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Lycklama A Nijeholt, Jelger A; Vietrov, Ruslan; Schuurman-Wolters, Gea K; Poolman, Bert

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Energy Coupling Efficiency in the Type I ABC Transporter GlnPQ

Jelger A. Lycklama a Nijeholt, Ruslan Vietrov,
Gea K. Schuurman-Wolters and Bert Poolman

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747, AG, Groningen, The Netherlands

Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747, AG, Groningen, The Netherlands

Correspondence to Bert Poolman: b.poolman@rug.nl

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Abstract

Solute transport via ATP binding cassette (ABC) importers involves receptor-mediated substrate binding, which is followed by ATP-driven translocation of the substrate across the membrane. How these steps are exactly initiated and coupled, and how much ATP it takes to complete a full transport cycle, are subject of debate. Here, we reconstitute the ABC importer GlnPQ in nanodiscs and in proteoliposomes and determine substrate-(in)dependent ATP hydrolysis and transmembrane transport. We determined the conformational states of the substrate-binding domains (SBDs) by single-molecule Förster resonance energy transfer measurements. We find that the basal ATPase activity (ATP hydrolysis in the absence of substrate) is mainly caused by the docking of the closed-unliganded state of the SBDs onto the transporter domain of GlnPQ and that, unlike glutamine, arginine binds both SBDs but does not trigger their closing. Furthermore, comparison of the ATPase activity in nanodiscs with glutamine transport in proteoliposomes shows that the stoichiometry of ATP per substrate is close to two. These findings help understand the mechanism of transport and the energy coupling efficiency in ABC transporters with covalently linked SBDs, which may aid our understanding of Type I ABC importers in general.

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Introduction

ATP binding cassette (ABC) transporters are found in all kingdoms of life and are responsible for the transport of a wide range of molecules like amino acids, ions, vitamins, lipids and antibiotics [1]. ABC transporters facilitate both uptake and export of compounds, but irrespective of their directionality, their core is composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The majority of ABC importers are recognized by the presence of a substrate-binding protein or domain (SBP or SBD), which captures the substrate and delivers the molecule to the TMD [2]. On the basis of protein structures and transport mechanisms, ABC importers are subdivided into Types I, II and III. Type I and II ABC importers employ extracytoplasmic SBPs or SBDs for substrate capture [3], and subsequently translocate the substrate via alternating access of the

substrate-binding pocket in the translocator domain. Type III importers, also named energy-coupling factor transporters, capture ligands via so-called S-components, which are small integral membrane proteins that associate with a transmembrane coupling protein and two NBDs to form a full transporter [4]. In this paper, we focus on GlnPQ, a Type I ABC importer with two different SBDs fused to each TMD and thus four SBDs in the functional complex.

GlnPQ has two identical canonical NBDs, named GlnQ, and two membrane domains (GlnP) to which the SBDs are linked (Fig. 1). The GlnP subunits are synthesized with a 26-amino-acid pre-sequence that serves to translocate the two SBDs across the membrane, followed by cotranslational insertion into the membrane of the TMD [5]. The domain order in GlnP (from N- to C-terminus) is signal sequence, SBD1, SBD2 and TMD; the latter three domains are connected by partly flexible linkers, which allow the

Fig. 1. Schematic of the domain organization of GlnP and GlnQ subunits that form the ABC importer GlnPQ; two GlnP and two GlnQ subunits are present in the functional complex. The signal peptide that is cleaved off from GlnP is shown in red; the subdomain structure of GlnP is indicated by SBD1, SBD2 and TMD, which are connected by linker sequences.

receptors to alternately deliver their substrate to the TMDs. The N-terminal SBD1 binds asparagine with a dissociation constant (K_D) below 1 μ M and glutamine with a K_D of ~ 100 μ M, whereas SBD2 is specific for glutamine [6]. GlnPQ imports asparagine, glutamine and glutamate but is inhibited by arginine [7]; the molecular basis for arginine inhibition is unknown but becomes apparent in this study. We have shown that SBD1 and SBD2 close by an induced-fit mechanism, and we have established a connection between the conformational dynamics of the SBDs and the transport of glutamine and asparagine [8]. Although GlnPQ and family members are unique in their domain organization and make use of a total of four (competing) substrate receptors, the available data suggest that the transporters operate as genuine Type I ABC importers. The GlnPQ system in *Lactococcus lactis* is always present at relatively high levels as glutamine or glutamate are essential amino acids for this and related Gram-positive bacteria. The *glnPQ* genes are under the control of a repressor, which allows tuning of expression in response to extracellular glutamine and ammonia [9].

Structural studies on the Type I ABC importer for maltose in *Escherichia coli* (MalFGK₂-MalE) have led to a model, the so-called Switch Model, wherein the translocation cycle starts with the TMDs in an inward-facing orientation and a pre-translocation state is triggered upon binding of the substrate-bound, closed-state of the SBP. The pre-translocation state allows binding of ATP to the NBDs, which results in a conformational change of the TMDs to an outward conformation, allowing the substrate to enter the TMD. Subsequent hydrolysis of ATP and release of inorganic phosphate and ADP resets the transporter in the inward conformation and releases the substrate

inside the cell. This model was built using biochemical data and X-ray crystal structures that provide snapshots of the transport cycle [10–13]. In an alternative view of the Switch Model, the open-unliganded MalE binds to the outward-facing transporter to trigger the hydrolysis of ATP. Subsequent binding to MalE of maltose triggers return of MalFGK₂ to the inward-facing state, which is accompanied by the release of substrate and inorganic phosphate [14,15]. A radically different model, known as Reciprocating Twin-Channel, proposes that the binding and hydrolysis of ATP and release of inorganic phosphate plus ADP in one NBD is directly coupled to substrate binding and translocation in one of two functionally separate substrate translocation pathways [16]. Thus, ATP hydrolysis alternates between the two NBD active sites, as does substrate translocation in two paths within the TMD. A mechanistic stoichiometry of two ATP per substrate is most consistent with the Switch Models, and one ATP per substrate is predicted by the Reciprocating Model; irrespective of the model, the actual stoichiometry could be higher if futile hydrolysis of ATP takes place.

