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Analysis of putative protomer crosstalk in the trimeric transporter BetP: The heterotrimer approach

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ABSTRACT

The homotrimeric, secondary active betaine carrier BetP from *Corynebacterium glutamicum* is a model system for stress-regulated transport in bacteria. Its activity responds to hyperosmotic stress and it harbors two different functions, transport catalysis (betaine uptake) and stimulus sensing, resp. activity regulation. Structural information from 2D and 3D crystals as well as functional analysis of monomerized BetP suggested the presence of conformational crosstalk between the individual protomers. To study whether the oligomeric state is functionally significant on a mechanistic level we generated heterooligomeric complexes of BetP in which single protomers within the trimer can be addressed. By testing dominant negative effects in a trimer of one active protomer combined with two protomers in which transport and regulation were abolished, we provide experimental evidence for the absence of functionally significant conformational crosstalk between the protomers on the level of both transport and regulation. This is supported by experiments using mutant forms of putative interacting signal donor and acceptor domains of individual BetP protomers. This result has important consequences for oligomeric transport proteins in general and BetP in particular.

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1. Introduction

Secondary transport systems frequently consist of several identical protomers forming homo-oligomeric complexes. Besides monomeric transporters, e.g. LacY [1] and LeuT [2], also dimeric, e.g. NhaA [3], or EmrE [4], and a number of trimeric carrier systems are known, e.g. Glt_{Ph} [5], AcrB [6], or Amt [7]. Although early speculations have been put forward for a conceptual advantage of oligomerization in transport systems [8], its possible functional significance is not understood. BetP, a glycine betaine uptake system from the Gram-positive microorganism *Corynebacterium glutamicum* shares the LeuT-like fold [2,9], and forms stable homotrimers [9,10].

BetP has been studied in detail in terms of transport function [11], regulation [12–14] and structure [9,15]. BetP catalyzes uptake of the compatible solute betaine under conditions of hyperosmotic stress. Upon reconstitution of the purified protein in proteoliposomes the carrier protein was demonstrated to comprise two functions, betaine transport on one hand, and sensing of hyperosmotic stress as well as regulation of transport activity in response to this stimulus, on the other hand [16]. Mutagenesis studies have revealed that in particular the C-terminal domain of BetP which faces the cytoplasm is critically involved in stimulus perception and subsequent activity regulation [12,17]. A rise in the cytoplasmic K^+ concentration was identified as the primary stimulus related to hyperosmotic stress, and the C-terminal domain was shown to be required for proper sensing of this stimulus [12,13,17].

For small number of oligomeric transport systems, a benefit due to a functional cooperation of protomers has been suggested, the most prominent example being the trimeric transporter AcrB [18,19]. If oligomerization is in fact functionally significant, experimental interference with protomer interaction should have functional consequences. Using a bioinformatic approach, the major trimerization sites of BetP protomers were identified. Mutagenesis of amino acid residues at this site led to stable BetP protomers in monomeric form [20]. Analysis of monomerized BetP revealed that the monomers retain their basic catalytic function, albeit showing very low transport activity, but, in difference to the trimeric protein, do not respond anymore to osmotic stress. This was taken as an indication that transport catalysis does not require the trimeric state, however, regulation does [21].

A number of results argue for the presence of conformational crosstalk between BetP protomers in the trimer. Trimeric BetP was found to be in a stable asymmetric form with respect to the conformation of its individual protomers based on structural data obtained in 2D and 3D crystals, respectively [10,15]. This unusual property of a homo-oligomeric protein suggested conformational crosstalk between protomers as a likely explanation. This hypothesis was supported by strong ionic interaction observed in atomic structures of BetP between individual C-terminal domains within the trimer and between the C-terminal domain and the cytoplasmic loop 2 of adjacent protomers, respectively [9,15,20]. This is an interesting analogy to Amt1, the

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trimeric ammonium transporter from *Arabidopsis thaliana* [22]. For Amt1, allosteric regulation of adjacent protomers by the trans-activating C-terminal domain interacting with cytosolic loops of the transporter has been shown [23]. The third argument in favor of inter-protomer crosstalk refers to the results of engineered monomeric BetP (see above), in which the loss of regulatory capacity was observed [21]. With respect to understanding protomer crosstalk, this interesting study left a number of questions open due to experimental restrictions involved in the experimental approach used. Mutational changes were required for generating BetP monomers, a possible direct impact of which on function cannot be discriminated from their structural effect leading to monomerization. This situation may be reflected by their low transport activity. On the other hand, the low activity observed could in principle also be used as an argument indicating the requirement of inter-protomer crosstalk for proper catalytic function.

In this work, we set out to study the fundamental question of a possible functional significance of oligomerization in transport proteins applying a new strategy, namely the generation of heterotrimeric BetP complexes. The basic idea is providing an experimental system, in which BetP protomers remain in their native surrounding, i.e. the trimer, and specifically interfering with catalytic and/or regulatory properties of individual protomers. For this strategy, experimental access to every individual protomer is required. Assuming a functional significance of conformational crosstalk between the three protomers, catalytically and/or regulatory incompetence of individual protomers should negatively affect the function of the adjacent protomer, a phenomenon called a 'dominant negative phenotype'.

Two different experimental strategies are available for generating hetero-oligomeric transport proteins; construction of large fusion products of linearly ordered individual subunits separated by appropriate linker peptides, on one hand, and complex formation from separately synthesized subunits which are rendered structurally individual, on the other hand. Both strategies would in principle allow for addressing every single protomer within the complex for experimental (mutational) variation. The first strategy is technically simpler and has successfully been applied to the trimeric carrier AcrB [19]. Because it is required to select the correct type of heterotrimer from the multitude of statistically formed combinations of protomers, the second strategy is experimentally more demanding. It has been successfully applied for the bacterial mdr protein EmrE [24] as well as the mitochondrial phosphate carrier [25]. So far, because of experimental complexity, this approach has not been applied for trimeric proteins.

Both strategies have their individual advantages and drawbacks. Fusion constructs can be analyzed under in vivo conditions in intact cells; however, besides difficulties with handling these large proteins, there are potential problems of interference by the artificially fixed N- and C-terminal domains and by unwanted effects of the linkers. The approach using individualized protomers needs experimentation in a reconstituted system, since the correct heterotrimer has to be sorted out after random expression. In this strategy, as an advantage, the structural properties of the transporter complex are close to the wild type protein.

We have applied both strategies to crosstalk analysis of BetP, but only the latter was finally successful. In spite of the fact that trimeric BetP was found to be structurally asymmetric in 2D and 3D crystals [10,15], using a BetP heterotrimer in which substrate transport and binding were abolished in two protomers by mutagenesis we were able to show that in this construct transport activity and regulation of the remaining active protomer do not require conformational crosstalk with its adjacent protomers.

2. Materials and methods

2.1. Cloning of BetP constructs and single site mutations

All strains and plasmids used in this work are listed in Tab. S1. An Age1 restriction site preceding the stop codon was introduced into the BetP coding sequence in the established BetP expression plasmid [16] via PCR. All linker and epitope tag coding sequences were introduced via subcloning into the locus between this new Age1 site and the original Hind3 site following the stop codon. The various constructs used in this work are explained in detail in Fig. 1. It has to be pointed out that all the constructs are based on the cys-less form of BetP and all carry a Strep-tag at its N-terminal end. For cloning the heterotrimer coexpression plasmids, derivatives of pASK-IBA5 encoding for the different BetP monomers were cut via Msc1/Pvu1 and Nae1/Pvu1, respectively [26]. These constructs were ligated resulting in an expression plasmid encoding for two separate BetP mRNAs each under control of their own promoter and terminator elements. Details of the construction of these plasmids are explained in Fig. S1. By repetition of this procedure the third subunit coding sequence was introduced. All single site mutations were introduced by the Stratagene QuikChange kit according to the manufacturer's recommendations. Mutation of the coding sequence for loop 2 of BetP was achieved by PCR with the respective mutation primers and subsequent restriction and ligation by the introduced Kpn1 site.

