Lipid-Protein Interactions in the Regulated Betaine Symporter BetP Probed by Infrared Spectroscopy*

Received for publication, October 27, 2014, and in revised form, November 4, 2015 Published, JBC Papers in Press, November 22, 2015, DOI 10.1074/jbc.M114.621979

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The Na+-coupled betaine symporter BetP senses changes in the membrane state and increasing levels of cytoplasmic K+ during hyperosmotic stress latter via its C-terminal domain and regulates transport activity according to both stimuli. This intriguing sensing and regulation behavior of BetP was intensively studied in the past. It was shown by several biochemical studies that activation and regulation depends crucially on the lipid composition of the surrounding membrane. In fact, BetP is active and regulated only when negatively charged lipids are present. Recent structural studies have revealed binding of phosphatidylglycerol lipids to functional important parts of BetP, suggesting a functional role of lipid interactions. However, a regulatory role of lipid interactions could only be speculated from the snapshot provided by the crystal structure. Here, we investigate the nature of lipid-protein interactions of BetP reconstituted in closely packed two-dimensional crystals of negatively charged lipids and probed at the molecular level with Fourier transform infrared (FTIR) spectroscopy. The FTIR data indicate that K+ binding weakens the interaction of BetP especially with the anionic lipid head groups. We suggest a regulation mechanism in which lipid-protein interactions, especially with the C-terminal domain and the functional important gating helices transmembrane helix 3 (TMH3) and TMH12, confine BetP to its down-regulated transport state. As BetP is also activated by changes in the physical state of the membrane, our results point toward a more general mechanism of how active transport can be modified by dynamic lipid-protein interactions.

The secondary active betaine transporter BetP is the major defense system for the Gram-positive soil bacterium Corynebacterium glutamicum to counteract hyperosmotic stress. Like all soil bacteria, C. glutamicum cells are often exposed to changes in osmolality resulting in instant water flux across the cell membrane. Hyperosmotic stress is followed by dehydration, shrinkage, and subsequent collapse of the cell. To survive, C. glutamicum counteracts the high external osmolarity by the activation of osmolyte uptake systems as BetP. BetP is a member of the betaine-choline-carnitine transporter (BCCT) family and imports exclusively the organic osmolyte betaine into the cytoplasm, a transport process that is energetically coupled to the electrochemical Na+ potential by co-transport with two Na+ ions (1–3). BetP is so far one of the best-characterized osmoregulated transporter proteins in terms of transport and regulation. BetP senses the increasing cytoplasmic K+ concentration as a measure of hyperosmotic stress via the C-terminal domain and regulates transport activity by a yet unknown interaction network with the N-terminal domains and cytoplasmic loops but also lipids (4–7). K+ activation is not the only stimulus; there is some evidence (8) that the physical state of the membrane that is affected by hyperosmotic stress also activates BetP. Moreover, the activation optimum depends strongly on the net charge of the membrane surface (9). In the entirely negatively charged C. glutamicum membranes (10) BetP is less sensitive than in Escherichia coli, where BetP reaches its activity optimum already at a much lower osmolality. Lipid-dependent regulation of BetP has been extensively studied in vitro by using various lipid combinations. Aside of the head group charge, the fatty acid composition has an impact on the transport activity of BetP (11). It was proposed that the osmosensing C-terminal domain of BetP harboring 14 positively charged arginine residues provides possible interaction sites with anionic lipids (12, 13), but the consequences of such an interaction could not be deduced without structural data.

Several atomic structures of the trimeric BetP (Fig. 1, A and B) in different transport states have been recently solved (12, 14, 15). The monomer of BetP (Fig. 1, C and D) consists of 595 amino acids comprising 12 transmembrane helices (TMH)3 that span the membrane as well as a curved α-helix (helix h7, Fig. 1A) at the periplasmic membrane surface (12). The positively charged osmosensing C-terminal domain protrudes into the cytoplasm (Fig. 1B). In the trimer, C-terminal domains interact with each other and with loops from adjacent protomers. The latest structure of BetP to 2.7 Å resolution (PDB entry code 4C7R) reveals specific binding of eight phosphatidylglycerol lipids to a hydrophobic cavity in the center of the BetP trimer as well as to the outer rim of BetP (Fig. 1) (16). Thereby,

* This work was supported by Collaborative Research Center SFB 807 “Transport and Communication across Biological Membranes” of Deutsche Forschungsgemeinschaft. The authors declare that they have no conflicts of interest with the contents of this article.

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3 The abbreviations used are: TMH, transmembrane helix; ATR, attenuated total reflection; νas, antisymmetric stretching; νs, symmetric stretching; PI, phosphatidylinositol; PG, phosphatidylglycerol; Cg, C. glutamicum; BetP ΔC45, C-terminal truncated BetP.
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**FIGURE 1.** Lipid-protein interactions in the BetP trimer (PDB entry code 4C7R). A, top view from the periplasmic side on the BetP trimer solved to a resolution of 2.7 Å in complex with 8 POPG lipids (16). The individual protomers are colored differently to highlight functional domains. In protomer A the bundle helices TMH3-TMH4 (first repeat) and TMH8-TMH9 (second repeat) are colored in red. TMH1-TMH2, which are not part of the transporter core acting as stabilizing scaffold, as well as the amphipathic helix h7, which mediates intratrimERIC contacts, are colored in light orange. In protomer B, the scaffold TMH5-TMH7 and TMH10-TMH12 is colored in blue. In protomer C, only the gating helices TMH3 (red) and TMH12 (blue) are colored. The lipids are depicted in sphere representation and colored similar to their interaction partners, e.g., helices or loops of either bundle, scaffold, or stabilizing domains. B, side view on protomer A and protomer C. Lipids are found in the center of the BetP trimer stabilizing the C-terminal domains, in between two adjacent protomers (orange), and at the perimeter of individual protomers. They span the whole bilayer reaching to the amphipathic helix h7 (blue) that is plunging in the upper part of at the periplasmic leaflet. C, top view from the cytoplasmic side on the BetP monomer (protomer C in A). Helices are color coded with bundle (red), scaffold (blue), and TMH1-TMH2 and h7 in light orange. Lipids are depicted as spheres. Positively charged residues in loop2 (Lys-121, Arg-126), loop4 (Lys-228), and loop8 (Arg-395) as well as in TMH12 (Lys-542) and the C terminal domain (Arg-554) are shown in stick representation. D, side view on protomer C with the cytoplasmic parts of the gating helices TMH3 (red) and TMH12 (blue) interacting with the fatty acyl chains of POPG lipids.

