Functional overexpression and in vitro re-association of OpuA, an osmotically regulated ABC-transport complex from *Bacillus subtilis*

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Abstract The osmotically regulated OpuA uptake system from *Bacillus subtilis* is a member of the SBP-dependent subfamily of ABC-transporters. The functional complex, OpuA(A_2B_2C), catalyzes the osmotically controlled import of the compatible solutes glycine betaine and proline betaine. Here, we describe the purification of the isolated TMS, OpuAB. Stimulated ATPase activity of OpuAA by OpuAB demonstrated that OpuAB adopts a functional fold. An interaction between all subunits could be verified in detergent solution with the highest ATPase stimulation determined for the dimeric NBS in the re-associated complex in the presence of all transport components plus substrate.

Keywords: ABC-transporters; Substrate stimulated ATPase activity; Membrane protein; Detergent solubilization; Transporter assembly

1. Introduction

Every cell is equipped with one or more mechanisms to maintain or restore cell volume, water content, and/or turgor pressure in response to changes in the extracellular media [1]. In the case of *Bacillus subtilis*, five osmotically controlled membrane transporters have been identified that function in the uptake of various compatible solutes for osmoprotective purposes [2]. Three of these osmo-protectant uptake (Opu) systems, OpuA, OpuB, and OpuC, are members of the ATP-binding cassette (ABC) transporter family.

ABC-transporters are ATP-dependent channels or pumps. In archea and prokarya, they perform import and export functions, whereas they function only as exporters in eukarya. By definition, an ABC-transporter is composed of two nucleotide-binding subunits (NBS) and two transmembrane subunits (TMS) [3]. The TMSs, which are divergent in sequence, are thought to form the translocation pore or channel and confer substrate specificity. Importers of the ABC-transporter family contain a fifth subunit, the substrate-binding protein (SBP) [4]. Recently, a novel class of this subfamily was identified [5], in which the SBP is fused to the TMS; an example is the protein OpuA from *Lactococcus lactis* [6].

The best-characterized SBP-dependent ABC-transporters are the histidine [7] and maltose permeases [8]. In the case of the histidine permease, it was shown that the whole transport complex could be overexpressed, but the NBS could only be extracted from the complex by urea treatment [9]. Similar results were obtained for the maltose permease [10]. However, over-expression of the TMSs was only achieved in the presence of the NBS implying a chaperone-like function [11]. To the best of our knowledge, RbsC, the TMS of the ribose importer, is the only example, where overexpression and purification of the isolated TMS was reported [12].

Here, we describe the purification of the isolated TMS of the OpuA transporter from *B. subtilis* [13], OpuAB. Proper folding of the isolated TMS was demonstrated by stimulation of the OpuAA-specific ATPase activity by OpuAB. Thus, a functional re-association of an SBP-dependent ABC-transporter starting from the isolated subunits was shown in detergent solution.

2. Materials and methods

2.1. Cloning of the *opuAB* gene

The *opuAB* gene was amplified from plasmid pBKB1 [14]. A 5′ *Nde*I and a 3′ *Hind*III restriction site as well as a 3′ extension (Strep-tag) were introduced using the following primers: 5′-GGA ATT CCA TAT GGA TAG ACT GCC TAG AAT ACC TT-3′ and 3′-ATA TAT AAA GCT TAT TAA CCA CCG AAC TGC GGG TGA CGC CAA GCG CTG GCA TTC CCC CTG CTT TTC TTC TT-5′. The PCR products were digested and cloned into the L(+)-arabinose-inducible vector pBAD33 [15] or the IPTG-inducible vector pET21a (Novagen). The resulting plasmids were used to transform various *Escherichia coli* strains.

2.2. Production of *OpuAB in* E. coli

One liter of LB media supplemented with the appropriate antibiotic was inoculated with 20 mL of an overnight culture and incubated at 25 ± 1 °C. Production of the Strep-tagged OpuAB proteins was induced at an $OD_{550\ nm} = 0.6$ with 0.1% L(+)-arabinose (pBAD33) or 1 mM IPTG (pET21a). After induction phase, cells were harvested (4000*g*, 15 min, 4 °C) and stored.