2.2. BetP expression

Expression of various BetP constructs was carried out in a Biostat B fermenter (Sartorius). *Escherichia coli* DH5a was precultured in LB (homotrimeric constructs) or TB medium (heterotrimeric constructs) supplemented with 100 μg/ml carbenicillin and inoculated in LB medium supplemented with 2% glucose, 50 μg/ml carbenicillin, 25 mM KPi pH 7.5; or TB medium supplemented with 2% glucose and 50 μg/ml carbenicillin to an OD_{600} of 2. The use of TB medium leads to higher yield of synthesized protein. Cells were stirred at 700 rpm, aerated with 3 l/min of air and kept at 37 °C throughout the fermentation. The pH was set to 6.7 using sulphuric acid/potassium phosphate. At an OD_{600} of 8, expression was induced by addition of anhydrotetracycline (AHT) to a final concentration of 1 μg/ml. Membranes were prepared and homo-trimeric BetP variants were purified as described [16].

2.3. Heterotrimer purification and reconstitution

Membranes were thawed and solubilized with 2% Triton at a protein concentration of 10 mg/ml in 50 mM potassium phosphate buffer at pH 7.5 supplemented with complete protease inhibitor (Roche). After debris centrifugation (109,000 ×*g*; 35 min; 4 °C), the solubilizate was diluted 1:4 with 50 mM KPi pH 7.5; 300 mM NaCl; 8.6% glycerol; 0.1% dodecylmaltoside; 10 mM imidazole, loaded on a 5 ml HiTrap Chelating HP column (GE Healthcare), and washed with 30 ml of the same buffer containing 50 mM imidazole. The protein was eluted in the same buffer containing 500 mM imidazole. Upon 1:10 dilution in 50 mM KPi pH 7.5; 200 mM NaCl; 8.6% glycerol; 0.1% dodecylmaltoside, and 1 mM EDTA, approx. 2 mg of the protein was loaded on a 2.5 ml α-FlagM2 affinity Gel column (Sigma-Aldrich). Upon washing with 10 ml (same buffer) the protein was eluted in the same buffer containing 0.2 mg/ml Flag peptide. Finally the protein was purified using Strep-Tactin as described previously [16]. Reconstitution and activity measurements were carried out as described previously [16] using radiolabeled ^{14}C-betaine (111–333 GBq/mol for measuring uptake in proteoliposomes).

2.4. Labeling of engineered cysteines

For single cysteine labeling the proteins were incubated with a 2-fold molar excess of Tris-carboxyethyl-phosphate (TCEP) in 50 mM KPi pH 7.5; 200 mM NaCl; 8.6% glycerol, 0.1% dodecylmaltoside, and 1 mM EDTA for 15 min on ice and subsequently with a 10 fold molar excess of the thiol reactive fluorescein derivative Bodipy_fl_IA dye (molecular probes) for 1 h. Free dye was removed after quenching with 10 fold excess mercaptoethanol by gel filtration on PD10 columns (GE-Healthcare).

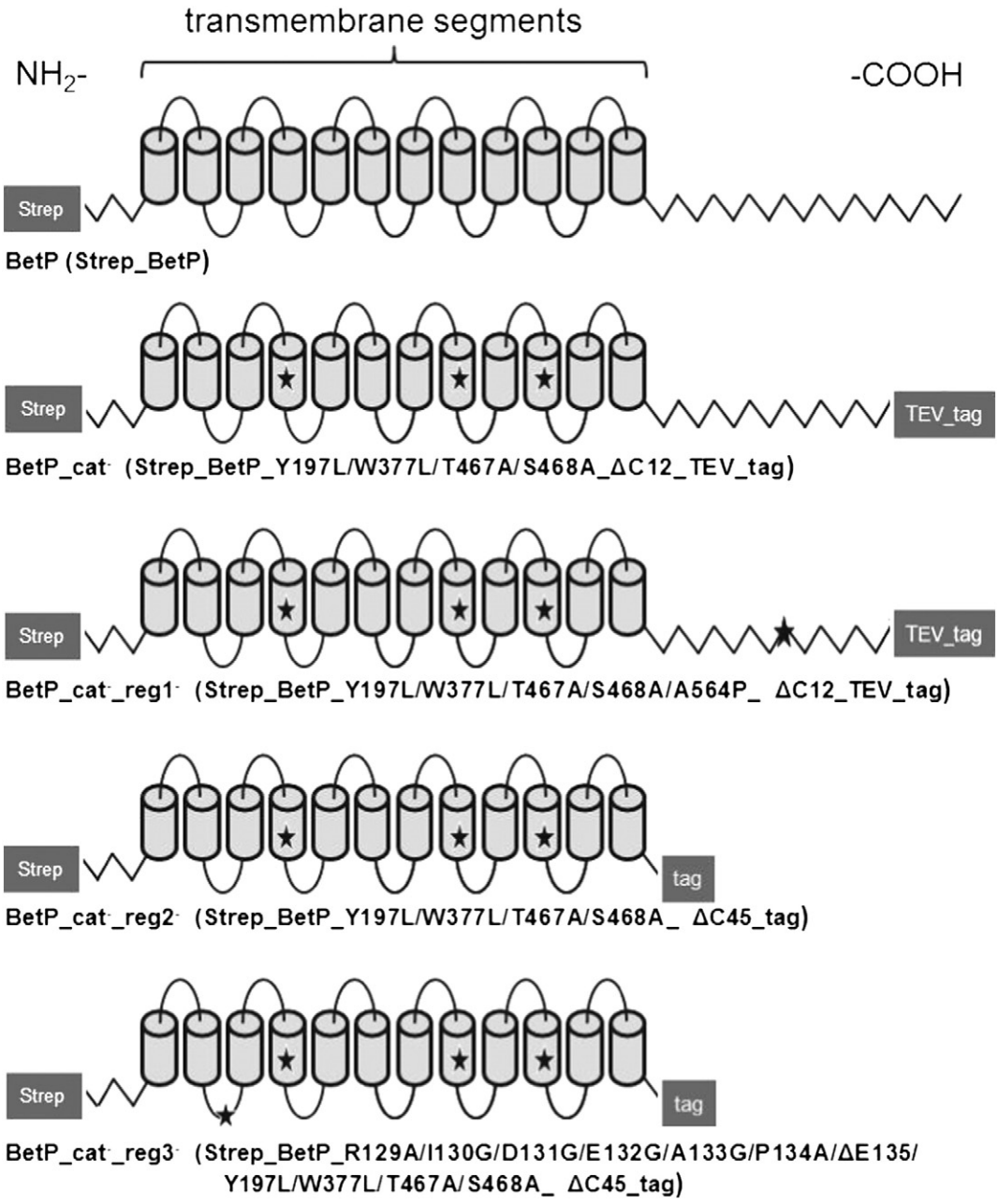

Fig. 1. Schematic representation of various BetP constructs used in this study. The constructs for which abbreviations are used in the text are listed below the respective symbol and the correct construction is given in brackets. All BetP constructs used in this study are based on the cys-less wild type protein, and all constructs start with a Strep-tag at the N-terminal domain. Amino acid replacements which lead to a catalytically inactive protein are indicated with cat$^-$, those resulting in a regulatory inactive protein with reg$^-$. The three ways to obtain regulatory inactive mutants (A564P mutation, C45-deletion, and a combination of C45-deletion and loop 2 mutation) are indicated with reg1, reg2 and reg3. The C-terminal domain is either untagged (first construct), or tagged with either His or Flag tag, marked in the figure as 'tag'. In some cases, a TEV cleavage site was introduced in front of the C-terminal tag. The positions of amino acid replacements are indicated by stars.

