to react to these changes by synthesis, as well as uptake and release of so-called compatible solutes [1]. One of the first responses of bacterial cells to hyperosmotic stress is uptake of compatible solutes which are accumulated to balance the increased external osmolality. For this purpose, the transport systems involved must be properly regulated, osmoreactive transport system(s) should thus be able to sense the extent of osmotic stress to respond with an adapted regulation of activity.

In the last years, a number of triggers have been described as stimuli for osmosensitive transport proteins in bacteria. Mechanosensitive channels, like MscL, open upon changes in membrane tension leading to release of intracellular solutes [2]. With respect to osmoregulated uptake carriers, three different systems have mainly been studied. ProP of *Escherichia coli*, a secondary carrier for zwitterionic compatible solutes, has first been proven by reconstitution to harbor both osmosensory and osmoregulatory properties [3,4]. For BetP of *Corynebacterium glutamicum*, a Na^+-dependent secondary betaine uptake carrier, changes in the lumenal K^+ concentration were shown to be an important stimulus related to osmotic stress when analyzed in proteoliposomes [5,6]. Finally,

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0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.10.012

OpuA of *Lactococcus lactis*, an ABC transporter, was found to be regulated in the dependence of the lumenal ion concentration, and this was correlated to changes in surface properties of the lipid membrane [7].

Once the hyperosmotic stress is compensated by accumulation of compatible solutes, active net uptake of these solutes should cease. Thus, an appropriate mechanism regulating the pool size of compatible solutes by regulation of uptake and/or efflux systems is necessary. In contrast to detailed studies on the kind of stimuli related to carrier activation in response to a hyperosmotic shock, down-regulation of uptake activity in response to osmotic compensation has not been studied equally well. In principle, two different mechanism can be imagined. Either the activity of the uptake carrier is reduced or a counteracting efflux mechanism mediated by other carriers or by efflux channels compensates the uptake carrier's activity.

Adaptation of net uptake after compensation of osmotic stress has been explained in terms of feedback regulation of the respective transporter. This mechanism was first postulated for the high-affinity, though not osmoregulated glycine betaine uptake system of *Staphylococcus aureus* [8,9]. In *Listeria monocytogenes* an osmosensitive secondary betaine uptake carrier was shown to be inhibited by internal glycine betaine and carnitine [10]. As an alternative mechanism, in *Lactobacillus plantarum* separate systems for (primary) uptake and efflux of betaine were discriminated and the efflux system was postulated to be inactive under hyperosmotic conditions [11]. Most of these studies, however, were done in cells harboring also other transport systems for these solutes in addition to those which were analyzed kinetically.

In this work, for studying the response of BetP to osmotic adaptation, we used cells of *C. glutamicum* in which BetP is the only carrier catalyzing uptake of compatible solutes. We determined betaine fluxes mediated by BetP in different phases of hyperosmotic stress by dissecting net flux, unidirectional influx and unidirectional efflux. In addition, steady-state betaine concentrations after different extents of hyperosmotic shock were investigated. A number of possible factors and mechanisms including feedback inhibition were studied in terms of their influence in triggering adaptation of transport activity. On the basis of these results, the response of BetP to increasing osmotic compensation in the cell was elucidated.

1. Experimental procedures

1.1. Bacterial strains and plasmids

For transport measurements of BetP, strain *C. glutamicum* DHPF (*betP*$^-$ *putP*$^-$ *proP*$^-$ *ectP*$^-$ *lcoP*$^-$) was used, which is totally deficient in compatible solute uptake. This strain was derived from *C. glutamicum* DHPE [12] by deletion of the *lcoP* gene, which codes for a fifth uptake system for compatible solutes with very low activity [13]. DHPF cells were transformed with plasmids pGTG (encoding BetP) or pC2 (encoding BetP lacking the last C-terminal 25 amino acids) as described previously [15]. pGTG and pC2 are derivatives of pEKEX2, in which the genes are under the control of the isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible *ptac* promoter. In the experiment reported in Fig. 2B, *C. glutamicum* strain Cgl-ProP was used, in which all carriers except ProP had been deleted (*betP*$^-$ *putP*$^-$ *ectP*$^-$ *lcoP*$^-$) [12–14]. For uptake measurements in *E. coli*, compatible solute uptake deficient *E. coli* MKH13 cells [16] were transformed with pASK-IBA5 *strep-betP* [6], in which the *betP* gene is fused in frame at its 5′ prime end to the sequence encoding the eight-amino acid strep tag II. The gene is under control of the *tet* promoter.

1.2. Growth, osmotic shock and transport assays

C. glutamicum DHPF pGTG cells were grown overnight in MM1 minimal medium [17], supplemented with 1 μg/ml desferoxamine, or BHI brain heart infusion medium (Difco, Detroit, MI, USA), each supplemented with 0.2 mM IPTG and 50 mg/L kanamycin. Cells were washed with buffer containing 25 mM KPi pH 7.5 and 25 mM NaPi pH 7.5 at 4 °C, resuspended in the same buffer at a cell density of about 0.1 g cdm/L (cell dry mass/L) including 10 mM glucose. In the experiments shown in Fig. 5, cells were washed and resuspended in MM1 medium. Cells could be stored for up to 4 h before application of hyperosmotic shocks by addition of NaCl at the indicated concentrations without loss of BetP transport activity. To avoid cell growth and changes in protein synthesis during the experiment, 25 μg/ml chloramphenicol was added, except in Fig. 5. *E. coli* MKH13 was treated like *C. glutamicum* with the following alterations: cells were grown overnight in Luria Bertani medium [18] and then transferred to fresh medium. At an OD600 of 1, anhydrotetracycline (IBA, Göttingen, Germany) was added for induction of *betP* for 3 h. K^+/Na^+ phosphate buffer was replaced by 100 mM Tris/morpholino ethane sulfonic acid (MES), pH 7.5. The co-solute Na^+ was provided by addition of 200 mM NaCl. For transport assays, 500 μM glycine betaine, [^{14}C]- or [^{3}H]-labeled betaine, [^{14}C]-labeled proline, or [^{14}C]-labeld glutamine, respectively, was added 30 s after the hyperosmotic shock. At time intervals of 15 s after betaine addition, samples of 200 μl were transferred to glass fiber filters (Schleicher und Schuell, Dassel, Germany) and washed twice with 2.5 ml 0.1 M LiCl (*C. glutamicum)* or 0.5 M sucrose plus 0.05 M MgCl2 (*E. coli*). The radio-

activity retained on the filters was determined by liquid scintillation counting.

1.3. Synthesis of labeled glycine betaine

Synthesis of [^{14}C]- or [^{3}H]-glycine betaine by oxidation of [^{14}C]- or [^{3}H]-choline chloride was performed as described earlier [19]. Radiolabeled [^{14}C]-choline chloride was purchased from Amersham International (Amersham, Buckinghamshire, UK), [^{3}H]-choline chloride was from Perkin Elmer, Köln, Germany.