2.3. Isolation of *OpuAB*

The cell pellet was thawed and re-suspended in buffer A (50 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA, and 10% glycerol), treated with 1 mg/mL lysozyme and 200 U Benzonase, stirred at 4 °C and

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Abbreviations: ABC, ATP-binding cassette; AP, alkaline phosphatase; NBS, nucleotide-binding subunit; Opu, osmo-protectant uptake; SEC, size exclusion chromatography; TMS, transmembrane subunit

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lysed by ultra sonication. Low spin centrifugation (10 000 × *g*, 20 min, 4 °C) was performed to remove cell debris and inclusion bodies [16]. Membranes were pellet by high-speed centrifugation (100 000 × *g*, 45 min, 4 °C) and re-suspended in 15 mL buffer A. The suspension was supplemented with 2% Triton X-100, incubated for 1 h at 4 °C and centrifuged (100 000 × *g*, 45 min, 4 °C). The supernatant was incubated 1:100 with avidin (2 mg/ml in 0.1 M Tris–HCl, pH 8.0) for 15 min at 4 °C and purified by Streptavidin-affinity chromatography. The column was equilibrated with 10 column volumes (CV) of buffer A supplemented with 0.05% (v/v) Triton X-100. First, unbound proteins were washed out with 5 CV. OpuAB was eluted with 2.5 mM D-desthiobiotin. Fractions were collected and analyzed by SDS–PAGE. Appropriate fractions were pooled and stored at 4 °C.

2.4. Isolation of OpuAA and OpuAC

The NBS (OpuAA) and the SBP (OpuAC) were purified as described [14,17]. OpuAA was cloned as an N-terminal His-tagged fusion protein. The protein was isolated via IMAC and displayed a dynamic monomer/dimer equilibrium (K_D = 6 μM) under physiological salt concentrations (150 mM NaCl). However, monomeric and dimeric species of OpuAA could be separated by size exclusion chromatography (SEC) under high salt conditions (1 M NaCl) [17]. A soluble form of OpuAC (residues 22–274) was produced as an N-terminal fusion protein with the maltose-binding protein and a factor Xa cleavage site. The fusion protein was isolated by amylose-affinity chromatography. OpuAC was liberated by factor Xa proteolysis and separated from MBP and factor Xa by anion-exchange chromatography. To obtain homogenous protein, an additional size-exclusion chromatography step was introduced.

2.5. Western blot analysis

Western blot analysis was performed using the Strep-tag via a streptavidin-alkaline phosphatase (streptavidin-AP) conjugate according to the protocol of the manufacturer. For cell analysis, samples of *E. coli* cultures were taken, centrifuged (4000 × *g*, 5 min, 4 °C), re-suspended in buffer A that was supplemented with 20 μL of 5× SDS–PAGE sample buffer and 1 μL Benzonase, and analyzed by SDS–PAGE after 1 h incubation. During detergent screening, cells were treated as described and subjected to Western blot analysis employing the individual detergents at a concentration 10× their critical micelle concentration.

2.6. SEC analysis of OpuAB

An OpuAB sample was loaded onto a Superdex 200 10/30 column. For molecular weight determination, the column was calibrated using the following proteins: carboanhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), amylase (200 kDa) and apoferritin (440 kDa) dissolved in buffer C.

2.7. Determination of protein concentration

Protein concentration was determined by the bicinchoninic acid assay according to the protocol of the manufacturer.

2.8. ATPase activity assay

ATPase activity of the isolated OpuA components was analyzed in appropriate combinations: OpuAA (0.5 μM); OpuAB (1 μM) in 0.05% Triton X-100; OpuAC (4 μM) at 5 mM Mg/ATP in the absence or presence of 1 mM glycine betaine using a PK/LDH enzyme-linked assay that was previously described in detail [17]. Values shown represent the average of at least three independent experiments with the errors reported as standard deviation.

3. Results and discussions

The work on the histidine [7] and maltose permease [18] has established that these two transporters can be overexpressed and isolated in their intact forms, i.e., in the presence of the NBS and TMS. Exchange of individual subunits was achieved by urea treatment in the case of the histidine transporter [7]. In the case of OpuA from *L. lactis*, Poolman and coworkers [19] developed a strategy to exchange subunits by changes in the glycerol content of the buffer. Since this strategy was not adaptable to OpuA from *B. subtilis*, we thought of developing a strategy, in which all components of the OpuA transport complex could be isolated separately and re-associated in detergent solution. Such an approach would open a new alternative to study asymmetric mutations within multi-component ABC-complexes, which are an important tool to understand the molecular principles of ATP hydrolysis [7,20–22] and might more important to understand NBS–TMS coupling.

To establish an overexpression system for OpuAB, various *E. coli* strains were transformed and subjected to Western blot analysis. Overexpression of OpuAB was only detectable in BL21(DE3)/C41 and C43 strains [16]. Interestingly, the amount of overexpression depended on induction time (Fig. 1). In the C41 strain, expression of OpuAB was detectable after 60 min, but after 90 min no protein was identified. This is likely due to proteolytic digestion of OpuAB. In the C43 strain, a different time-dependence was detected. OpuAB was visualized after 30 min; maximum levels of overexpression were reached between 60 and 90 min; and complete degradation of OpuAB was observed after 120 min. Thus, C43 strains containing a plasmid coding for Strep-tagged OpuAB were used for further studies restricting the induction time to 90 min. The second band visible in the Western blot analysis (Fig. 1) corresponded to a cross-reactivity of the antibody system, because it was already detectable before induction. We did not investigate the nature of this band further, because it did not interfere with OpuAB purification (Fig. 2).