3. Results

3.1. Generation and verification of heterotrimeric BetP constructs

Since only the availability of fusion protein constructs would allow the hetero-oligomeric complex to be studied in intact cells, we first applied this strategy. A large fusion gene was constructed, starting with the N-terminal domain of the first protomer, ending with the C-terminal domain of the third protomer, and carrying linker peptides of varying lengths and sequences connecting the three individual protomers. The fusion constructs were expressed in *C. glutamicum* DHPF which is devoid of all five transport systems for compatible solutes and in *E. coli* MKH13 which is unable to take up or synthesize betaine [27]. In the recombinant strains, we measured effective betaine uptake. However, these constructs could not be used, since they turned out to be not stable and to form aggregates upon purification. Trimeric fusion constructs of BetP seem to have a strong tendency to form super-oligomers, probably trimers of the trimeric fusion proteins, in which specifically the first protomer of each individual trimer in the aggregate associates to a trimeric BetP protein. We did not succeed in eliminating aggregation as well as protein degradation by variation of both type and length of linker peptides and thus discarded this approach.

In the second type of strategy heterotrimers were generated from three different, individually expressed protomer genes. The technical complication of this approach originates from the fact that the three independently synthesized protomers statistically form trimeric complexes in

any possible combination. Not considering positional effects, this leads to 10 basically different types of combinations. Consequently, this strategy requires a method to select the exclusive interesting type of oligomer, i.e. the heterotrimer composed of three different protomers. For this purpose, we fused different molecular tags to the individual protomers, with which, upon a combination of subsequent affinity chromatography steps the desired type of construct should be accessible. Consequently, in this case transport activity can only be measured upon functional reconstitution of the purified heterotrimeric construct.

In the published procedure for isolation of functionally active BetP a Strep-tag fused to the N-terminus of the protein was used [16]. We explored fusion of altogether 15 different types of tags with various types of linkers at the N-terminal end, however, with the exception of the originally established Strep-tag, no other tag was successful. In several cases, the tag rendered BetP inactive, in others the affinity column purification did not work (missing tag exposure), or the tag induced aggregation of the fusion protein. Primarily, we tried to avoid tagging BetP at the C-terminus since this part of the protein is required for stimulus sensing [11,17], but we found that both activity and regulation of BetP were not significantly affected by tags fused to this site. Fusion of peptide tags to the C-terminal end, however, gave rise to another problem. Whenever the C-terminal domain of BetP was extended by a peptide tag, the fusion protein became sensitive to proteolytic degradation in the C-terminal domain after expression in *E. coli*. Since neither a variation of linker length between the C-terminus and the peptide tag, nor the use of *E. coli* strains with low proteolytic activity prevented degradation, we stepwise truncated the C-terminal end of BetP before fusing it to the peptide tag. Finally, BetP truncated by 12 C-terminal amino acids turned out to be stable upon expression in *E. coli* (Fig. 2A). We controlled whether the truncated version of BetP retains its functional properties and found, in accordance with previous results, that the truncated protein is fully active and still regulated albeit with a somewhat shifted profile [17] (Fig. 2B). It should be pointed out that C-terminally truncated forms of BetP were only used in the catalytically ore regulatory inactive protomers and not in the active protomers of heterotrimeric forms.

Using this truncated form of BetP as a basis, we tested possible C-terminal tags for their suitability. Exclusively the His-tag and the Flag-tag were found to lead to active protein not prone to aggregation and suitable for isolation by affinity chromatography. Since a strategy using the N-terminal Strep-tag as a third tag fused to one of the protomers only failed as a tool for isolation, heterotrimers had to be generated with two suitable C-terminal tags only, in addition to the N-terminal Strep-tag attached to each protomer. This was achieved by high expression of the gene coding for the third protomer which is devoid of a C-terminal tag and only harbors the N-terminal Strep-tag (1–1.5 mg of BetP from a 1 l *E. coli* culture), in comparison to a rather low expression of the genes coding for the other two protomers tagged at the C-terminus (0.2 mg of BetP from a 1 l *E. coli* culture), thus

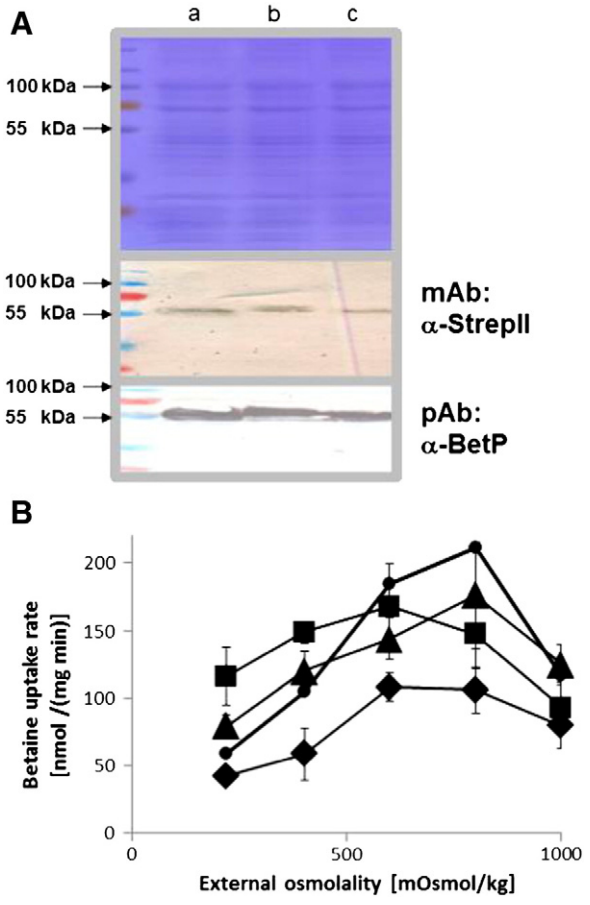

Fig. 2. Stability of C-terminally tagged and truncated constructs of BetP. BetP was C-terminally truncated by 12 amino acid residues and fused to a Flag-tag and a His-tag, respectively at its new C-terminal end. (A) SDS-PAGE (upper panel) and Western blot analysis (lower panels) of membrane extracts of *E. coli* MKH13 cells expressing the different BetP constructs indicated at the top of panel A of the figure. The Western blot was developed using anti-Strep and anti-BetP antibodies, respectively (a, Strep_BetP_DC12_TEV; b, Strep_ BetP_DC12_TEV-Flag; c, Strep_BetP_DC12_TEV-His). (B) Betaine uptake activity in *E. coli* MKH13 cells expressing the indicated BetP constructs in dependence of external osmolality (circles, cys-less wild type BetP; squares, Strep_BetP_DC12_TEV-Flag; triangles, Strep_BetP_DC12_TEV-His; rhomboids, Strep_BetP_DC12_TEV). All experiments were done in triplicate, error bars indicate SD. Abbreviations used: mAB, monoclonal antibody against StrepII tag; pAB, polyclonal antibody against BetP protein.