1.4. Kinetic dissection of betaine fluxes

To discriminate between net betaine flux v_{net} and unidirectional betaine uptake rate v_{up}, which is the sum of v_{net} and unidirectional efflux rate v_{ef}, according to the equation $v_{net}=v_{up}-v_{ef}$, double label experiments were performed. When cells were grown in MM1 medium, at the beginning of the experiment no internal betaine is present, thus, after addition of [^{14}C]-betaine, v_{net} equals v_{up}. Even after prolonged time, i.e., in the adapted state, v_{net} can be determined correctly, since internal and external [^{14}C]-betaine will always have the same specific radioactivity. Under these conditions, however, v_{up} cannot be determined, since internal label accumulates and v_{ef} is not known. To discriminate all three fluxes, carrier-free ^{3}H-betaine was added to aliquots at certain time points during the transport experiments. When added [^{3}H]-betaine is present only externally, v_{up} can be directly determined. Since v_{net} is known from the simultaneous flux of [^{14}C]-label, v_{ef} can be calculated.

1.5. Determination of internal potassium

Cells were grown, washed and shocked as described for the transport experiments, except that cell density was 2 g cdm/L and glycine betaine was 5 mM. At the indicated time points, 1-ml samples were transferred in triplicate to glass fiber filters and washed twice with 2.5ml MOPS buffer pH 7.5 with an osmolality corresponding to that of the medium. After lysis with 0.1% (w/v) cetyl trimethyl ammonium bromide (CTAB), cell debris was sedimented and the K^+ concentration in the supernatant was determined with a flame photometer (ELEX 6361, Eppendorf, Hamburg, Germany).

1.6. Volume measurements

Cells were grown and shocked as described for K^+ determination. For volume determination according to Rottenberg [20], 670-μl aliquots of the cell suspension (1.5 g cdm/L) were incubated with 20 mM [^{14}C]-taurine (4 μCi/mmol) and [^{3}H]-H_2O (0.39 μCi/ml) for 1 min at discrete times after hyperosmotic shock and addition of betaine. Cells were separated by centrifugation through silicone oil (d=1.06; Wacker, Germany) into 20% (v/v) HClO4. After careful removal of the supernatant the cell pellet was centrifuged again. Radioactivity in the sediment and the first supernatant fraction was determined by scintillation counting.

1.7. Determination of internal solutes

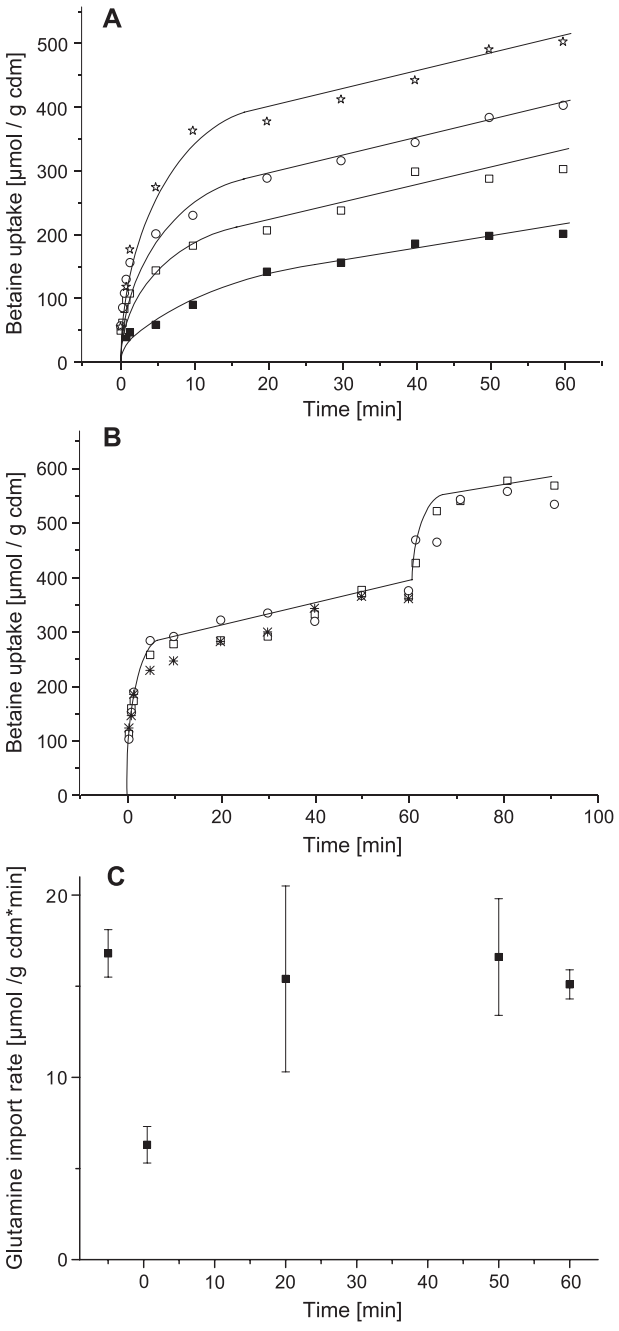
Cells were treated as described for determination of K^+. Samples of 2 ml were centrifuged and the pellet was washed once with 1 ml of buffer. For trehalose determination, the cell pellet was suspended in 1 ml methanol and incubated for 20 min at 70 °C. After centrifugation of cell debris, 500 μl or less of the supernatant was collected, supplied with myo-inositol as an internal standard and dried under nitrogen. After addition of 65 μl of 20 mg/ml methoxamine in pyridine, the samples were incubated for 1 h at 30 °C. Then, 35 μl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was added, followed by incubation for 1.5 h at 65 °C. Trehalose in the samples was determined by split injection capillary gas chromatogratography and flame ionisation detection with a Trace GC gas chromatograph (ThermoFinnigan, San Jose, CA, USA). Injection and detection devices were kept at 280 °C. Separation was achieved by a linear temperature gradient from 160 to 280 °C with a heating rate of 12 °C/min, starting at 2 min post-injection. The final temperature was kept constant for 3 min for complete elution.

For proline determination, the cell pellet was suspended in 1 ml 0.1% (w/v) CTAB for 5 min and cell debris was separated by centrifugation. The supernatant was analyzed by reverse phase high-performance liquid chromatography (HPLC) (HP 1090 liquid chromatograph, HP1046A fluorescence detector; Hewlett Packard) with automated precolumn derivatization. An EC 125/4 Nucleodur 100-5 C18 ec column (Macherey & Nagel, Düren, Germany) was used. Proline was derivatized with 9-fluorenyl-methyl-chloroformiate/acetonitrile. Solvent A contained 33.25 mM sodium acetate, pH 6.5, 2.5% (v/v) acetonitrile and 2.5% (v/v) methanol, and solvent B was a mixture of acetonitrile/methanol (1:1). A solvent gradient was used ranging from 5% solvent B to 100% solvent B.

For glycine betaine determination, the method described by Galinski and Herzog [21] was used with slight modifications. Briefly, cell pellets were resuspended in a mixture of methanol/chloroform/water (10:5:4) which was shaken for 15 min. Addition of chloroform, followed by incubation for 15 min and centrifugation, led to phase separation. The betaine-containing aqueous phase was analyzed by isocratic HPLC on an NH_2-column in 70% acetonitrile with a refractive index detector.