These lipids are bound to essential parts of the transporter core (Fig. 1, C and D). The transporter core in BetP shares the so-called LeuT-like-fold of two inverted structurally related repeats of five TMHs. An iris-shaped 4-TMH bundle is formed by the first two helices of each repeat (Fig. 1, A and C, in red in protomer A). Two long and highly tilted helices and the third helix of each repeat together with two V-shaped hairpins from the fourth and fifth helix, respectively, form a scaffold adjacent to the bundle (Fig. 1, A and C, in blue in protomer B). The central pathway for betaine and sodium runs in between these two domains (Fig. 1, C, black cross, and D). From the eight lipids observed in the trimeric structure, at least five lipids are located at functional important helices, mainly close to the cytoplasmic parts of the bundle helix TMH3 and the scaffold helix TMH12 (Fig. 1D, lipids are colored similar to the helices with which they are interacting). These two helices are crucial in betaine transport (17) and often are referred to as gating helices. Both perform an alternating hinge-like movement to change the accessibility of the central substrate binding site. The cytoplasmic part of TMH3 (Fig. 1C, in red) moves away from the center of the transporter core to enable the release of betaine and sodium. The periplasmic part of TMH12 moves toward the center in the transition from outward open to inward open when the substrates are bound. In addition, TMH12 is directly followed by the osmosensing C-terminal domain, which is also involved with its first eight amino acids (Tyr-550 –Arg-558) in lipid-protein coordination.

Despite the fact that the majority of lipids interacts to some extent with helices from the transporter core, it was not possible to assign unambiguously a functional role to the observed lipid-protein interactions mainly because of the unknown activation state the individual BetP structures represent. Interestingly, although positively charged residues are found in close proximity to the negatively charged PG head groups (Fig. 1C) the coordination is less specific than anticipated from the specific PG binding, which was tested previously by thin layer chromatography and mass spectrometry (16).

However, the crystal structure suggests that the rather loose coordination of lipids might involve another parameter, e.g., the presence of K⁺ or lipid-lipid interaction. Answering the question if activation requires transient lipid binding will be an important step toward understanding of the role of lipids in the molecular regulation mechanism of BetP.

Therefore, in the present study the lipid-protein interactions of BetP upon K⁺ activation have been investigated on a molecular level by using FTIR spectroscopy equipped with an ATR accessory to identify specific changes under activating conditions. IR spectroscopy is a perfect tool to selectively probe the perturbations on the hydrophobic lipid tails, the interfacial region, or on the polar head groups of lipids by a membrane protein (18, 19). To investigate BetP in a native-like environment we have reconstituted the transporter in two-dimensional crystals formed from native C. glutamicum lipids (1:1:1 PG:PI:cardiolipin). Two-dimensional crystals provided a controlled and ordered arrangement of the transporter in the lipid bilayer for the FTIR measurement. For BetP reconstituted in two-dimensional crystals of native C. glutamicum lipids, it could recently be shown that the transporter remains fully functional after two-dimensional crystallization in comparison to its transport properties in proteoliposomes (40). The lipid signals in the IR spectrum can be followed in width and position to yield information regarding the lipid-protein interactions as an external parameter. Besides, observation of the accompanying spectral alterations in the individual amino acid side chains from the protein environment as well as in the amide modes from the protein backbone can help to reveal the interactions of the lipids with specific sites of the proteins (20–22).

We observe that under activating conditions phospholipid head groups undergo K⁺-dependent changes in their electrostatic and H-bonding properties favoring weak H-bonds or unbonded head groups whereas the hydrophobic tails become more mobile and even disordered upon K⁺-induced activation of BetP.
Experimental Procedures

Preparation of the BetP Samples

Cell Culturing and Protein Purification—Cell culture and protein preparation methods have been described previously (9). E. coli DH5αmcr (23) was used for the heterologous expression of strep-betP. Cells were grown at 37 °C in LB medium supplemented with carbencillin (50 μg/ml), and induction was initiated with anhydrotetracycline (200 μg/liter). Cells were harvested at 4 °C by centrifugation and resuspended in buffer containing 100 mM Tris-HCl (pH 7.5) and protease inhibitor Pefabloc 0.24 mg/ml. Membranes were isolated from disrupted cells and solubilized with 1% β-dodecyl-maltoside. The protein was then loaded on a StrepII-Tactinmacroprep® column, washed with 50 mM Tris-HCl (pH 7.5), 200–500 mM NaCl or only 200 mM NaCl if used for two-dimensional crystallization, 8.6% glycerol, 0.05% β-dodecyl-maltoside, and eluted with 5 mM desthiobiotin, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 8.6% glycerol, and 0.05% β-dodecyl-maltoside.

Two-dimensional Crystalization of WT-BetP and C-terminal-truncated BetP (ΔC45)—A lipid mix of PI, PG, and cardiolipin (1:1:1) dissolved in chloroform was dried by evaporation and solubilized into 1% decylmaltoside (Glycon) at a final concentration of 4 mg/ml. Protein-lipid-detergent mixtures were adjusted to contain 2.5 mg/ml protein, 0.15% decylmaltoside, and a lipid-to-protein ratio of 0.2 (w/w). After incubating overnight, the sample was placed into Mini Slide-A-Lyzer10K dialysis devices (Pierce) and dialyzed at 37 °C for 2 weeks against 500–600 ml of dialysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 5% 2-methyl-2,4-pentanediol, 2.5 mM CaCl₂, and 3 mM NaN₃) with BioBeads added.

Protein Samples Used for IR Spectroscopy and Activating/Inactivating Buffer Conditions

The blank C. glutamicum lipids in the absence of protein (C₉-liposomes) as well as samples of BetP two-dimensional crystals reconstituted in C. glutamicum lipids described above were used without further processing. The buffer of the samples was exchanged to the desired sodium phosphate buffer at pH 7.5 either by centrifugation (6000 rpm for ~30 min, at +4 °C) in an Eppendorf tube, or samples of the protein were directly placed on the ATR diamond to achieve the buffer exchange by means of a continuous-flow perfusion technique (24) overnight at +4 °C.

