Biochimica et Biophysica Acta 1838 (2014) 1288–1295

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Stimulus analysis of BetP activation under in vivo conditions

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 18 December 2013
Accepted 23 December 2013
Available online 30 December 2013

Keywords:
BetP
Transport
Stimulus
Osmoregulation
Reconstitution
Corynebacterium

ABSTRACT

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

1. Introduction

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

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

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

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

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http://dx.doi.org/10.1016/j.bbamem.2013.12.017

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

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strains, plasmids, and growth conditions

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

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

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

2.4. Transport assays

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

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

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

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

2.6. Western blot analysis

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