2. Results

BetP responds to hyperosmotic shocks with instant activation [22]. Fig. 1A shows the response of BetP in *C. glutamicum* cells to various osmotic upshifts by the addition of NaCl. At an osmolality below the activation threshold, which is around 300 osM/kg [12], the initial velocity of betaine uptake was low (cf. also Table 1). Upon osmotic upshift, BetP became instantly activated and catalyzed fast betaine uptake. Ten to twenty minutes after the upshift, the measured net uptake decreased again and remained at a low level (adaptation phase).



3. Adaptation to different levels of hyperosmotic stress

To characterize the shift between the active and the adapted state of BetP in detail, different osmotic shocks were analyzed, and a second osmotic shock was applied after compensation of the first shock was achieved. The level of betaine accumulation reached in the adaptation phase increased with increasing extent of osmotic stress (Fig. 1A), and the internal betaine concentration in the adaptation phase depended linearly on the osmotic stress applied (cf. Fig. 5A). Application of a second osmotic shock in the adaptation phase resulted in reactivation of BetP and led to further increased levels of internal betaine (Fig. 1B). Again, some time after the repeated activation, betaine uptake slowed down and reached a second adaptation phase.

A number of control experiments were necessary for the correct interpretation of these results. It could be argued that a decreasing driving force was the reason for the observed decrease in net uptake of betaine after a few minutes. To rule out this possibility, an uptake system depending on the same energy source, the Na^+ coupled glutamine uptake system [23], was tested under identical conditions, i.e., in the presence of unlabeled betaine (Fig. 1C). Besides with a sudden decrease in uptake activity directly following NaCl addition, the glutamine uptake rate remained essentially unchanged in the phase in which net betaine uptake ceased. It has previously been shown that BetP, in the presence of low external substrate concentrations, is in principle able to accumulate betaine to ratios exceeding 106 [22]. It may further be argued that the increase in the chemical Na^+ potential due to addition of NaCl was responsible for the observed reactivation of BetP (Fig. 1B), since betaine uptake is in fact coupled to co-transport of Na^+. The results shown in Fig. 1B, however, prove that a closely similar time course and extent of activation was observed if KCl, which does not increase the driving force, was added instead of

Fig. 1. Glycine betaine and glutamine uptake in DHPF pGTG cells of *C. glutamicum*. (A) Glycine betaine uptake. Starting with a basal osmolality of 116 mosM/kg (25 mM sodium and potassium phosphate pH 7.5 each, 10 mM glucose), uptake was measured at unchanged osmolality (closed squares) and upon hyperosmotic shocks to 593 mosM/kg (open squares), 897 mosM/kg (open circles), and 1564 mosM/kg (asterisks) by the addition of 250, 400, and 740 mM NaCl, respectively. For betaine uptake measurement, 0.5 mM of [^{14}C]-glycine betaine was added 30 s after the hyperosmotic shock or after transfer to medium without shock. (B) Betaine uptake upon two consecutive hyperosmotic shocks. The first upshift was from 116 to 593 mosM/kg, followed by a shift to 1564 mosM/kg by adding NaCl or KCl in the following combinations: both shocks with NaCl (squares), first shock with NaCl, second shock which KCl (circles), and only the first shock with KCl (asterisks). All other conditions as in A. (C) Glutamine uptake. Conditions exactly as described in A except that only unlabeled betaine was added. A hyperosmotic upshift of 116 to 593 mosM/kg with NaCl was applied and 0.5 mM unlabeled glycine betaine was added 30 s later. At the indicated time points, 0.1 mM [^{14}C]-glutamine was added to aliquots of the suspension and the initial velocity of glutamine uptake was determined. All values shown in A, B, and C are means of experiments carried out at least in triplicate.

Table 1
Dissection of betaine fluxes measured at different phases of osmotic activation with cells expressing either wild-type BetP or the truncated variant BetP Δ25 grown in different media

Protein	Medium	Experimental situation (time of measurement)	Osmolality [mosM/kg]	v_{net}	v_{up}	v_{ef}	v_{ef}/v_{up}
				[nmol. min^{-1}. mg cdm^{-1}]			
BetP	MM1	I no shock, initial phase (0–5 min)	116 (constant)	$=v_{up}$	6.4±0.8	0.0	0.0
		I no shock, adaptation phase (40–60 min)	116 (constant)	1.4±0.2	3.6±0.9	2.2±1.1	0.61±0.10
	MM1	II 1st osmotic shock (activation, 0–45 s)	116→593	$=v_{up}$	62.1±8.2	0.0	0.0
	MM1	III adaptation phase (40–60 min)	593 (constant)	3.2±0.4	17.2±1.0	14.0±1.5	0.81±0.03
	MM1	IV 2nd osmotic shock (51.25–52 min)	593→1564	17.1±4.3	48.2±11.8	31.2±16.1	0.65±0.07
BetP Δ25	MM1	I no shock, initial phase (0–1 min)	116	$=v_{up}$	42.2±6.3	0.0	0.0
		no shock, adaptation phase (40–60 min)	116 (constant)	1.2±0.29	6.6±1.75	5.5±2.0	0.80±0.05
	MM1	II osmotic shock (t=0)	116→593	$=v_{up}$	70.8±23.5	0.0	0.0
	MM1	III adaptation phase (40–60 min)	593 (constant)	0.68±0.17	9.2±2.9	8.5±2.7	0.92±0.02
BetP	BHI	I no shock (0–30 min)	116 (constant)	n.d.	2.0±0.7	n.d.	
	BHI	II 1st osmotic shock (activation, t=0)	116→593	n.d.	72.4±2.7	n.d.	
	BHI	III adaptation phase (15–25 min)	593 (constant)	3.9±1.0	14.8±1.9	10.9±2.9	0.74±0.29
	BHI	IV 2nd osmotic shock (20 min)	593→1564	64.5±7.6	168±12.9	104±20.5	0.62±0.17

n.d., not defined, because of the presence of internal betaine.

NaCl. Consequently, the change in external osmolality, and not in chemical Na^+ potential, caused both activation and reactivation of BetP. Moreover, uncharged solutes, e.g., sorbitol or proline, were equally effective in increasing the initial velocity of betaine uptake (results not shown).

In the following detailed analysis of BetP-mediated transport, we focused on betaine fluxes observed in representative states of activity (cf. Figs. 1 and 2, Table 1), namely (i) BetP activity before the first osmotic shock, (ii) initial transport activity right after the first osmotic shock, (iii) activity in the time range of 30–60 min after salt addition, i.e., in the state of adaptation, and (iv) BetP activity right after the second osmotic shock.

4. Dissection of betaine fluxes

In transport experiments using labeled betaine, in principle only net uptake of label can be measured, i.e., the difference between label taken up and exported. It correctly reflects total betaine uptake flux only under conditions when no internal betaine is available. In the presence of internal substrate, the observed uptake of label is difficult to assign to a defined flux. This is particularly important if not only uptake is considered, but also efflux via the uptake carrier, i.e., exchange, as well as efflux through other transporters or channels.