As a solvent, 10 mM sodium phosphate (NaPi) buffer in water (H₂O) and in deuterium oxide (99.9% D₂O) at pH/pD 7.5 was utilized. The desired buffer conditions were adjusted by adding the salts of potassium chloride (KCl) and sodium chloride (NaCl) into the 10 mM NaPi buffer to activate and inactivate the protein, respectively. The final pH value was controlled with a pH meter. The formula pD = pH + 0.4 was used to measure the pD value of the deuterated buffer as the pD values are 0.4 digit higher than the pH meter reading (25, 26).

In Situ Activation of BetP by Using ATR-FTIR Spectroscopy

In the present study a Bruker Vector 22 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with an ATR accessory and a liquid N₂-cooled MCT detector was used to record the infrared spectra. The combination of a home-built microdialysis cell system with ATR-FTIR spectroscopy allows us to perfuse the protein sample continuously with the buffer of interest (24). Thereby, in situ changes in a protein can be followed diligently during a long term experiment by using only one sample. Titration experiments with increasing K⁺ concentration and in situ activation/deactivation of BetP samples were performed by using this microdialysis perfusion method (24). As already reported, BetP has a cation-specific activation mechanism, with an increase in the cytoplasmic K⁺ (and Rb⁺ or Cs⁺) concentration acting as a trigger mechanism to activate BetP. However, BetP is not significantly activated in response to an increased in the cytoplasmic Na⁺ concentration (2). Therefore, in this study the BetP sample was activated with a buffer containing K⁺ ions, whereas a buffer containing Na⁺ ions was used as inactive buffer conditions.

A 5-μl sample of protein (~2.5 mg/ml) was placed on top of the ATR element and dried by monitoring the O-H stretching band (3000–4000 cm⁻¹) in the spectrum. This was repeated until a desired absorbance was reached to obtain a concentrated, semi-dried sample (~25 mg/ml final concentration). After the sample was sealed with a dialysis membrane with a 25-kDa molecular mass cut-off (Spectra/Pore 6), the BetP sample was equilibrated by a continuous flow of inactivating H₂O buffer (10 mM NaPi (pH 7.5), 500 mM NaCl) with the help of the inlet and outlet tubing system. After stable IR absorbance spectra were obtained, the sample was subsequently titrated with increasing K⁺ while keeping the total ionic strength constant in H₂O buffer. Because Na⁺ ions do not activate the BetP protein, the final concentration of ionic strength of the buffer was adjusted to totally 500 mM by replacing NaCl with KCl. The concentration of K⁺ was increased from 0 mM (inactive state condition) to 500 mM (active state condition) in 50 mM K⁺ intervals. Then the concentration of K⁺ was switched back to 0 mM again (2nd circulation of the inactivating buffer, which does not include K⁺ ions) so that a stepwise activation of BetP is provided. Totally 14 buffers with different ionic strength of K⁺ (from 0 to 10, 50, 100, 150, 200, 220, 250, 300, 350, 400, 450, 500 mM and back to 0 mM) were perfused across the protein sample at a constant speed (0.32 ml/min) provided by a pulse-free peristaltic pump. Each buffer was perfused for 100 min to equilibrate the buffer of the sample on the ATR crystal. The spectra were recorded every 2.5 min at a resolution of 4 cm⁻¹. A total of 256 scans were averaged before Fourier transformation, taking the spectrum of the empty ATR cell as background. The temperature of the sample was adjusted to +4 °C with the help of a circulating water bath and controlled routinely with a thermocouple. Additionally, the buffer to be perfused across the protein sample was always kept in ice during the perfusion process to ensure the temperature of the sample on ATR unit as +4 °C.

In the absence of protein the blank lipid samples and the buffers were titrated with increasing K⁺ concentrations under otherwise identical conditions (i.e. time, temperature, salt concentration, pH, number of scans, and nominal resolution) to compare the spectral alterations. It is also noteworthy to address that each buffer used for K⁺ titration experiments was 35 ml, from which 15 ml is discarded to ensure that the buffer in the closed compartment and in the tubing systems is com-
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completely exchanged to new buffer. Later on, the remaining 20 ml of buffer were used for the closed circulation through the protein sample.

Analysis of the IR Spectra—Spectra processing and visualization were carried out using the spectrometer OPUS software 4.2 (Bruker). The last five spectra for each buffer condition were averaged to be used for data processing. Because atmospheric water vapor absorption is difficult to avoid in long term experiments and is superimposed on the protein spectra (27, 28), the contribution of atmospheric water vapor to the protein sample was removed by subtracting a water vapor spectrum from the sample spectrum. To do so automatically, a software program was used (29). Afterward, absorption of the corresponding H2O buffer was subtracted from the sample spectrum (BetP or pure lipids) by taking the O-H stretching region at $\sim 3400 \text{ cm}^{-1}$ as reference.

Infrared Difference Spectra—By means of the ATR perfusion cell technique, we obtained activation-induced IR difference spectra that represent any small spectral alterations such as shift of band position or changes of band intensity, all of them related to the reaction induced. The difference spectrum was calculated taking the protein as reference in 10 mM NaPi (pH 7.5), 500 mM NaCl (inactive state) so that the resultant absorbance difference corresponds to the difference spectrum active – inactive. In the difference spectrum the positive signals demonstrate the final state (active state) of the sample in the presence of K+ ions, whereas the negative ones correspond to the initial state (inactive state) in the absence of K+ ions. Difference spectra of the blank lipid sample as well as buffer difference were used for comparison. Difference spectrum of the buffer in the absence of the lipids and BetP was obtained by subtraction of the absorbance spectrum of buffer recorded at 0 mM K+ (inactivating buffer condition) from the absorbance spectrum recorded at 500 mM K+ (activating buffer condition).

Statistics—Statistical analysis was performed using the SAS 9.3 TS Level 1M2 program. The values of the mean ± S.D. as well as the degree of significance (denoted as $p$ value) of the differences between mean values of two groups (for activation and inactivation conditions) was calculated by using the Wilcoxon Scores (Rank Sums) test.