To determine a detergent suitable for extracting OpuAB from the membrane, a minimal detergent screen was set-up including Triton X-100 and X-114, decyl-maltoside, dodecyl-maltoside, CHAPS, octyl-glucoside, $C_{12}E_8$, taurocholate, Zwitter3-12, and lauryl-dimethylamine-oxide (data not shown). After preparation of membrane fractions [16], the sample was split into equal parts and incubated with the appropriate detergents. Interestingly, OpuAB could be solubilized at nearly equal efficiencies by all detergents, which were tested, except taurocholate and octyl-glucoside.

As outlined in Section 2, crude membranes were prepared after inclusion bodies were separated and OpuAB solubilization was achieved by incubation with 2% Triton X-100. After Strep-tag affinity purification, fractions containing OpuAB were pooled. As shown in Fig. 2A, purity of the protein was around 90% as judged from a SDS gel.

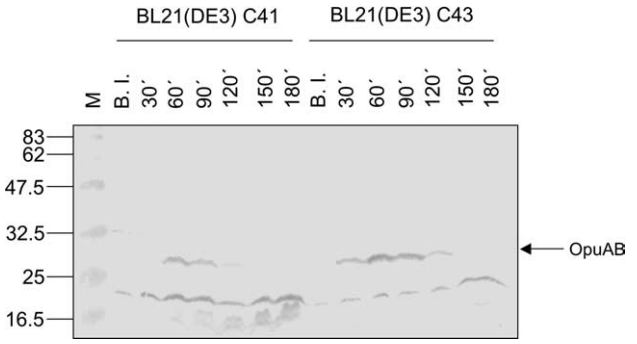

Fig. 1. Time-dependent overexpression of OpuAB. The arrow indicates OpuAB. M, marker proteins (molecular weights are given to the left), B.I., before induction. Time points are given in minutes. The lower molecular weight band very likely represents a cross-reactivity product.

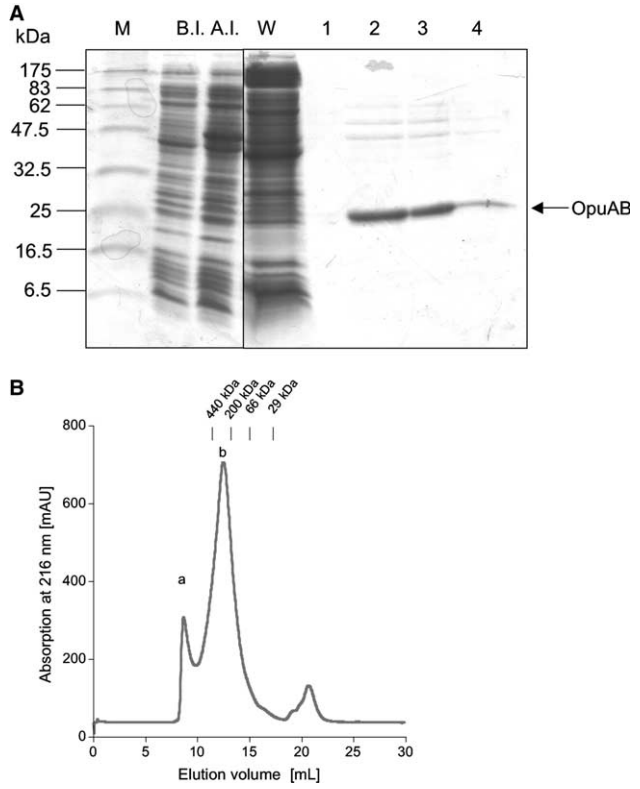

Fig. 2. (A): SDS–PAGE (15%) stained with Coomassie blue. Individual lanes are labeled, M, marker proteins (molecular weights are given to the left); B.I., before induction; A.I., after induction with 0.1% arabinose; W, wash fraction; 1–4, elution fractions. (B) SEC of OpuAB after Strep-tag purification. Molecular weight of the marker proteins used are indicated.