We experimentally dissected all three individual betaine fluxes, net flux v_{net}, which normally means net influx, unidirectional uptake rate v_{up}, and unidirectional efflux rate v_{ef} (see Section 2). Flux dissection by means of double label experiments is more accurate than an alternative method often used by adding a large amount of unlabeled substrate at the outside thus quenching influx of label, since the latter approach interferes with the substrate equilibrium across the plasma membrane. In the experimental setup applied here, we can safely assign all betaine import fluxes to BetP, since we used recombinant *C. glutamicum* cells in which all other transport systems relevant for betaine uptake had been deleted. Betaine efflux, on the other hand, could be mediated by BetP, by other yet unknown carrier(s), or by the activity of mechanosensitive channels [17,24].

Cells were subjected to an osmotic shock from 116 to 593 mosM/kg by addition of 250 mM NaCl; 30 s later [^{14}C]-betaine was added (Table 1 and Fig. 1). In the initial phase, uptake of [^{14}C]-labeled betaine represents both the unidirectional uptake rate v_{up} and net uptake v_{net}. At various time points, v_{up} was determined by adding carrier-free [^{3}H]-betaine to an aliquot of the cell suspension. For analyzing betaine fluxes in response to a second shock, [^{3}H]-betaine and 490 mM NaCl were added simultaneously, resulting in an osmolality of 1564 mosM/kg. The measured fluxes are summarized in Table 1. A parameter, v_{ef}/v_{up}, was derived which is a practical measure for the extent of efflux, or exchange, respectively. This ratio varies numerically between zero (pure uptake mode) and 1 (pure exchange mode or balanced uptake and efflux). In the adapted phase, this parameter (v_{ef}/v_{up}) reached values around 0.7.

Values for v_{net}, v_{up}, v_{ef}, and v_{ef}/v_{up} have been measured and calculated for a set of experiments in *C. glutamicum* DHPF/pGTG expressing wild-type *betP* (first part of Table 1). Prior to osmotic upshift, only low BetP-mediated betaine influx of 6.4 nmol min^{-1} mg cdm^{-1} was observed. Upon an osmotic shift from 116 to 593 mosM/kg, BetP was activated and catalyzed betaine influx at a rate of 62 nmol min^{-1} mg cdm^{-1}. In the adapted state, uptake activity slowed down to very low v_{net} values around 3 nmol min^{-1} mg cdm^{-1}. In this phase, a significant extent of exchange, or balanced influx (by BetP) and efflux (by another system), respectively, was determined. Importantly, v_{net} was not only reduced because of an increasing efflux activity, but in particular due to a reduced unidirectional uptake activity v_{up}. Consequently, besides changing to the exchange mode,

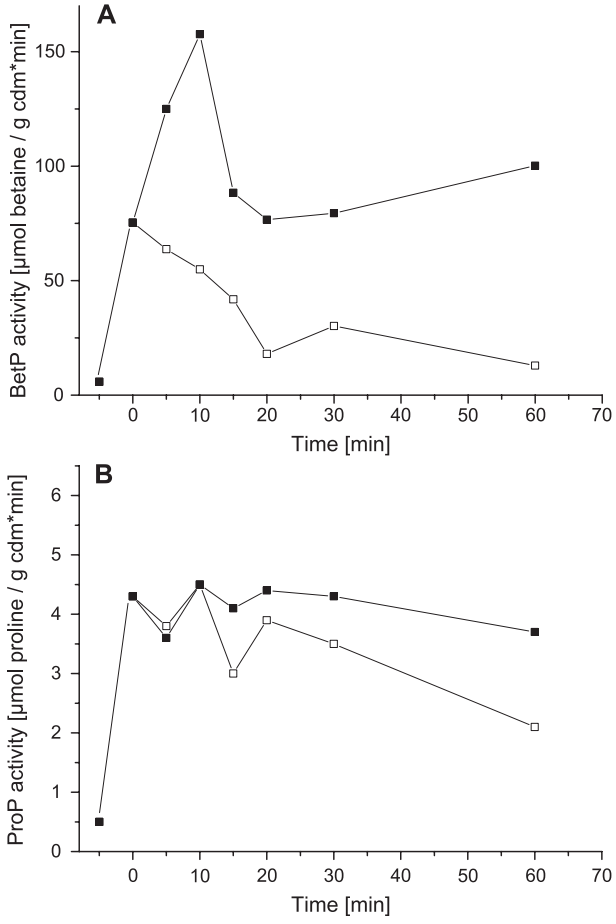


Fig. 2. Time course of betaine and proline uptake activity by strains *C. glutamicum* DHPF pGTG and Cgl-ProP. (A) Betaine uptake by strain DHPF pGTG. Lower curve: uptake of betaine in the presence of 0.5 mM betaine (open squares). Conditions as described for Fig. 1A; a hyperosmotic shift to 897 mosM/kg with NaCl was applied. In one part of the experiment (results not shown), ^{14}C-labeled betaine (0.5 mM) was added at the beginning. The external label was monitored in the time course of the experiment thus providing the value for the actual external betaine concentration necessary for the second part of the experiment. In the experiment shown in the lower curve, 0.5 mM unlabeled betaine was added at the beginning, and carrier-free [^{14}C]-betaine was added to aliquots of the cell suspension at the indicated times. Thus, unidirectional betaine uptake rate (v_{up}) was determined in individual experiments, and corrected according to the concomitantly determined specific activity (see above, results not shown). Upper curve: uptake in the absence of added betaine (solid squares). Same as described above, except that no betaine was added at the beginning and 0.5 mM [^{14}C]-betaine was added to aliquots of the cell suspension at the indicated times. Thus, in contrast to the experiment described in the lower part, no internal betaine was present when measuring BetP activity. (B) Proline uptake by strain Cgl-ProP. Same experiment as described in part A, except that unlabeled and ^{14}C-labeled proline were used instead of betaine. Values shown in A and B are means of experiments carried out in duplicate.

BetP activity is down-regulated when reaching the adapted state.

The surprisingly effective down-regulation of BetP activity in response to betaine accumulation was further elaborated. In Fig. 2A the time course of betaine unidirectional uptake activity (v_{up}) after an osmotic upshift is shown. Activation of BetP in response to hyperosmotic stress started from a very low level (before osmotic shock). It was immediate but only transient, if betaine was present (Fig. 2A, lower curve). Upon increasing internal concentrations of betaine (please refer to Fig. 1) in response to the osmotic shock, i.e., when approaching osmotic compensation after 10–20 min, BetP activity was down to low levels again. In contrast, after showing a transient burst to high activity, BetP stayed active within the first hour of measurement, if no betaine was added externally (Fig. 2A, upper curve). In order to find out whether this behavior is typical for osmoresponsive uptake systems, we also studied ProP of *C. glutamicum*, a proline uptake carrier which is osmoregulated in a very similar manner as BetP [12]. Interestingly, ProP showed a different response to osmotic upshifts. Proline uptake by ProP only slightly decreased after application of an identical hyperosmotic shock even in the presence of added substrate proline (Fig. 2B).