ExperimentsPerformed with FTIR Transmission Spectroscopy

Infrared spectra were recorded in the transmission mode on a Vector 22 FTIR spectrometer (Bruker) equipped with a DGTs detector. The O—P—O stretching modes of lipid head groups give rise to the absorbance bands in the spectral region 1265–1000 cm$^{-1}$. IR band positions depend on the hydration state of the lipids. Because the lipid head groups are sensitive to hydrogen bonding as well as to electrostatic interactions, recording of this spectral region in both H2O and D2O allows us to compare the IR bands and spectral alterations (i.e. shifting of vibrations of O—P—O modes upon K+-induced activation) observed in D2O with those observed in H2O. Therefore, BetP two-dimensional crystals and Cg-liposomes were incubated in 2H2O buffer (10 mM NaPi, pH 7.5) in the absence (0 mM K+) and presence of K+ ions (200 mM and 500 mM) and kept 3 days at $+4^\circ \text{C}$. A 1.7-µl sample of BetP was loaded on the CaF2 cells (30 with a path length of 8 µm. The cuvette was diligently sealed with PTFE paste (Carl Roth, Karlsruhe, Germany) to avoid drying of the sample upon heating and subsequently mounted into a thermostated sample holder. After the sample was equilibrated at $+4^\circ \text{C}$, the temperature was increased stepwise from $+4^\circ \text{C}$ to $+94^\circ \text{C}$ in 2 °C intervals using a water bath circulating through the sample holder. For both sample and reference spectra 128 interferometer scans were averaged at 4 cm$^{-1}$ spectral resolution. The blank lipid samples in the absence of protein as well as the corresponding buffer solutions were measured under the same conditions. Absorption of the corresponding 2H2O buffer was subtracted from the each sample spectrum by taking the O-D bending region at around $1200 \text{ cm}^{-1}$ as reference.

Results

C. glutamicum membranes comprise only anionic glycerophospholipid species, PG, PI, and cardiolipin (each at $\sim 1/3$) (11) (Fig. 2A, inset). Therefore, two-dimensional crystallization was performed with exactly the same lipid composition, yielding vesicular closely packed two-dimensional crystals (Fig. 2B) as tested by freeze fracture of the crystals (Fig. 2B). The use of closely packed BetP two-dimensional crystals provides an elevated amount of protein but ensures a sufficient interaction cross-section of lipids (Fig. 2B). Moreover, the two-dimensional crystals used in this study oppose a minimum of crystal contacts, as it can be anticipated from the projection structure of wild type BetP in Cg-lipids (lower panel, Fig. 2B, Ref. 31).

The polar head groups and the nonpolar acyl chains provide reporter groups for different depths in the lipid bilayer that lead to distinct signatures in the IR spectrum due to CH$_3$, CH$_2$, C=O, and PO$_2^-$ stretching modes that serve to analyze the physical properties of membrane lipids and lipid–protein interactions (Fig. 2A). The IR spectrum of C. glutamicum liposomes, i.e. protein free liposomes prepared from the C. glutamicum lipids (PG, PI, and cardiolipin), which will in the following be denominated as Cg-liposomes and serve as a control, was recorded in H2O buffer in the absence of K+ ions (10 mM NaPi, (pH 7.5), 500 mM NaCl). The infrared signatures of the phos-
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Band positions and assignments of phospholipid signals in the IR spectrum for Cg-liposomes and BetP two-dimensional crystals in H$_2$O buffer

The peak positions were determined from the absorbance spectrum in Fig. 2A as well as from the IR second derivative spectra to resolve the band positions.

| Assignment                  | Position (cm$^{-1}$) for Cg-liposomes | Position (cm$^{-1}$) for BetP |
|-----------------------------|--------------------------------------|-----------------------------|
| CH$_3$ stretching (antisymmetric) | 2957                                | 2957                        |
| CH$_2$ stretching (antisymmetric) | 2900                                | 2917                        |
| CH$_2$ stretching (symmetric)   | 2873                                | 2972                        |
| CH$_3$ bending, scissoring     | 2851                                | 2849                        |
| C=O stretching (esters)        | 1741, 1720                          | 1742                        |
| CH$_2$ bending, scissoring     | 1466                                | 1469                        |
| CH$_2$ bending, scissoring     | 1453 (shoulder)                     | 1454                        |
| CH$_3$ bending (symmetric)      | 1415                                | 1417 (shoulder)             |
| CH$_3$ bending, deformation (symmetric) | 1378                          | 1366, 1368                  |
| PO$_2^-$ stretching, double bond | 1206                                | 1239                        |
| (antisymmetric)                |                                      |                             |
| C–O stretching                 | 1172                                | 1172                        |
| C–C stretching                 | 1148                                | 1150                        |
| PO$_2^-$ stretching, double bond (symmetric) | 1091                        | 1092                        |
| R–O–P–O–R$'$                  | 1070                                | 1071                        |

C–H Stretching Modes from the Hydrophobic Tails of Lipids—
The IR second-derivative spectra of the Cg-liposomes and BetP two-dimensional crystals were recorded at K$^+$ concentrations increasing stepwise from 0 to 500 mM (Fig. 3). The carbonate stretching modes from the hydrophobic tails of bilayer lipids give rise to the strongest absorbance bands in the spectral region 2800–3100 cm$^{-1}$ with their position, depending on the acyl chain packing and conformation. However, the bending vibrations (scissoring, wagging) of those molecules result in weak signals in the 1350–1500 cm$^{-1}$ region (Fig. 2A) and overlap with a number of amino acid side chain absorbance. Henceforth, one can analyze the potential interactions of the hydrophobic part of the lipid bilayer with the protein by following these signals. In particular, the C–H stretching vibrations are used to obtain information regarding the freedom of motion and fatty acyl order (34). Cg-liposomes (Fig. 3B) exhibited band positions at 2920 and 2851 cm$^{-1}$ attributed to the antisymmetric and symmetric CH$_2$ stretching vibrations, respectively, and at 2957 and 2873 cm$^{-1}$ attributed to the antisymmetric and symmetric CH$_3$ stretching vibrations, respectively. These peak positions observed for Cg-liposomes (Fig. 3B) are downshifted regardless of K$^+$ concentration in BetP two-dimensional crystals (Fig. 3A) 2920 → 2917 cm$^{-1}$, 2957 → 2849 cm$^{-1}$ for the CH$_2$ modes and 2957 → 2956 cm$^{-1}$, 2873 → 2872 cm$^{-1}$ for the CH$_3$ modes). This indicates that the membrane lipid acyl chains in the hydrophobic region reveals a higher order in the presence of BetP, resulting in a favorable alteration in the lipid chain packing for the protein-lipid system.