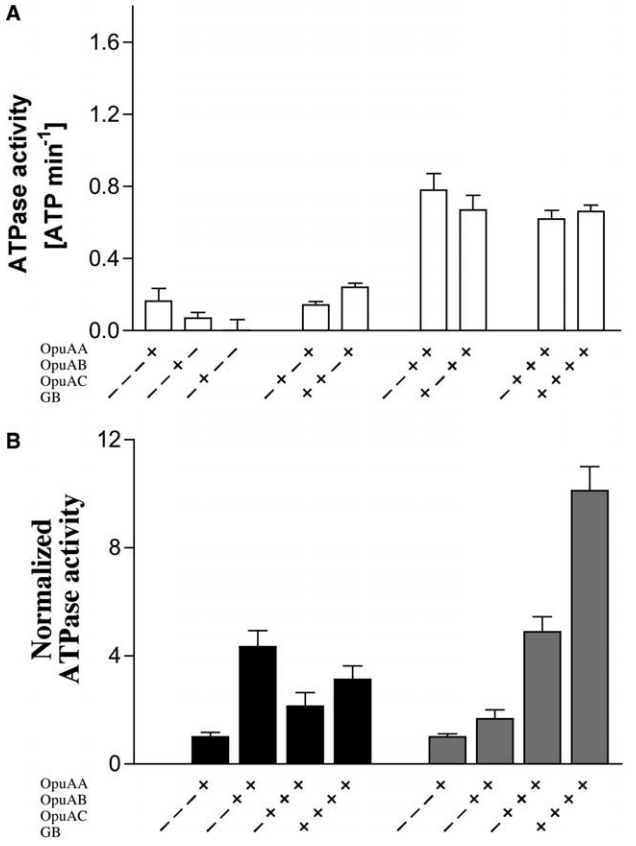

Fig. 3. ATPase activity of the OpuA transport complex in detergent solution in dependence of the absence or presence of subunits or GB. (A) shows the ATPase activity of OpuAA without separating monomeric and dimeric species, while (B) shows the results of separating monomeric (black bars) and dimeric (grey bars) OpuAA prior to the assay.

To further determine the oligomeric state of OpuAB, SEC was applied. OpuAB eluted in two peaks (Fig. 2B). Peak a corresponded to aggregated protein, while the elution volume of peak b corresponded to a higher oligomeric species of OpuAB plus associated detergent. As calculated from the elution times of soluble protein markers, this species might represent dimeric, trimeric, or tetrameric OpuAB. However, the determination of the oligomeric state of a membrane protein by SEC is error prone and further experiments are required to determine the exact oligomeric state. Nevertheless, the results of the SEC (Fig. 2B) implied that OpuAB retained a non-monomeric state. Furthermore, the additional purification step increased the purity of OpuAB to roughly 95% (data not shown), enabled a separation of aggregated protein, and resulted in an overall yield of roughly 1 mg/L *E. coli* culture.

However, the major question of whether OpuAB adopted a functional fold remained unsolved. Therefore, the OpuA transport complex was ''re-associated'' in detergent solution. The individual subunits and glycine betaine (GB) were mixed, and ATPase activity of OpuAA was used as a read-out for possible subunit interaction. To distinguish the relative role of individual combinations of the subunits and the relative contribution of each subunit, different combinations were analyzed (Fig. 3A). The activity of isolated OpuAA was comparable to the activity of the NBS in the absence of detergent. Surprisingly, a small but detectable activity was detected for OpuAB. Although this is reminiscent of HisM, a TMS of the histidine permease [23], we believe that the activity is due to a contaminant ATPase. A 4-fold stimulation of ATPase activity was observed in the combinations OpuAA/OpuAB. This stimulation is clearly larger than the sum of the individual components. Within experimental error, stimulation did not change due in the presence of OpuAC with or without GB. Since OpuAA undergoes a dynamic monomer–dimer equilibrium [17], addition of the NBS without separating the monomeric and dimeric species might influence ATPase stimulation if the dimeric form of OpuAA is the catalytic relevant species. Since we have no knowledge about the affinities of the individual components, one can easily envision that dimerization of OpuAA is the limiting factor. To investigate this hypothesis further, we analyzed the influence of monomeric and dimeric NBS separately (Fig. 3B). Now, a much clearer picture of the stimulation of ATPase activity emerged. For monomeric OpuAA (Fig. 3B, black bars), a 4-fold stimulation was observed for the OpuAA/OpuAB combination that did not change significantly upon addition of OpuAC with or without GB.

In striking contrast was the ATPase stimulation for dimeric OpuAA (Fig. 3B, grey bars). Adding OpuAB to dimeric OpuAA resulted in hardly any stimulation. The combination OpuAA/OpuAB/OpuAC showed a 4-fold stimulation, while addition of GB resulted in a nearly 10-fold stimulation of ATPase activity. This is in clear contrast to the isolated NBS, where the dimer showed a lower activity [17], but resembles the trend observed for the maltose importer [10]. Further-

more, the modulation by GB implies a functional response already in detergent solution. Such a behavior was observed for example for the histidine permease, but only in its reconstituted form [24].

These data demonstrated that isolated OpuAB can be overexpressed in a functional form and that dimeric OpuAA induced the highest degree of ATPase stimulation in the presence of OpuAC plus substrate. Furthermore, the obtained pattern of stimulation suggests that even in detergent solution an OpuA transport complex can be ''re-associated'' at least transiently.

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