For a more detailed characterization of the state of activity of BetP after reaching osmotic compensation, *C. glutamicum* cells were exposed to a second hyperosmotic shock. As expected, the second shock led to a significant increase in both unidirectional influx rate v_{up} and net influx rate v_{net} (Fig. 1B and Table 1). These two rates, however, differed strongly, indicating that exchange is a prominent activity of BetP also under the conditions of a second upshift. As a consequence, in spite of the fact that activation and thus an increased unidirectional uptake rate leading to higher accumulation of betaine was observed, also the efflux rate was found to be significantly increased in response to a second hyperosmotic shift as compared to the preceding adapted state. For the interpretation of these results, it is important to note that under all circumstances measured, the calculated v_{eff}/v_{up} values were around 0.7. Consequently, also betaine efflux was increased in the second osmotic upshift.

5. Participation of cytoplasmic K^+ in BetP down-regulation

The raise of internal K^+ in response to osmotic shock was shown to be an activating trigger for BetP in proteoliposomes [6]. Vice versa, a decrease of internal K^+ may be responsible for down-regulation of BetP activity. In intact cells, an increase in internal K^+ upon hyperosmotic shock may be caused by two reasons. First, all solutes are concentrated due to cell shrinkage, and second, K^+ is accumulated in *C. glutamicum*, similar to *E. coli* [25], in response to a hyperosmotic shock. To measure the change of internal K^+ concentrations, determination of cytoplasmic volumes (correctly: free cytoplasmic water) is indispensable. In these experiments, we realized that the response of internal K^+ significantly depended on the growth history of the cells, namely whether they were grown in minimal (MM1) or complex

(BHI) medium. Complex medium contained traces of betaine and growth in this medium thus led to the presence of low internal betaine concentrations in the cells at the beginning of the experiment. The response of the two differently grown cells, however, was not significantly different with respect to regulation of BetP activity (Table 1). As expected, decreasing cell volumes were observed after hyperosmotic shocks in both MM1 and BHI grown cells (Fig. 3). The adaptation phase after the osmotic upshifts, however, was different. In contrast to cells grown in complex medium, minimal medium-grown cells reached pre-shock cytoplasmic volumes. In both cell types, the amount of cytoplasmic K^+ was measured and the internal K^+ concentration was calculated. In all cases, the internal K^+ concentration increased after the shock followed by a decrease in minimal medium grown cells (Fig. 3A). In BHI grown cells, cytoplasmic K^+ did not decrease during the time in which BetP activity reached an adapted state (Fig. 3B).

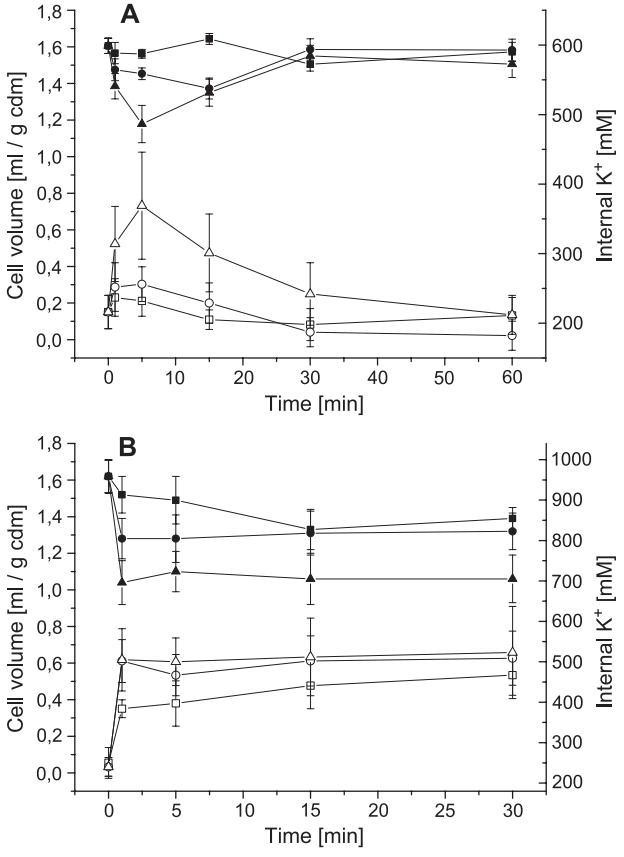


Fig. 3. Free cytoplasmic water (closed symbols), which is a measure for cell volume, and internal potassium concentration (open symbols) after hyperosmotic shock with 250 mM NaCl (squares), 400 mM NaCl (circles) or 740 mM NaCl (triangles). Cells were pre-grown overnight in MM1 medium (A) or BHI medium (B) and washed with buffer containing 25 mM potassium phosphate, 25 mM sodium phosphate pH 7.5 and 10 mM glucose. Glycine betaine at 5 mM was added 30 s after the hyperosmotic shock. All values shown in A and B are means of experiments carried out at least in triplicate.

6. Internal solute accumulation and the adapted state of BetP

Several explanations can be put forward for the observed down-regulation of BetP activity in the adapted state. In previous publications, product inhibition due to internal accumulation of substrate has been suggested for *S. aureus* [8,9], *L. plantarum* [26], and *L. monocytogenes* [10]. We first measured internal betaine concentration in the adapted state as a consequence of hyperosmotic shocks of various extent (Figs. 1A and 5A). The betaine concentration was found to be linearly related to the extent of hyperosmotic shock. The observed down-regulation of BetP can thus not be a consequence of simple product inhibition, but the internal betaine pool in the adapted state seemed to be directly influenced by the extent of osmotic stress. This can in principle be explained by a model in which product inhibition would work only under osmotic compensation, as it has been suggested for *L. plantarum* [26].

In order to test this possibility, we measured unidirectional betaine uptake v_{up} at different times after the osmotic upshift in the absence of betaine (Fig. 4). In contrast to the other experiments, a medium including C- and N-source was applied and protein synthesis was not inhibited, since we had to allow for osmotic adaptation by induction of biosynthesis of compatible solutes other than betaine which cannot be synthesized by *C. glutamicum*. Consequently, this experiment is fundamentally different from those described in the previous figures. In response to a significant osmotic stress, cells stopped growing for some time until they resumed growth with more or less unchanged rate, indicating adaptation to osmotic stress (Fig. 4A). Measurement of internal compatible solutes revealed an increase of trehalose within the first 1–2 h, which was then followed by massive synthesis of proline (Fig. 4B), as had been observed in *C. glutamicum* [27] before. BetP was found to be down-regulated at a much slower time scale (Fig. 4C) as observed under conditions of internal betaine accumulation (cf. Fig. 2). The slower response is explained by the fact that cells solely depend on internally synthesized compatible solutes for osmotic compensation (Fig. 4), whereas in previous experiments (Fig. 2 and Table 1) cells were supplied with external betaine which is accumulated by BetP. The time course of the decrease in BetP activity upon hyperosmotic shock was reflected by a concomitant increase of the internal concentration of trehalose and proline. Since down-regulation of BetP indicating adaptation was reached also in the absence of internal betaine, this solute can be excluded as a specific inhibitor for BetP.