Cg-liposomes and BetP two-dimensional crystals, respectively, were stepwise titrated with increasing K$^+$ concentration in H$_2$O buffer from 0 mM (1st cycle of the inactive state) to 500 mM (active state) and, afterward, switched again to 0 mM K$^+$ (2nd cycle of the inactive state). The total ionic strength was kept constant to prevent the ion shielding effect in the protein. According to previous studies, BetP was shown not to be activated by increasing internal Na$^+$ concentrations (2); therefore, NaCl within the NaPi buffer was replaced with KCl to adjust the final concentration of ionic strength to totally 500 mM. An increase in the K$^+$ concentration perturbs the fatty acid moieties in the IR spectrum of the BetP two-dimensional crystals (Fig. 3A). Both CH$_2$ and CH$_3$ stretching vibrations of the fatty acid chains are slightly upshifted upon K$^+$-induced activation of BetP (2848.63 → 2848.76 cm$^{-1}$ for the v$_s$(CH$_2$) modes and 2871.92 → 2872.23 cm$^{-1}$ for the v$_s$(CH$_3$) modes) suggesting minor conformational disordering of the acyl chains in the hydrophobic part of the lipid-BetP system. Although these changes are small, the shift is relevant, and this can be measured
at the precision required. The experiments were repeated three times for inactivating (0 mM K\(^+\)/H\(_2\)O) and activating (500 mM K\(^+\)/H\(_2\)O) buffer conditions to confirm the reproducibility of the FTIR data. In all cases small spectral alterations were observed, and the tendency was the same: the C-H stretching vibrations of the fatty acid chains were slightly upshifted upon activation of BetP with K\(^+\). This confirms that the results are reproducible and realistic. The degree of significance of the differences between the two groups (for activation and inactivation conditions) is 10\% (the \(p\) value was found as 0.0684). When BetP two-dimensional crystals were again perfused with inactivating buffer for the second time, which does not involve K\(^+\)/H\(_2\)O ions (2nd cycle of inactivation), these IR signals shifted to a slightly lower position again, close to the position recorded during the 1st circulation of inactivating buffer without K\(^+\) ions. On the other hand, the frequency of the CH\(_2\) stretching modes for the blank lipid samples downshifts (2920 → 2919 cm\(^{-1}\) and 2851 → 2850 cm\(^{-1}\)), whereas its CH\(_3\) stretching modes located at around 2957 and 2873 cm\(^{-1}\) do not change significantly in response to a rise in the K\(^+\) concentration (Fig. 3B).

The spectral shift determined from the IR second-derivative spectra was drawn as a function of increasing K\(^+\) concentration (Fig. 4, C and D). The plots of the change in the bandwidth are very similar to those of wave number shifting. Both antisymmetric (data not shown) and symmetric stretching vibrations of CH\(_2\) and CH\(_3\) modes for BetP two-dimensional crystals broaden slightly with increasing K\(^+\), which was not observed for Cg-liposomes. This indicates that K\(^+\) ions induce to some extent an increment in the dynamics of the fatty acyl chains. Even though a minute change is monitored in the FTIR spectra for the C-H stretching modes of the lipid-protein system, characteristic for the non-H-bonded hydrophobic groups, the frequencies of those modes have a tendency to upshift and to broaden bands in the presence of K\(^+\) ions. Particularly, the position and bandwidth of the CH\(_3\) stretching modes for the BetP-lipid system are exactly the same during perfusion of the 1st and 2nd cycle of inactivating buffer without K\(^+\) ions, becoming a narrower band and having a lower frequency as compared with the recorded spectra under high K\(^+\) concentrations. Altogether the FTIR data strongly suggest that the hydrophobic tails of the lipids involved in lipid-protein interactions become more mobile and even disordered upon K\(^+\)-induced activation of BetP.

**PO\(_2\) Stretching Modes from the Phospholipid Head Groups**—Among the reporter groups for probing the lipid-protein interactions near to the membrane surface are the infrared signatures of the phosphate moiety found in the hydrophilic region of the phospholipids. The O—P—O antisymmetric and symmetric stretching vibrations are located in a wide range at ~1190–1265 cm\(^{-1}\) and around 1100–1050 cm\(^{-1}\), respectively, and are sensitive to hydrogen bonding. The number of these bands and their exact frequency strongly depends on the hydra-
tion state of the lipids, i.e. hydration of phosphate head groups results in a downshift of the frequencies of the O—P—O stretching modes (32, 35).

The antisymmetric stretching vibration \( \nu_{as}(PO_2^-) \) modes show a broad band at around 1239 cm\(^{-1} \) for BetP two-dimensional crystals (Fig. 5A, Table 1), whereas the band position of pure lipids appears at 1217 cm\(^{-1} \). This might indicate that BetP interacts with lipid head groups resulting in less stable H-bonding/electrostatic interactions and thereby altering the electronic microenvironment of the phosphate head groups. In fact the x-ray structure revealed an interaction of the lipid phosphate groups with positively charged residues (Fig. 6B). Splitting of these bands into seven positions absorbing at 1260, 1246, 1238, 1225, 1213, 1203, and even 1192 cm\(^{-1} \) for the BetP-lipid system can be noticed in the second-derivative spectrum (Fig. 5B). The second-derivative spectrum of the pure lipids (Fig. 5D) reveals only four band positions absorbing at 1264, 1244, 1223, and 1208 cm\(^{-1} \) (at 0 mM K\(^+ \)) as compared with the BetP-lipid system. Therefore, when the protein is interacting with phospholipids, at least three different forms of PO\(_2^-\) groups gain a distinct position due to a change in the H-bonding properties and/or electrostatic interactions, i.e. PO\(_2^-\) groups of hydrated phospholipids are in the polar environment and, therefore, maintain H-bonding interactions with the solvent. The strength of H-bonding to the O—P—O bond alters the electron density around the O—P—O bond of the phosphate head groups, inducing a change in the vibrational frequency of the O—P—O bond. The frequencies of the PO\(_2^-\) groups of hydrated phospholipids are observed at a lower frequency in comparison to anhydrous phospholipids. Therefore, the observed three different frequencies reflect a different hydration status and H-bonding strength when BetP is reconstituted into the phospholipid bilayer. This suggests that the lipid-protein interactions substantially influence some PO\(_2^-\) groups, which are located in the polar environment of the protein (periplasmic and/or cytoplasmic sites), resulting in a change of the hydration status of the PO\(_2^-\) groups and in the H-bonding strength disregarding of K\(^+\) ions.