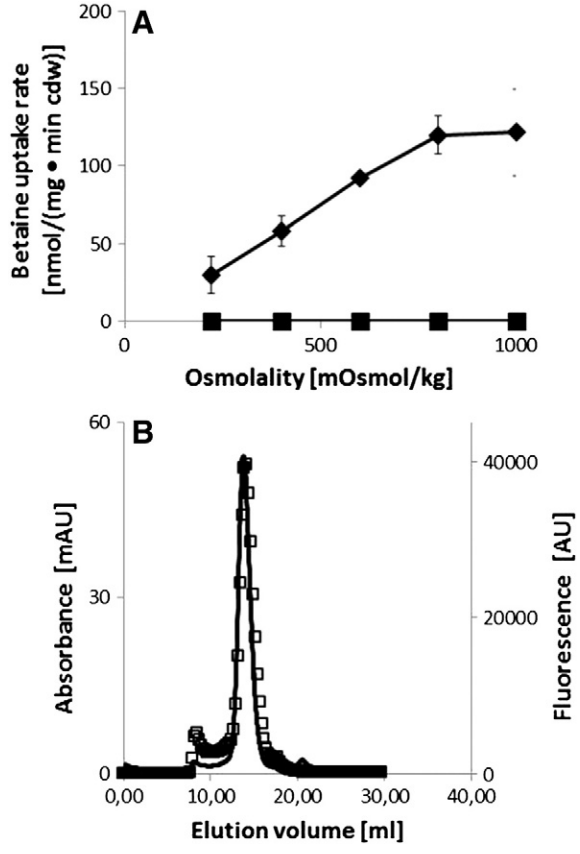

Fig. 3. (A) Betaine uptake activity of BetP upon expression in *E. coli* cells in dependence of external osmolality. Homotrimeric forms of BetP E577C (rhomboids) and the construct BetP_cat$^-$ (squares) were tested. (B) Gel filtration chromatography of the heterotrimeric cys-less BetP construct composed of a BetP_cat$^-$_His, a BetP_cat$^-$_Flag (Fig. 1) and a Strep_BetP_E577C protomer. The purified heterotrimeric protein was subjected to labeling with Bodipy-fl-IA at the single cysteine and to gel filtration. Absorbance at 280 nm (protein concentration, solid line) and fluorescence (Bodipy label, 485/520 nm, squares) were recorded. All experiments were done in triplicate; error bars indicate SD.

providing a high probability for the presence of the protomer lacking a C-terminal tag in the heterotrimeric construct. In order to prove the validity of this approach, we introduced a cysteine at position 577 of the third protomer lacking the C-terminal tag in the otherwise cys-less BetP [14]. This construct, when tested as a homotrimer, was shown to be fully active and regulated (Fig. 3A). By labeling the single cysteine in the heterotrimer with the fluorophor Bodipy-fl-IA (fluorescein iodoacetamide) we found a degree of labeling of 35 ± 7% based on the protein content of the sample and the molecular mass of BetP. As a control, we also labeled both the homotrimeric form of E577C variant of BetP and the cys-less parental form, which led to 95 and 4.8% labeling, respectively. Consequently, the labeling efficiency found in the heterotrimer perfectly fits with a share of the C-terminally untagged protomer in the isolated heterotrimer of one third thus proving the generation of a correct heterotrimeric construct. The final procedure for isolation and characterization of the heterotrimeric BetP protein included membrane preparation, detergent solubilization, followed by three consecutive chromatographic steps on Ni-NTA, α-FlagM2, and Strep-Tactin affinity matrices (see Materials and methods section). Isolation and purification of the heterotrimer are documented in Fig. S2. Because of the complex procedure and the required predominant expression of one of the protomers, the yield of purified and active heterotrimers was very low. When starting from 60 g of *E. coli* cells strongly overexpressing BetP, the final yield of heterotrimer was about 500 μg of protein.

We performed a number of tests for controlling the quality and the properties of the heterotrimeric product. Monodispersity of heterotrimeric BetP in DDM detergent solution was shown by size-exclusion chromatography (Fig. 3B). Combinations of the different constructs being homomeric with respect to BetP and heteromeric in terms of the tags used were shown to be fully active and regulated. Importantly, this holds true for the truncated form of BetP (Fig. 4), although the