7. Betaine accumulation in the C-terminally truncated mutant BetPΔ25

We have previously shown that BetPΔ25, a mutant which is C-terminally truncated by 25 amino acid

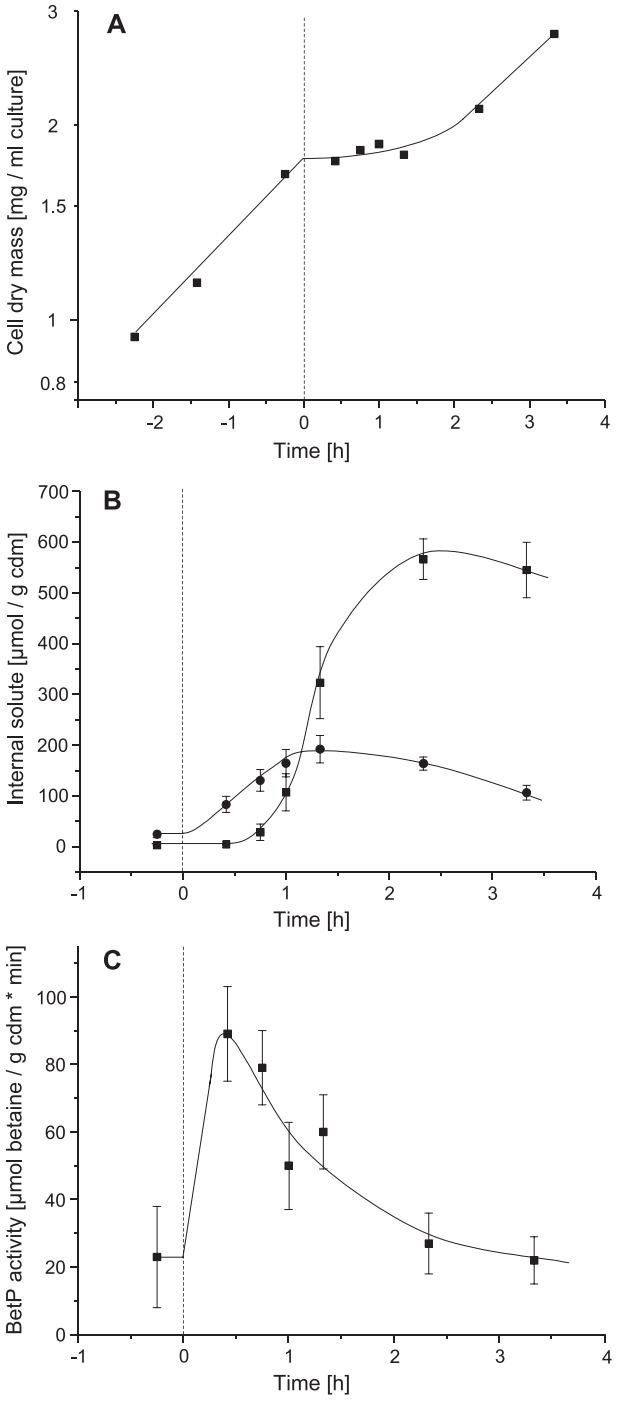


Fig. 4. Adaptation of DHPF pGTG cells to a hyperosmotic shock in the absence of betaine. Cells were pre-grown and washed in MM1 medium. In difference to the other experiments, MM1 medium was used during uptake measurement and addition of chloramphenicol was omitted to allow cells to adapt to hyperosmotic stress by synthesis of compatible solutes and by protein synthesis. Cells were subjected to a hyperosmotic shock with 740 mM NaCl at t=0, 2.5 h after the washing procedure. (A) Growth curve, (B) internal amount of the compatible solutes trehalose (circles) and proline (squares), (C) betaine uptake activity, measured by addition of 0.5 mM [^{14}C]-betaine to aliquots of the culture. Other conditions as described in Figs. 1 and 2. All values shown in A, B, and C are means of experiments carried out at least in triplicate.

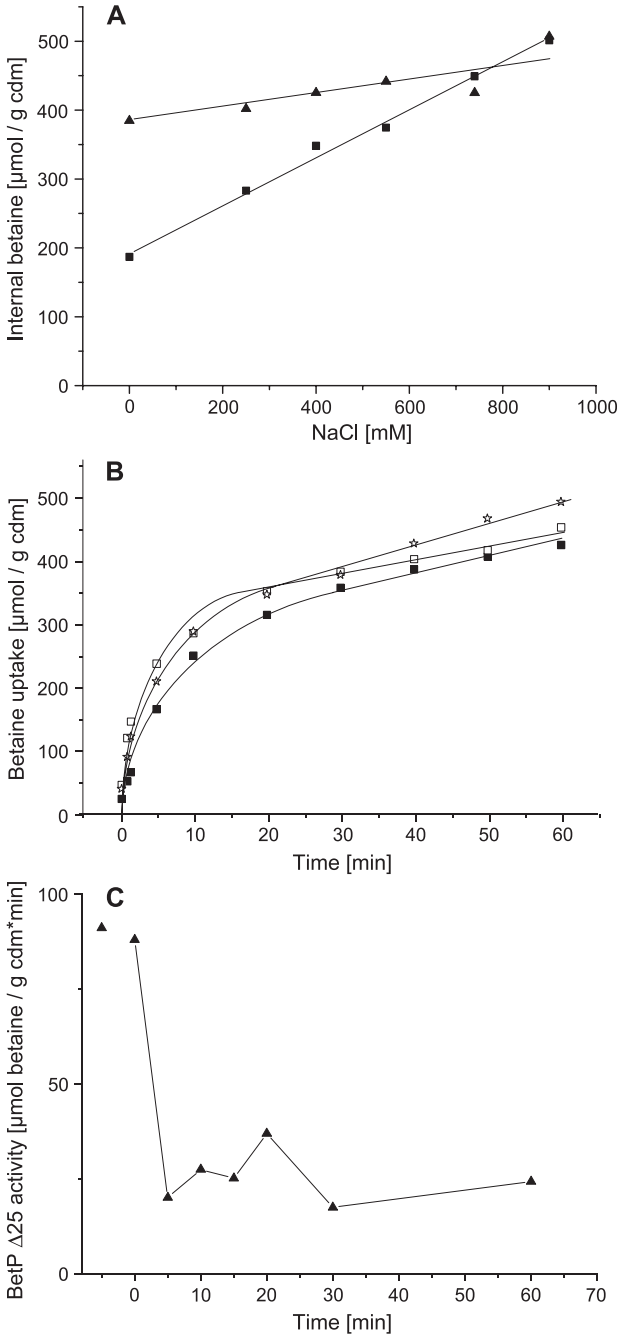


Fig. 5. Adaptation to osmotic stress in BetP wild-type and BetPΔ25. (A) Internal betaine after adaptation to hyperosmotic conditions. DHPF pGTG and DHPF pC2 cells, respectively, were subjected to hyperosmotic shocks with different amounts of NaCl. The cells harbored either wild-type BetP (squares) or the mutant BetPΔ25 (triangles). Betaine uptake was measured as described in Fig. 1; the values plotted for internal betaine represent the average of samples taken at 30, 40 and 50 min after betaine addition (see Fig. 1). (B) Glycine betaine uptake of DHPF pC2 cells expressing *betPΔ25*. The cells were either exposed to an osmotic upshift from 116 to 1564 mosM/kg (stars), from 116 to 897 mosM/kg (open squares) or not exposed to osmotic stress (solid squares). Other conditions as described in Fig. 1. (C) Same experiment as described in Fig. 2A in the presence of betaine except that BetPΔ25 was used. All values shown in A, B, and C are means of experiments carried out at least in triplicate.

residues, shows a deregulated pattern of osmoregulation [15]. This recombinant form of BetP is constantly active, i.e., even in the absence of stimuli related to hyperosmotic stress. At the same time, its affinity for the cosubstrate Na^+ is drastically decreased. We observed a K_m of 134 mM as compared to 4 mM measured in *C. glutamicum* for wild-type BetP [22,28].