However, upon a rise in the K\(^+\) concentration, the intensity at 1225 and 1213 cm\(^{-1} \) attributed to the strongly H-bonded phosphate head groups decreases concomitantly with a shift toward higher wave numbers (1213 → 1214 cm\(^{-1} \)), whereas the intensity at 1260, 1246 and 1238 cm\(^{-1} \) ascribed to the free/
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state of the membrane lipid head groups, BetP two-dimensional crystals and Cg-liposomes were incubated in D2O buffer in the absence (0 mM K⁺) and presence (200 mM and 500 mM) of K⁺ ions and kept 3 days at +4 °C. Afterward, they were analyzed by FTIR spectroscopy in the transmission mode at +4 °C (Fig. 8A). Obviously, the protein-lipid system again harbors three more components for the phospholipid PO₄⁻ modes (both antisymmetric and symmetric stretching modes) as compared with pure lipids, whereas pure lipid head groups have only one component at 1213 cm⁻¹ (with 0 mM K⁺); protein reconstitution leads to bands absorbing at 1262, 1244, and 1224 cm⁻¹ (with 0 mM K⁺), indicating the presence of both free/weak and strong H-bonds. This determination matches definitely with the spectra recorded in H2O buffer. These components are perturbed in the presence of K⁺. BetP two-dimensional crystals exhibit a band at 1212 cm⁻¹ (strongly H-bonded) in the deuterated buffer without K⁺ ions, whereas this band position shifts upward to 1222 cm⁻¹ and 1232 cm⁻¹ (free/weak H-bonded) in the presence of 200 mM and 500 mM K⁺ concentrations, respectively (Fig. 8A). This large upshifting of both antisymmetric and symmetric stretching vibrations of O—P—O modes (1212 → 1222 → 1233 cm⁻¹ for 0 → 200 → 500 mM K⁺ ions) together with the appearance of a strong band at 1233 cm⁻¹ of free/weakly H-bonded head groups is an unequivocal indicator that proves the release of some anionic phospholipids head groups formerly bound to BetP under activating high K⁺ conditions.

Two-dimensional crystals of BetP ΔC45 (C-terminal truncated form of BetP) were also analyzed with FTIR spectroscopy in the active (500 mM K⁺) and inactive state (0 mM K⁺) conditions to figure out the possible interactions of the C-terminal domain with the environment. A weak interaction of the C-terminal domain either with adjacent domains of BetP or with the lipid bilayer was suggested by EPR studies (36). The C-terminal truncated BetP two-dimensional crystals exhibit a clear signal at around 1218 cm⁻¹ both in the active and inactive states (Fig. 8B). This low frequency mode is attributed to the antisymmetric stretching vibrations of O—P—O modes of the phospholipid head groups, which are strongly H-bonded. The peak positions of the phospholipids νas(PO₂⁻) modes are not significantly altered with K⁺. This implies that the phospholipid head groups surrounding BetP ΔC45 two-dimensional crystals do not respond to K⁺-induced activation in the absence of the C

FIGURE 7. ATR-IR difference spectrum of BetP showing the antisymmetric and symmetric stretching vibrations of O—P—O modes in H₂O buffer. The difference spectrum of BetP (solid line) was recorded taking the reference as inactive state of BetP: (active)-(inactive). The blank lipid difference (dashed line) and H₂O buffer difference (dotted line) were recorded taking the reference as inactivating buffer condition: (buffer or Cg-liposomes with 500 mM K⁺)-(buffer or Cg-liposomes with 0 mM K⁺).
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FIGURE 9. Effect of the temperature on the PO\textsubscript{2}\textsuperscript{−} modes of phospholipid head groups. A, the shift of the peak positions for the phospholipids \( \nu_{\text{as}}(\text{PO}_2^-) \) modes with respect to increasing temperature \((4 \rightarrow 94 \, ^\circ\text{C})\) for C\textsubscript{g}-liposomes and for BetP two-dimensional (2D) crystals in \( ^2\text{H}_2\text{O} \) buffer. The fraction of the low frequency \((b)\) and high frequency \((c)\) \( \nu_{\text{as}}(\text{PO}_2^-) \) modes for C\textsubscript{g}-liposomes and for BetP two-dimensional crystals in \( ^2\text{H}_2\text{O} \) buffer is shown. To calculate the relative percentage of the components, the \( \nu_{\text{as}}(\text{PO}_2^-) \) bands from the absorbance spectra were integrated for the spectral range 1275–1219 cm\(^{-1}\) (free/weakly H-bonded), 1219–1184 cm\(^{-1}\) (strongly H-bonded), and 1275–1184 cm\(^{-1}\) (total \( \nu_{\text{as}}(\text{PO}_2^-) \) band). Afterward, the relative fraction of each component was calculated with respect to the total band area.

The thermal stability of anionic phospholipid head groups—Fig. 9A represents the thermal stability of lipid head groups for the blank lipids and for BetP two-dimensional crystals. The peak positions of the phospholipids \( \nu_{\text{as}}(\text{PO}_2^-) \) modes were determined from the second-derivative spectra in \( ^2\text{H}_2\text{O} \) buffer monitored with FTIR spectroscopy in the transmission mode and were plotted with respect to increasing temperature from 4 \(^\circ\text{C}\) to 96 \(^\circ\text{C}\) recorded in 2 \(^\circ\text{C}\) intervals. The plot clearly shows that in the presence of 500 mM K\textsuperscript{+} BetP two-dimensional crystals exhibit a sharp phase transition at 49 \(^\circ\text{C}\) that is lower than for pure lipids (57 \(^\circ\text{C}\)). On the other hand, the lipid-protein system in the presence of 0 mM and 200 mM K\textsuperscript{+} did not show a transition state but underwent a slight instability only in the range 10–28 \(^\circ\text{C}\). This suggests that high K\textsuperscript{+} concentrations (500 mM) induces a dramatic change in the H-bonding strength of the membrane lipid head groups for reconstituted BetP and results in more accessible anionic lipid head groups exposed to the solvent. Fig. 9, B and C, presents the percentage of the \( \nu_{\text{as}}(\text{PO}_2^-) \) modes for pure lipid and BetP two-dimensional crystals in \( ^2\text{H}_2\text{O} \) buffer. The relative fraction of each component was calculated with respect to the total band area from the absorbance spectra and was drawn as a function of increasing temperature. C\textsubscript{g}-liposomes without K\textsuperscript{+} display 49% of lipid head groups in the high frequency region (1275–1219 cm\(^{-1}\)), whereas the lipid-protein system harbors 80% (500 mM K\textsuperscript{+}), 60% (200 mM K\textsuperscript{+}), and 50% (0 mM K\textsuperscript{+}) of those modes. Here, we can conclude that the population of free/weak H-bonded lipid head groups increase under the high K\textsuperscript{+} concentration, whereas the amount of the stronger H-bonded ones decreases.