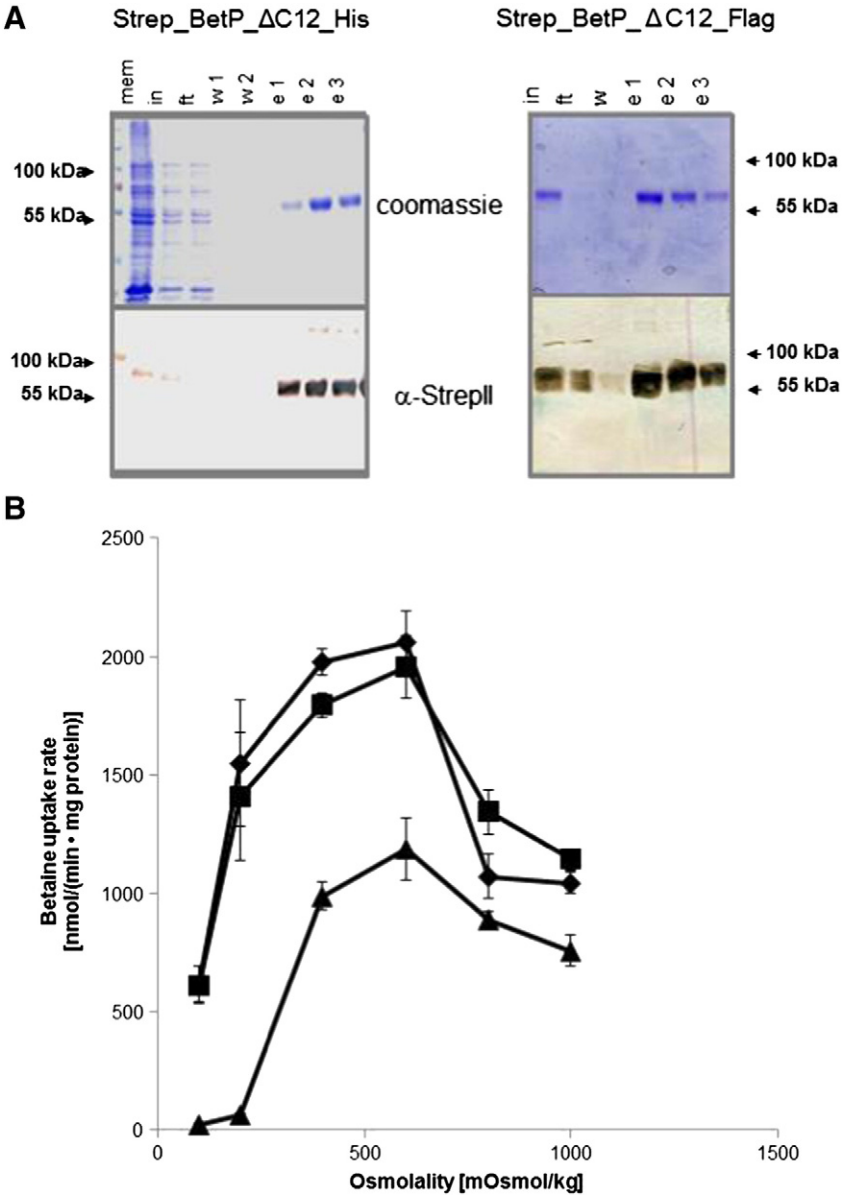

Fig. 4. SDS-PAGE/Western blot analysis of affinity purifications of differently tagged homotrimeric BetP constructs which were truncated by 12 amino acids at the C-terminal domain, Strep_BetP_DC12_His (A, left side) and Strep_BetP_DC12_Flag (A, right side) proteins. The indicated BetP variants were purified from crude membrane extracts (His tag) or pre-purified using a Strep-Tactin column (Strep tag). The fractions tested applied were solubilizate (in), column flow through (ft), wash fractions (w), and elution fractions (e). For Western blotting monoclonal antibodies against StrepII-tag were used. (B) Betaine uptake activity of the reconstituted BetP constructs was analyzed in dependence of the external osmolality. Homotrimeric forms of Strep_BetP_DC12_His (squares) and Strep_BetP_DC12_Flag (rhomboids) were used. For comparison, activity regulation of reconstituted wild type BetP is shown (triangles). All experiments were done in triplicate; error bars indicate SD.

activation profile is slightly shifted, as has been noticed before [17]. It is not clear why the truncated versions of BetP have a higher maximum activity. However, this is not relevant for the results since only inactivated forms of this construct are used in the studies described below. It should be noted that an experiment using differently tagged wild type proteins as a control is not possible, since wild type BetP protomers with fusions at the original C-terminal end were found to be proteolytically degraded (see above). A further, very important control concerns the question whether the oligomeric forms of BetP are stable with respect to their subunit composition, i.e. whether a dynamic exchange of subunits between the oligomeric complexes does occur. Subunit exchange would prevent any meaningful conclusion to be drawn from the activity measurements. For this purpose, we expressed His-tagged and Flag-tagged BetP separately in *E. coli*. From these cells, membranes were prepared, subjected to three freeze–thaw cycles to guarantee effective mixing of all components, and incubated for 6 h. From this mixture BetP was solubilized and isolated by Ni-NTA affinity chromatography. No Flag-tagged protein was detected upon elution of the His-tagged BetP proving the absence of protomer mixing in membrane embedded BetP (Fig. 5).

3.2. Analysis of protomer crosstalk in catalysis (transport)

The observation of a stable asymmetric conformation of BetP trimers by both electron crystallography (2D) and X-ray crystallography (3D) argues for the presence of conformational crosstalk between the individual protomers as an essential feature of this transporter [10,15]. This conception was challenged by results obtained with BetP constructs monomerized by mutation, which showed transport activity, albeit at a significantly lower level than the WT [21]. To study this question in detail with BetP in its natural trimeric surrounding, we constructed heterotrimeric BetP complexes being composed of one single catalytically active and two catalytically inactive protomers which completely lack transport function. If conformational crosstalk is a prerequisite for function, the two inactive protomers should be dominantly negative over the single active protomer.

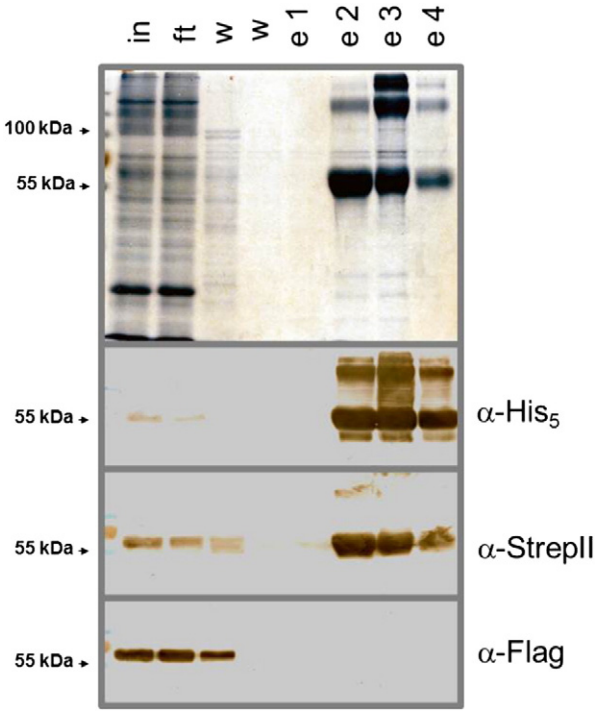

Fig. 5. SDS-PAGE (upper panel)/Western blot (three lower panels) analysis of separately expressed BetP_cat$^-$_His and BetP_cat$^-$_Flag constructs (Fig. 1). The two constructs were expressed separately, mixed as described in the text, and subjected to chromatography on a Ni-NTA affinity column. Affinity chromatography was performed as described for isolation of BetP heterotrimers. The fractions tested applied were solubilizate (in), column flow through (ft), wash fractions (w), and elution fractions (e). For Western blotting monoclonal antibodies against His-tag, Flag-tag, and StrepII-tag were used, as indicated on the right hand side of the blot.

For this purpose, we replaced two aromatic amino acids in the substrate binding site by leucine (Y197L and W377L) which strongly reduces transport [9]. In order to render the transporter fully inactive, we further replaced threonine and serine, respectively, by alanine (T467A and S468A), which was shown to abrogate the catalytically essential Na-2 binding site [28] (Table 1). This BetP variant BetP_cat$^-$, when expressed as a homotrimer, was found to be inactive upon expression in *E. coli* MKH13 cells (Fig. 3). The heterotrimeric BetP composed of both a His-tagged and a Flag-tagged version of the catalytically inactive protomer, together with an active BetP protomer lacking a tag at the C-terminal domain but harboring a cysteine at position 577 as a labeling site, was shown to be correctly assembled and selectable from the heterogeneous mixture of possible protomer combinations (see above). A purified heterotrimer composed of two catalytically inactive protomers (BetP_cat$^-$_His and BetP_cat$^-$_Flag and an active BetP) (Fig. 6B) was reconstituted in proteoliposomes and analyzed for betaine transport in the presence of increasing sorbitol concentrations (osmotic stress) according to previously established procedures [16]. The results in Fig. 6C demonstrate that this BetP heterotrimer is active, with respect to both its catalytic as well as its regulatory properties. Its maximum transport activity, when measured under exactly the same conditions (reconstitution procedure, osmotic stress) as the wild type protein, reaches only about 30–40% of that of homotrimeric wild type BetP (Figs. 4, 6C). This result is in accordance with the fact that only one of the three protomers is active in the heterotrimeric BetP. Notably, also the profile of the activation pattern is highly similar to that of the wild type protein (Figs. 4, 6C). We conclude from this result that functional crosstalk on the level of transport catalysis is absent in the heterotrimeric construct and that the remaining active protomer in the heterotrimeric construct is fully functional both in transport and regulation.

3.3. Analysis of crosstalk in regulation (activation)

Upon analysis in proteoliposomes, BetP has been shown to harbor two different functions, transport catalysis, on one hand, as well as sensing and regulation in dependence of osmotic stress, on the other hand. Moreover, the intact C-terminal domain of BetP was identified as being required for sensing and regulation in dependence of the cytoplasmic K^+ concentration [12,17]. After having proven that functional crosstalk is not essential on the level of transport catalysis, and in order to analyze putative conformational crosstalk on the regulatory level, a BetP construct in which sensing and regulatory elements are absent was required. We have previously shown that proline residues when placed at strategic positions within the C-terminal domain may render BetP incompetent for regulation in response to K^+ (Fig. 7A) [17,29]. We thus tested an A564P construct of BetP, which carries a proline residue right in the middle of the C-terminal domain and found that its regulatory properties are strongly compromised within the range of

Table 1
Activity of cys-less Strep-BetP constructs.