We studied betaine fluxes in *C. glutamicum* DHPF cells expressing BetPΔ25 and observed a significant basic transport activity of 42.2 nmol min^{-1} mg cdm^{-1} at low external osmolality (Table 1). Addition of Na^+ increased uptake activity due to it function as co-substrate, which has previously been shown [15]. Both in the absence and in the presence of a hyperosmotic shock, BetPΔ25 reached adaptation (Fig. 5B), similar to the situation observed with wild-type BetP (Fig. 1), and unidirectional uptake v_{up} decreased significantly in both cases (Figs. 5C and 2A). Exchange was observed to an extent closely similar to that of wild-type BetP as indicated by the value of v_{eff}/v_{up}. Hence, the response to the increase in internal betaine is not abolished in BetPΔ25, which has lost its capacity for regulation of activation.

On the other hand, the correlation between the extent of osmotic stress and betaine accumulation seems to be impaired in BetPΔ25 (Fig. 5A). High internal betaine concentrations were reached already at low osmotic stress and the increase at higher osmolality was not pronounced. Taken together, BetPΔ25, which is deregulated with respect to its response to activating osmotic stimuli, is still able to reach the adapted state by down-regulating its transport activity, however, the correlation of stimulus (osmotic stress) and response (regulation of pool size) seems to be less tightly coupled as compared to wild-type BetP.

8. Down-regulation of heterologously expressed BetP in E. coli MKH13

Besides the factors studied so far which may trigger BetP activity in the adapted state, also a chemical modification or the interaction with a regulatory protein can be responsible for this type of activity regulation. To investigate this in more detail, we heterologously expressed *betP* in *E. coli* MKH13, a strain devoid of all known uptake systems for compatible solutes [16]. Osmotic shocks of lower extent (from 100 to 486 mosM/kg) were used because of the lower osmotic resistance of *E. coli*. BetP was immediately activated upon hyperosmotic shock, similar to the situation in *C. glutamicum* DHPF (Fig. 6). The high initial uptake rate of 42 nmol min^{-1} mg cdm^{-1} decreased to low values of v_{net} (3 nmol min^{-1} mg cdm^{-1}) in the adaptation phase (20–40 min). Also unidirectional uptake v_{up} decreased significantly to 19 nmol min^{-1} mg cdm^{-1} in this phase, however, the decrease was less pronounced as compared to *C. glutamicum* DHPF/pGTG. Hence, BetP shows a similar regulation pattern in terms of its response to increasing internal betaine when present in a heterologous host.

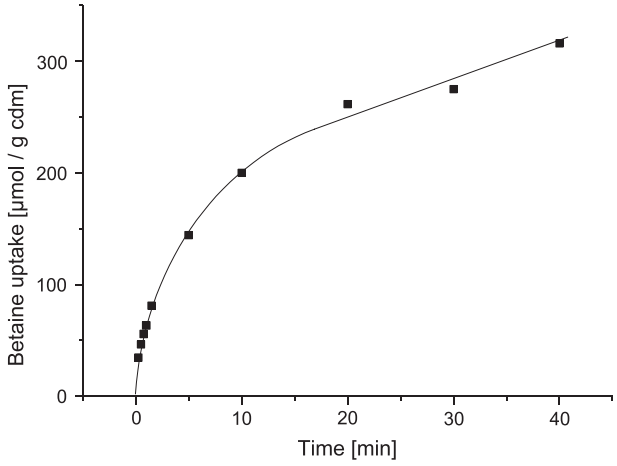


Fig. 6. Betaine uptake by *E. coli* strain MKH13 transformed with a plasmid encoding for strepBetP. Betaine uptake was assayed in 100 mM Tris/MES buffer after the addition of 200 mM NaCl. Other conditions as described in Fig. 1. All experiments were carried out at least in triplicate.

9. Discussion

In order to regulate the pool size of osmotically active solutes in the cytoplasm, bacterial cells activate uptake systems for compatible solutes in response to hyperosmotic stress and down-regulate solute uptake when osmotic compensation is reached [29]. We have studied activation of BetP, the betaine carrier in *C. glutamicum*, in response to hyperosmotic shifts [6,14]. Here we describe another type of regulation, namely how BetP adapts to the situation of increasing internal betaine in the course of compensation after hyperosmotic stress. Our results indicate that this regulatory mechanism is fundamentally different from BetP activation and also different from previously suggested mechanisms for adaptation of osmoregulated carriers. It does not involve activity of efflux channels, and it is furthermore demonstrated that the stimulus responsible for adaptation is not related to the internal accumulation of betaine or of K^+. The latter result, together with the finding that the deregulated mutant BetPΔ25 does also show the phenomenon of adaptation, furthermore clearly indicates that BetP does not simply fall back into the pre-activation state when adapting its activity to increasing internal concentration of compatible solutes.

Adjustment of transporter activity upon osmotic compensation has been studied for several ATP-driven transport systems in intact cells [8,10,26,30]. This is the first study on osmotic adaptation of an osmoregulated carrier in a cell which is devoid of any other uptake system for compatible solutes. We used a recombinant *C. glutamicum* strain in which all these systems (BetP, EctP, ProP, LcoP, and PutP)

had been deleted and *betP* was expressed from a plasmid. Consequently, betaine uptake flux can be attributed to BetP only. This does not necessarily hold for outward fluxes, which could be mediated by yet unknown efflux carriers as well as by channels, known to be present in *C. glutamicum* [17,24]. More precisely defined systems, e.g., proteoliposomes in which BetP activation was elucidated [6], were not used here, since a stable, long-lasting electrochemical potential, which would be necessary for studying the adaptation phase of a secondary carrier, is difficult to establish.

In response to a hyperosmotic shock, BetP is instantly activated in *C. glutamicum* [5,22], and lumenal K^+ was shown to play a major role as a stimulus in proteoliposomes from *E. coli* lipids [6]. We show here that net uptake of compatible solutes ceases once an appropriate cytoplasmic betaine pool is reached, and we called this the adaptation phase. Unidirectional uptake was largely down-regulated under these conditions, although the driving force for betaine uptake was found to be undiminished as indicated by the undiminished rate of Na^+ coupled glutamine uptake. This result gives rise to several questions, concerning (i) the mechanism responsible for betaine efflux in the adaptation phase, (ii) the way how betaine import and export are mutually regulated, i.e., whether activation and adaptation are regulated similarly, and (iii) concerning the kind of triggers responsible for adaptation of BetP. Whereas the first question concerns the kind of transport system(s) involved in efflux, the second addresses osmoregulation and the last question osmosensing.