According to the plots in Fig. 9, B and C, BetP two-dimensional crystals exhibited the most dramatic change with increasing temperature in the presence of high K\textsuperscript{+} (500 mM). Its low frequency fraction in the range 1219–1184 cm\(^{-1}\) increased from 20% to 54%, whereas its high frequency percentage decreased from 80% to 46%. This indicates that 34% of lipid headgroups became more H-bonded upon increasing temperature from 4 to 94 \(^\circ\text{C}\) due to an increased dynamics of lipid head groups upon K\textsuperscript{+}-induced activation of BetP.

C–O stretching modes from the phospholipid carbonyl groups—The C–O stretching modes from the interface part of the membrane gave rise to a broad band between 1760 and 1700 cm\(^{-1}\) (33). This spectral region is influenced by the strong absorption of Asp/Glu residues. The C–O modes from those charged residues are downshifted by a few cm\(^{-1}\) (5–10) in \( ^2\text{H}_2\text{O} \), whereas the vibrations arising from lipid ester carbonyl groups were not affected by the \(^1\text{H} \rightarrow ^2\text{H}\) isotope exchange (37). Taking this advantage into consideration, one can easily distinguish these signals in an infrared spectrum.

In the second-derivative spectrum at the higher end of the C–O range (toward 1750–1735 cm\(^{-1}\)), minute spectral alterations in the presence of high K\textsuperscript{+} concentration were observed (Fig. 10A). Because the band positions in this region did not respond to hydrogen/deuterium exchange (data not shown), these vibrations can be assigned to the ester carbonyl groups, or they are most likely internal groups of Asp/Glu residues that are not on the surface of the protein. From this point of view, we can conclude that the double bond character of the C–O modes from ester carbonyl groups is slightly perturbed in response to K\textsuperscript{+}-stimulated activation of BetP.

A broad single C–O band that appears around 1735 cm\(^{-1}\) in the absorbance spectrum (Fig. 2A) is split into two positions in the second-derivative spectrum depending on its hydration status (Fig. 10A). The second-derivative spectra represent the C–O stretching region (1760–1700 cm\(^{-1}\)) in \( ^2\text{H}_2\text{O} \) buffer recorded for the inactive (with 0 mM K\textsuperscript{+}) and active state (500 mM K\textsuperscript{+}) of BetP and for blank lipid. C\textsubscript{g}-liposomes exhibit two components at around 1741 cm\(^{-1}\) (non-H-bonded) and around 1720 cm\(^{-1}\) (H-bonded) due probably to two different
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FIGURE 10. C=O modes from ester carbonyl groups. A, the IR second-derivative spectra for the C=O stretching region (1760–1700 cm⁻¹) in the absence (Cg-liposomes) and presence of BetP in H₂O buffer. B, lipid C=O stretching vibrations at around 1743 cm⁻¹ with respect to increasing temperature (4–94 °C) for Cg-liposomes and BetP two-dimensional crystals in D₂O buffer.

The wealth of structural data (in complex with lipids and in different conformations of the alternating access cycle) implies that binding of POPG lipids will stabilize and consequently restrict the flexibility of TMH3, TMH6, TMH7, TMH11, and TMH12 as well as of cytoplasmic loops 2, 4, and 8 under down-regulated conditions. These loops were previously assigned by peptide array analysis to be involved in osmoregulation (7). TMH6 and TMH7 form the cytoplasmic gate, whereas TMH11 and TMH12 are assigned as the periplasmic gate. Both elements play a crucial role in the isomerization from outward- to inward-facing conformations and vice versa; therefore, we assume that regulatory lipid-protein interactions will strike most likely at these positions, especially as they are more exposed to the lipid bilayer than other helices from the transporter core. The orientation of the osmosensing C-terminal domain (16) was stabilized by lipids as well, facing the adjacent protomer (Figs. 1A and 6B). Conformational changes during the transport cycle of BetP occur dominantly at the cytoplasmic part of TMH3 and at the periplasmic part of TMH12. If interaction with lipids restrict the movement of these gating helices directly via hydrophobic interactions and indirectly via ionic interactions with the preceding cytoplasmic loops (7, 12, 16, 40), the modulation of lipid-protein interactions might play an important role in regulation. Here, the orientation of the C-terminal domain with a ladder-like arrangement of several arginines might contribute to sequester negatively charged lipid molecules around the BetP trimer and by doing so enhance lipid-lipid interactions. To go one step further toward a molecular mechanism of regulation, the tight/weak lipid-protein interactions have to be assigned to inactive/active states, and second, the role of the C-terminal domain in the modulation of lipid-protein interactions has to be defined.

In fact, the non-perturbing biophysical FTIR measurements reveal an important missing puzzle piece by enabling an investigation on the direct molecular effect of K⁺ activation on an anionic lipid membrane that contains BetP. The FTIR data show unambiguously that during activation, the interaction with both hydrophobic tails as observed for lipids bound to TMH3, TMH9, and TM12 as well as with the head groups bound to cytoplasmic loops and C-terminal domains are signif-

Discussion

The osmo-regulated betaine transporter BetP is activated by increasing amounts of potassium ions and has shown a strong dependence of its activation profile on the amount of anionic lipids found in the surrounding membrane (5). Functional data indicated that the more anionic lipids compose the membrane that accommodates BetP the more K⁺ is required to activate BetP (9). A recent x-ray structure (16) has revealed lipid-protein interacting sites of both hydrophobic tails and negatively charged head groups (Fig. 1). Although the interaction of the fatty acyl chains with hydrophobic residues along TMH2, TMH3, TMH9, h7, and TMH12 appears to be rather unspecific (Fig. 1D), there are several positively charged residues, namely Lys-121 in loop 2 preceding TMH3, Lys-228 in loop 4, Arg-395 in loop 8 preceding TMH9, and Arg-542 and Arg-554 in TMH12 and the C-terminal domain that coordinated negatively charged head groups of PG (Fig. 1C). However, the binding lengths between, e.g. the amine group of the arginines and phosphate groups of PG range from 4.3 to 5.4 Å (Fig. 6B), which suggest that these lipids might be further coordinated by either water or by more dynamic lipid-lipid interaction.
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Significantly reduced. Up-regulation of the BetP transport rate apparently requires a weakening of lipid-protein interactions. With FTIR we detect the effect on all lipids, i.e. lipids associated with BetP as well as bulk lipids. Here, the fact that experiments were performed in two-dimensional crystals is advantageous, as two-dimensional crystals provide a minimized amount of bulk lipids compared with the layer of lipids interacting directly with protein. It was shown in a previous study that BetP reconstituted in such two-dimensional crystals maintains its regulatory and transport properties (40). Moreover, the control measurements in liposomes showed that there is no K⁺ effect on the bilayer in the absence of BetP (Fig. 3B), whereas after reconstitution of BetP into the membrane, the mobility of both head groups and tails of the lipids decreased significantly (Fig. 3). This can only be explained by the fact that specific lipid-protein interactions also affect lipid-lipid interactions in the closely packed environment of two-dimensional crystals.