BetP construct	Betaine uptake (μmol/(g cdm min)$^{-1}$)
Wild type	68.5
Y197L	12.5
W377L	10.0
Y197L, W377L	2.0
Y197L, W377L, T467A, S468A	0.0

Betaine uptake activity of various BetP constructs in *E. coli* MKH13 cells in the presence of optimal conditions for activation (800 mOsmol/kg external osmolality).

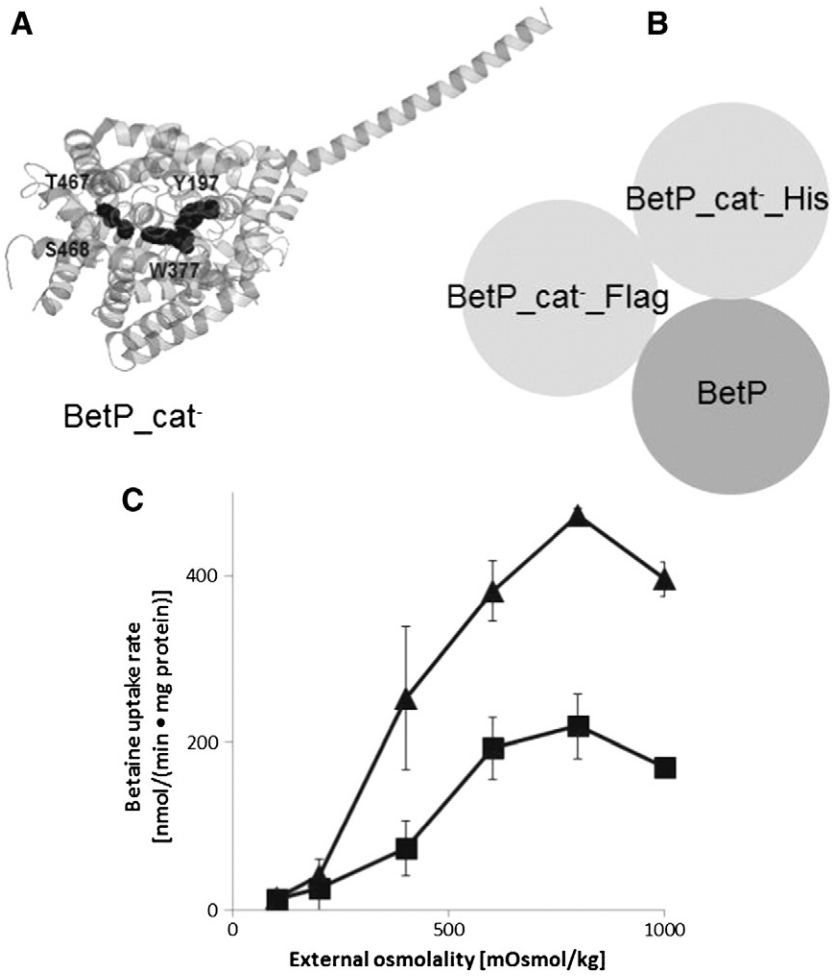

Fig. 6. Analysis of catalytic cross-talk. (A) Graphic representation of the BetP protomer which is inactive in substrate (Y197L/W377L) and sodium binding (T467A/S468A). (B) Schematic drawing of the BetP heterotrimer used in this experiment; top view. (C) Betaine uptake activity of the reconstituted heterotrimeric BetP construct composed of a BetP_cat$^-$_His, a BetP_cat$^-$_Flag, and a Strep_BetP (triangles) or a Strep_BetP_E577C protomer (squares), respectively. All experiments were done in triplicate; error bars indicate SD.

stimuli which can be tested in proteoliposomes (Fig. 7C). We then constructed BetP_cat$^-$_reg1$^-$ (Fig. 1), a heterotrimeric form of BetP which is comprised of both a His-tagged and a Flag-tagged version of the transport-inactivated (Y197L, W377L, T467A, S468A) and deregulated (A564P) BetP combined with an active and a regulated form of BetP as the third protomer (see above). The same control experiments for assembly and isolation of the correct heterotrimer as described above were successfully applied. Stimulation analysis of this heterotrimer in proteoliposomes surprisingly revealed that also this form of BetP is functional, with respect to both its catalytic as well as its regulatory properties (Fig. 7D). The maximum transport activity was found to be at about 10% of the corresponding homotrimeric form (wild type homotrimeric BetP, Fig. 4). Although the reduction in V_{max} cannot be explained, the result of this experiment concerning the fully maintained regulatory capacity of the orphan protomer within the heterotrimer is clear. We conclude from this result that functional crosstalk between protomers does not seem to be an essential requirement also on the level of sensing and regulation for the heterotrimeric construct, and that the single active protomer is functional both in transport and regulation in the BetP trimer.

However, the presence of the (mutated) C-terminal domain from the adjacent protomer could still provide a necessary basis for physical interaction. In a further experiment, we thus removed the C-terminal domain of the two functionally inactive protomers in the heterotrimeric construct by truncating the last 45 amino acids which leads to BetP_cat$^-$_reg2$^-$ (Figs. 1 and 7D). Truncation of the C-terminal domain has been shown to render the homotrimeric BetP not activatable [17]. The results in Fig. 7D demonstrate that even the removal of the C-terminal domain in the two adjacent inactive protomers does not abolish regulation of the remaining active protomer and leads to a functional heterotrimer.

Although the previous results indicate the absence of a regulatory input originating from the C-terminal domain of the adjacent subunits into the active protomer, they do not exclude a possible requirement of crosstalk based on an interaction of the C-terminal domain of the active protomer with its interaction site at the adjacent protomer. This cannot be tested by simply removing the C-terminal domain from the active protomer. Besides leading to a deregulated form of BetP [17], the expression of this construct is low and thus not appropriate for the experimental strategy applied here which requires overexpression of the active protomer in comparison to the two inactive protomer partners (see above). To study this question, we replaced amino acid residues 129–135 of the two adjacent inactive, c-terminally truncated, protomers of BetP by a completely different sequence (RIDEAPE replaced by AGGGGAΔ), shortened by one amino acid residue (construct BetP_cat$^-$_reg3$^-$). This stretch of amino acids in loop 2 of BetP was shown to be the site of interaction of the C-terminal domain [9]. The results described in Fig. 8 indicate that in spite of a compromised interaction between the C-terminal domain and loop 2 the regulation profile is very similar to that obtained for the heterotrimer harboring a wild-type like protomer.