The first question could be answered by analyzing betaine transport in the adaptation and consecutive reactivation phase. Although net uptake ceased after activation, it never stopped completely, indicating that this phase is not a true steady state. BetP could be instantly reactivated by a second hyperosmotic shock. Reactivation depended on osmotic stress and not on changes of the electrochemical Na^+ potential, since Na^+ could be replaced by K^+ or by uncharged solutes. In kinetic terms, at least three different mechanisms may be responsible for adaptation of BetP activity. (a) BetP may switch from a productive (net) uptake mode to an unproductive exchange mode, (b) BetP uptake activity may be down-regulated, or (c) betaine efflux may occur via other carriers or channels simultaneous to betaine uptake by BetP.

To discriminate between these models, we dissected betaine transport into three different fluxes, namely net flux v_{net}, unidirectional uptake v_{up}, and unidirectional efflux v_{ef}. Except at the beginning of the experiment where no efflux occurs, the v_{ef}/v_{up} value indicates the presence of either an exchange or a balanced uptake by BetP and efflux by different carriers/channels. Any separate efflux system, in particular an efflux channel, will be switched off after a hyperosmotic shock. As we measure similar v_{ef}/v_{up} values both in the adaptation phase and right after the second upshift, and even a strongly increased v_{ef} in response to the second shock, separate efflux systems are thus very unlikely to be responsible for the observed betaine export, in contrast to other examples [11]. Consequently, betaine efflux is most likely be ascribed to BetP, acting in the exchange mode. In kinetic terms, this is a reasonable explanation in view of high internal betaine concentrations under these conditions. A final argument against specific export systems being involved in pool size regulation in *C. glutamicum* is provided by the observation that betaine accumulation was significantly different in strains carrying the truncated form BetPΔ25, although this strain harbors essentially the same efflux systems as the wild type (Fig. 5A).

The experimental dissection of betaine fluxes provided answers to the first two questions raised above. These results led to the identification of systems involved in betaine pool size regulation, and they are indicative for the mechanism how BetP reaches the adaptation phase. It turned out that two different effects are responsible for the decrease in net uptake activity during adaptation. BetP switches to the exchange mode and, more interestingly, the V_{max} of the transporter, i.e., the measured v_{up} value, is down-regulated to low levels. The latter mechanism has not been described before for osmotic adaptation of any other osmoregulated carrier. It is furthermore not common for osmoregulated carrier systems in *C. glutamicum* either, as was shown by the analysis of ProP, which reacted differently to adaptation, although its response to an osmotic upshift is closely similar to BetP. Interestingly, in an experiment similar to that of Fig. 5C, it has previously been shown for ProP from *E. coli* that a decrease of activity is observed if ProQ was deleted, a protein of unknown function present in this organism [31].

The third question regarding the nature of the trigger(s) being responsible for BetP down-regulation is more difficult to answer. There are in principle a large number of parameters available as candidates for regulatory signals [1,29,32,33], namely (a) external osmolality, ion concentration or water activity, (b) the same parameters in the internal compartment, (c) turgor pressure, (d) specific ions or molecules, (e) membrane strain, and (f) molecular crowding.

Several factors could be ruled out as triggers for down-regulation of BetP activity. Activation of BetP in proteoliposomes was shown to depend on lumenal K^+ [6]. This correlation was also observed in *C. glutamicum* cells here, at least for BHI-grown cells. The fact that no decrease in internal K^+ was detected during adaptation argues against K^+ as a trigger for down-regulation of BetP. This result is corroborated by the observation that the truncated mutant BetPΔ25, which is insensitive to lumenal K^+ (unpublished observations), can still be down-regulated in its uptake activity, although the mutant form of BetP seems to respond by down-regulation already at lower concentrations of internal betaine as compared to the wild-type. Furthermore, the fact that adaptation was also shown for BetP when heterologously expressed in *E. coli* argues against the involvement of so far unknown protein factors. External

conditions, e.g., Na^+ or betaine concentration, were also ruled out as possible stimuli since they were kept constant throughout the experiment.

Finally, down-regulation of BetP activity may be discussed in view of the above mentioned hypothesis suggesting that internal betaine directly inhibits transport. The fact that betaine accumulation linearly depends on the extent of osmotic stress rules out both simple feedback regulation by betaine and inhibition by the increasing outward-directed chemical potential of betaine. Both parameters would not depend on the extent of osmotic stress. For carrier systems in *S. aureus* [8,9], *L. plantarum* [26] and *L. monocytogenes* [10], a modified model for feedback regulation has been suggested. To explain the systems' adapted response to osmotic stress, it was supposed that feedback inhibition is released under hyperosmotic stress.

Two results argue against this modified model of feedback inhibition as the explanation for down-regulation of BetP activity. Most importantly, BetP activity is decreased upon osmotic compensation also in the absence of internal betaine. Furthermore, although both BetP and BetPΔ25 catalyze betaine uptake, identical osmotic conditions lead to different betaine accumulation levels. If feedback inhibition was the trigger for BetP down-regulation, it would have to act on the two BetP forms at different concentrations of internal solutes. As a very remote explanation, inhibition by internal proline or trehalose in principle still remains a possibility to explain the results of Fig. 4. Both trehalose and proline, however, are not substrates of BetP, nor are they closely related to betaine in terms of structure.

It should be pointed out here that the modified feedback inhibition model has actually never been proven or disproven also in organism such as *L. plantarum* or *L. monocytogenes*, since a second model, which we prefer because of its simplicity, has not been ruled out. Down-regulation of BetP activity in the adaptation phase could be due to a trigger acting on the protein directly and indicating increasing osmotic compensation. This trigger may be related to the physical state of the membrane (membrane strain) or, alternatively, to the hydration state of BetP as influenced by the amount of free water in the cytoplasm. The latter explanation, however, does not seem to be very likely since the measured change of free water under the conditions studied here was not impressive (Fig. 3). On the other hand, osmoregulated carrier proteins have in fact been shown to be influenced by the surrounding membrane [6,35]. It has also to be emphasized that a modified model of feedback inhibition is necessarily more complicated. It essentially requires, besides the presence of a second, regulatory binding site, also a second stimulus (signal input), which is responsible for the release of feedback inhibition under hyperosmotic stress. The release of feedback inhibition, then, has to depend linearly on the extent of stress. It furthermore requires the affinity of the putative regulatory site for solutes which are not substrates of the carrier. Notably, the feedback inhibition model was recently found not to be valid for osmosensitive betaine uptake in intact cells of *L. lactis* [30] as well as for reconstituted OpuA from *L. lactis* [34].

Acknowledgement

We thank Dr. J. Kunte, M. Stein, and A. Wirtz for help in the HPLC analysis of betaine. This work was financially supported by the DFG (SP 1070).

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