Therefore, we are confident to draw the conclusion that changes in lipid spectra reflect mainly the effect on the lipid-protein binding sites, e.g. those observed in the BetP structure. As these binding sites do not differ so much in their coordination for the eight individual lipids, we assume that there might not be only one specific regulatory lipid binding site but a combined regulatory effect of the lipids interacting with the transporter core and the osmosensing C-terminal domain. In light of K⁺ binding being cooperative, requiring at least 220 mM K⁺ for activation, the scenario of one specific binding site is rather unlikely.

The PG lipids in the BetP structure in the absence of K⁺ and presence of 300 mM Na⁺ showed straightened fatty acid tails due to hydrophobic protein interactions (Fig. 1D). As K⁺ induced a significant conformational disordering of the acyl chains, i.e. an increase in dynamics of the hydrophobic lipid tails (Figs. 3 and 4), we assume that we observed an increased flexibility in the cytoplasmic parts of the bundle helices TMH3 and TMH9 and TMH12. In terms of transport regulation, this would mean that the transporter is now able to cycle through the transition of outward open to inward open in an unrestricted way. K⁺ binding might replace lipid-protein interactions by, most likely, competing with positively charged residues. This would to some extent explain the fact that elevated Na⁺ concentration does not act as a trigger, as Na⁺ requires a tight coordination of at least five ligands (40).

The central lipids stabilize the trimeric assembly. Alterations in the mobility of acyl chains would also result in a more flexible trimeric architecture. In fact we have observed in two-dimensional crystals that a constitutively active mutant of BetP, as a slightly different orientation of individual protomers within the trimer (41) and a re-arrangement of protomers, would also be a suitable explanation for recent dynamic investigations by EPR (36), which reported on differences of distances in DEER measurements at high K⁺ concentrations compared with the structural data that were obtained in the absence of potassium.

The hydrophilic region of the bilayer lipids was also affected by BetP reconstitution, causing a dramatic change in the hydration status of the phosphate moiety (Fig. 5). A high K⁺ concentration results in a pronounced change in the H-bonding strength of lipid head groups and renders anionic lipid head groups more accessible to the solvent (Fig. 9). The population of free/weakly H-bonded lipid head groups (~1238 cm⁻¹) increased significantly with elevated K⁺ concentrations, reflecting weakening or release of the membrane lipid head groups from the bound state. Based on the head group interactions observed in the structure, we conclude that the FTIR data dominantly describe an effect on lipids that interact with the cytoplasmic loops and with the osmo-sensing C-terminal domains.

The C-terminal domain of BetP comprises a number of positively charged arginine residues that were identified as interaction partners with anionic lipid head groups but also with negatively charged side chains (Asp/Glu) from charge clusters found in the N-terminal domain and in cytoplasmic loops. Recently, the ionic interaction of terminal domains with cytoplasmic loops via charged residues has been described (42). Here again, there is an important difference between two-dimensional crystals used in this study and three-dimensional crystals. The C-terminal domains in three-dimensional crystals cannot interact with the membrane surface, as there is none. In the artificial bilayer of two-dimensional crystals, an interaction all along the C-terminal domain is possible. A change in the intensity of the arginine residues as well as activation-induced alterations in the population and H-bonding properties of C=O stretching modes from the protonated Asp/Glu residues absorbing in the range of 1700–1730 cm⁻¹ (for band assignments see Refs. 30 and 43) were detected in the difference spectra of the deuterated sample (active = inactive) for BetP two-dimensional crystals under an elevated K⁺ concentration (Fig. 10A). The wave number positions of the phospholipids modes are not significantly altered with K⁺ in the case of the C-terminal truncated form of BetP. This implies that the phospholipid head groups surrounding BetP ΔC45 do not respond to K⁺-induced activation in the absence of the C-terminal domain, as compared with wild type of BetP, which exhibits a pronounced change in the electrostatic and H-bonding properties of the phospholipid head groups (Fig. 8). Starting from this point we can postulate that negatively charged phosphate lipid head groups are released from the positively charged Arg residues of the C-terminal domain upon K⁺ binding and might undergo a change in interaction toward loops or the N-terminal domains. This brings another molecular detail for the regulation mechanism. We suggest that the lipid-protein interactions restricting TMH3 and TMH12 are enabled by an interaction of the C-terminal domain with the bilayer surface. Upon activation, e.g. under elevated K⁺ concentrations, the C-terminal domain is detached from the lipid-bound state, also affecting binding of lipids to the gating helices.

To some extent, the FTIR data finally confirm the molecular switch model (7), which hypothesized that due to K⁺ activation the positively charged C-terminal domains switch their interaction partner from lipids (under down-regulating conditions) to protein interactions, which could also involve intratrimetric interactions between C-terminal domains as suggested by structural studies (12) and the fact that monomeric BetP is

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down-regulated (6). However, K⁺ is not a general stimulus for other secondary transporters, but a recent study as shown that the main stimulus for BetP originates in changes of the physical state of the membrane, which might be a much more general mechanism, although for other secondary transporters with long charged terminal domains, e.g. the mammalian sodium proton exchanger NHE3 (44).

Author Contributions—G. G., W. M., and C. Z. designed the study and wrote the paper. R. M. G. and C. Z. purified and crystallized the BetP protein. G. G. designed, performed, and analyzed the experiments. R. M. G. and C. Z. contributed to the preparation of the figures that represent the three-dimensional structures of BetP. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Winfried Haase and Friedericke Joos for providing the Freeze-Fracture image shown in Fig. 1. We thank Reinhard Krämer and Caroline Koshy for careful reading of the manuscript.

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