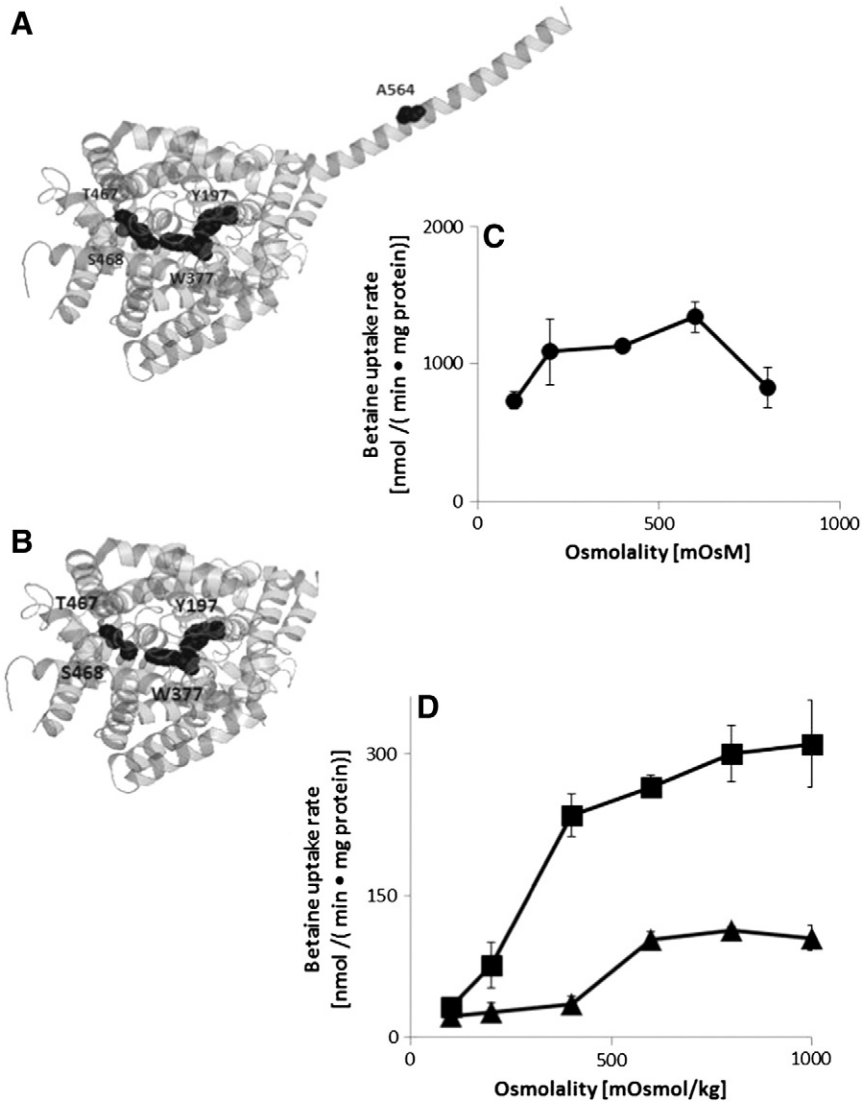

Fig. 7. Analysis of regulatory cross-talk. Graphic representation of BetP protomers which are inactive in substrate (Y197L/W377L) and sodium binding (T467A/S468A), and, in addition, (A) in K^+ dependent regulation by mutation (A564P) or (B) by C-terminal truncation (DC45). Top views of protomers are shown. (C) Betaine uptake activity of the reconstituted homotrimeric BetP Strep_BetP_A564P construct. (D) Betaine uptake activity of the reconstituted heterotrimeric BetP construct composed of a BetP_cat$^-$_reg1$^-$_His, a BetP_cat$^-$_reg1$^-$_Flag, and a Strep_BetP protomer (triangles), or a BetP_cat$^-$_reg2$^-$_His, a BetP_cat$^-$_reg2$^-$_Flag, and a Strep_BetP protomer (squares). All experiments were done in triplicate; error bars indicate SD.

4. Discussion

Whereas generation of a fused construct of three BetP protomers did not turn out to be a useful tool for analyzing protomer crosstalk within the trimeric BetP protein, we were successful with heterotrimeric constructs composed of protomers individualized by specific tags. In control experiments these constructs were shown to be monodisperse, stable in their composition, and, when homotrimeric with respect to the BetP moiety, fully active and regulated. Since the N-terminal Strep-tag was required for the primary purification step, and elaborate experimental efforts led to the identification of only two further functional tags (His-tag and Flag-tag) at the C-terminal end, we did not succeed in constructing a heterotrimer harboring three different C-terminal tags for protomer individualization. This experimental restriction, however, turned out not to be relevant for the present study. For the analysis of conformational crosstalk, we applied the dominant negative approach, which requires, in the case of a trimeric protein complex, the presence of two inactive and one active protomers. The presence of the two different inactive protomers in the complex was proven by the tag-specific isolation procedure, that of the third, active protomer by specific labeling of an engineered cysteine, and also by the observation of its high transport activity.

We did not detect functionally significant conformational crosstalk in the BetP heterotrimer. Each single protomer was found to be able to function properly with respect to both transport catalysis (activity) and regulation (activation). This result is in contrast to a number of previous observations indicating the presence of functional interaction between the adjacent protomers in the BetP trimer. The strongest argument in favor of crosstalk is derived from structural data obtained with 2D and 3D crystals of BetP, in which a stable asymmetry with respect to the conformation of the three individual protomers in the trimer was shown [10,15]. This observation refers to similar results obtained for the trimeric transporter AcrB, where evidence for conformational crosstalk between individual protomers based on structural asymmetry has been provided [18]. Atomic structures of the BetP trimer revealed a twofold contact between the individual protomers. Upon artificial monomerization of BetP by amino acid replacement at the periplasmic side, not only oligomerization was lost but also the capacity for regulation in response to osmotic stress. This was taken as an indication for the fact that BetP requires the trimeric state for activity regulation [20]. It

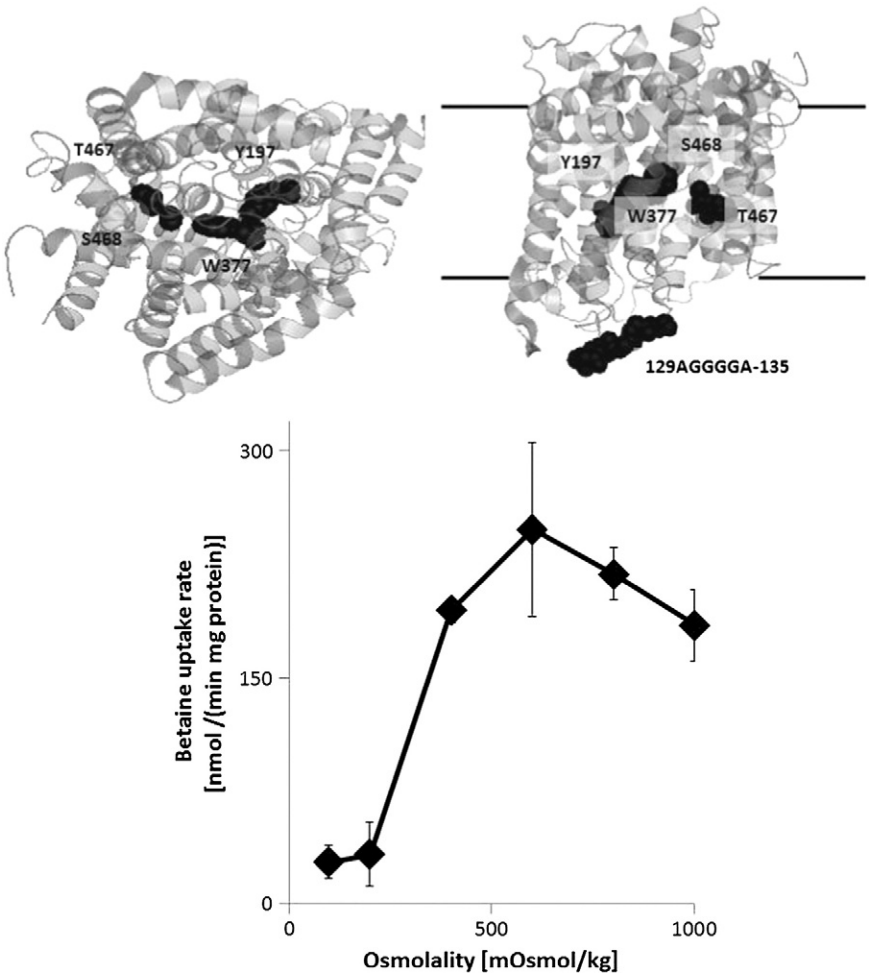

Fig. 8. Analysis of regulatory cross-talk. Graphic representation of the inactivating single site mutations and the mutated loop 2 in the BetP model (PDB ID: 2WIT) (A) top view, (B) side view (membrane boundary indicated). For simplicity, only two protomers are shown. (C) Betaine uptake activity of the reconstituted heterotrimeric BetP construct is identical to that used in Fig. 7B with respect to the C-terminal truncation of the inactive monomers by 45 amino acids, in which the two inactivated BetP constructs carried, in addition, a mutated loop 2 sequence (BetP_cat$^-$_reg3$^-$, see text). All experiments were done in triplicate; error bars indicate SD.

was now an intriguing question how trimerization per se affects regulation in BetP. Furthermore, the linearly extending C-terminal domain of each single protomer forms direct physical contact mainly to loop 2 of the adjacent protomer [9]. This feature, which is reminiscent of a similar situation in the Amt1 transporter from plants [23], in particular gave rise to interpretations suggesting a functional significance of this remarkable kind of contact between the individual protomers.

According to the results presented here, a single protomer of BetP, when being in the trimeric environment, harbors the capacity both of active betaine uptake and of responding to osmotic stress even when the adjacent protomers are inhibited to function in transport and/or regulation. Consequently, BetP protomers function independent of the actual conformational state of the adjacent subunits. Admittedly, the results from biochemical studies reported here do not provide any information to explain the presence of asymmetric conformations of trimeric BetP observed in both 2D and 3D crystals in mechanistic terms.

Beyond the clear-cut statement of absence of conformational crosstalk in the heterotrimer, a number of further interesting conclusions can be drawn from these results. We have tested both possible directions of conformational information transfer. Abrogation of protein dynamics related to substrate translocation (suppression of betaine and Na^+ binding) in the two transport-inactive protomers does not affect the neighboring active protomer. The same result was obtained for loss of regulatory competence even in complete absence of the C-terminal domain. Interestingly, this seems to hold true also in the other direction, i.e. for a putative crosstalk directed from the active to the inactive protomers in the heterotrimer. The C-terminal domain, which is required for sensing and regulation, does not need the presence of its 'docking site' at loop 2 of the adjacent (inactive) protomer, which was investigated by mutational analysis of the respective cytoplasmic loop of this protomer. On the other hand, it has been shown previously that an A564P mutation in the C-terminal domain as well as the deletion of this domain leads to loss of regulation and/or activity of the protomer to which this domain is linked [17,29]. Consequently, these results indicate that the regulatory competence of the C-terminal domain seems to be relevant in terms of an interaction with its own catalytic domain, i.e. the membrane part of the BetP protomer. The interpretation of a self-sufficient catalytic and regulatory competence of an individual BetP protomer with functional substrate binding sites and an intact N- and C-terminal domain reflects the first functional model of BetP regulation which was derived solely from biochemical results prior to the availability of structural data [12,17]. This model suggested interaction of the C-terminal domain with the cytoplasmic surface of the same protomer. However, this model did not take the trimeric nature of BetP into account and is not supported by the current structural data.

Based on another aspect of this work, the location of the terminal part of the C-terminal domain, which is not fully resolved in the X-ray structure [9,15,21], seems to be even more speculative than before. Previous work has already indicated the inaccessibility of this stretch of amino acids for labeling [30]. The fact that any peptide fused to the C-terminal domain rendered this part of the protein highly sensitive to proteolytic degradation in the heterologous host indicates that the

accessibility and thus the conformation of the terminal part of the C-terminal domain becomes significantly changed by an attached peptide extension. Notably, the final 12 C-terminal amino acids, the truncation of which rendered the protein insensitive to proteolytic degradation, do not seem to be involved in the physical contact between the protomers as derived from the crystal structure [9].

It should be mentioned that a possible contribution of the N-terminal domain of BetP to protomer interaction has not been studied in detail yet. We were not able to generate heterotrimeric BetP complexes in which the active protomer was truncated at its N-terminal end because of low expression of this construct under the conditions applied here. Consequently, we cannot exclude a contribution of this domain to physical interaction of the protomers in BetP. The functional significance of the N-terminal domain has been demonstrated [12] and indications for its interaction with the C-terminal domain have been provided [29]. Unfortunately, this domain had to be partially truncated in the crystallized form of BetP for reasons of stability [9].

Oligomerization into homodimeric and homotrimeric complexes is a frequent structural feature of transport systems which strongly argues for a functional advantage of this configuration. Even protomer linking via terminal domains is observed in several other cases, e.g. Amt proteins [23], FocA [32], and KtrAB [33]. Experimental evidence for a functional significance of oligomerization in terms of conformational crosstalk, however, is scarce. A dominant negative phenotype has been reported for dimeric EmrE [24] and the mitochondrial phosphate carrier [25], and results indicating regulatory crosstalk of trimeric Amt proteins have also been interpreted in this direction [23]. Based on the observation of a dominant negative phenotype, results obtained with a covalent AcrB trimer indicated conformational crosstalk and a directional cycling mechanism in this transporter, although some experimental problems due to proteolytic degradation of the fusion product have to be taken into account [19]. On the other hand, conformational crosstalk was excluded for trimeric GltP on the basis of crosslinking studies [34].

Our results exclude a basic functional significance of conformational inter-protomer crosstalk for both transport catalysis and regulation in the heterotrimeric construct. However, these results do not exclude subtle alterations in the mechanism of regulation, which may be indicated by small but significant changes in the activation profiles of the wild type protein and C-terminal modifications, e.g. the A564P or the C45 truncation mutant.

Importantly, our results furthermore do not exclude a benefit of oligomerization for other aspects of carrier structure and function, e.g. stability, structural assembly, or dynamic flexibility. An adjacent protomer could function as an evolutionary optimized passive bearing providing sites of interaction more favorable for catalytic action as compared to a direct contact to the surrounding phospholipid bilayer. Interestingly, this idea is in agreement with a previous study in which artificially monomerized BetP was analyzed [21]. Monomeric BetP was found to be active, albeit at a low level, and not regulated. In contrast to this result, the orphan active protomer in the heteromeric construct investigated in the present work is fully active and retains the capability of osmo-dependent regulation. Although possible direct effects on activity of the two amino acid replacements required for artificial monomerization in the previous study cannot be completely ruled out, monomerization seems to result in a significant decrease of activity as a consequence of the absence of the adjacent protomer partner(s). Although crosstalk is not required for catalytic nor for regulatory function, i.e. every protomer is self-sufficient and can function independently in both qualities, the significant differences in the behavior of isolated monomers [21] and orphan monomers in a trimeric complex (this study) strongly indicate that the physical neighborhood of the individual protomers is beneficial for optimal activity. We believe that this concept, developed on the basis of studies using heterotrimeric forms of BetP may be relevant for other oligomeric transporters, too. In particular, a detailed mechanistic comparison with those oligomeric carrier systems may be promising, where experimental results indicate a functionally significant conformational interaction between individual protomers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.03.002.

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