Intuitively, one would expect that ATP hydrolysis and substrate import are tightly coupled, to avoid unnecessary draining of ATP from the cell. Although it was found for the Type I ABC transporter OpuA that two ATP molecules are needed for one substrate [17], several other studies show a much higher use of ATP and a high basal ATPase activity when no substrate is present [18–20]. A recent study proposes that the basal ATP hydrolysis in the Type II ABC importer BtuC₂D₂ gives rise to a hyperactive state; during prolonged basal ATP hydrolysis in the absence of substrate, the ABC importer undergoes a slow conformational change after which it shows a $\sim 60\%$ higher uptake rate of vitamin B₁₂ [21]. Here,

we investigate the basal and substrate-dependent ATPase and translocation activity of GlnPQ by taking advantage of nanodiscs and proteoliposomes as membrane model systems and single-molecule Förster resonance energy transfer (smFRET) as method to probe the conformational states of the receptor domains. The goal of the research is to determine the energy coupling efficiency of the ABC importer GlnPQ, which includes the quantification of the ATP/substrate stoichiometry and the mechanistic basis for the futile hydrolysis of ATP. We find that the ATP/substrate stoichiometry is close to two and that docking of the closed-unliganded state of the SBDs increases the basal ATP hydrolysis.

Results

GlnPQ nanodisc assembly and characterization

To analyze the ATP hydrolysis of full-length GlnPQ in a lipid environment, we reconstituted the protein complex in nanodiscs. In our initial analyses, we used two different membrane scaffold proteins (MSPs), namely, MSP1D1 and MSP1E3D1, resulting in nano-

disc with diameters of 9.5 and 12.1 nm, respectively [22,23]. Because we did not observe discernable differences in ATPase activity (data not shown), we continued with the smaller MSP1D1 nanodiscs. We tested different lipid compositions to identify the optimal conditions for GlnPQ, but always using lipids with two oleoyl (*cis*- Δ^9 -octadecanoate) chains. We found that the ratio DOPE:DOPC:DOPG of 50:12:38 gives rise to the highest activity, and this mixture was used in all subsequent experiments (Fig. 2a). A typical size-exclusion chromatography profile of full-length GlnPQ in nanodiscs and subsequent SDS-PAGE analysis of the peak fraction are shown in Fig. 2b and c, respectively. A schematic representation of GlnPQ in a nanodisc is shown in Fig. 2d.

The closed state of SBD initiates the ATP hydrolysis cycle

As shown in Fig. 2a, we observe significant ATPase activity in the absence of substrate, indicating that full-length GlnPQ is either hydrolyzing ATP with no apparent trigger or initiated by an unknown factor. Our previous work on the conformation dynamics of the SBDs in GlnPQ, combined with transport data, indicated that the closed state of the SBD interacts

Fig. 2. Lipid-to-protein ratios for reconstituting GlnPQ in nanodiscs. (a) ATPase activity of GlnPQ nanodiscs at a GlnPQ:MSP1D1:lipid ratio of 1:5:500. Except for the reconstitution in *E. coli* lipids, the mol% of DOPE was kept at 50% and the fractions of DOPC and DOPG were varied. The ATPase activity is shown without (white bars) and in the presence (black bars) of 1 mM of glutamine. (b) A typical size-exclusion chromatography profile of GlnPQ nanodiscs composed of DOPE:DOPC:DOPG at ratios of 50:12:38; the peak fraction used for further analysis is indicated in gray. (c) The peak fraction was analyzed by 12.5% polyacrylamide SDS-PAGE and showed the presence of GlnP, GlnQ and the scaffold protein MSP1D1. (d) A schematic representation of GlnPQ in nanodiscs.

Fig. 3. Basal ATPase activity of GlnPQ and derivatives in nanodiscs. (a) After assembly, the nanodiscs mixtures were concentrated and loaded on a Superdex 200 10/300 GL column. The resulting chromatograms are normalized to the highest peak. (b) The peak fractions were analyzed by 12.5% SDS-PAGE analysis. The protein bands visible between the MSP and GlnQ proteins are contaminations in the MSP preparation. (c) The basal ATPase activity (no substrate present) of GlnPQ and derivatives reconstituted in nanodiscs: (1) without GlnPQ; (2) GlnPQ without SBDs but the TMD-SBD2 linker still present (GlnPQ-L-ΔSBD); (3) GlnPQ with only SBD2 (GlnPQ-SBD2); (4) GlnPQ with only SBD2(D417F); (5) GlnPQ with only SBD1 (GlnPQ-SBD1); (6), GlnPQ with only SBD1 (E184D, V185E); (7) wild-type GlnPQ; (8) full-length GlnPQ with D417F in SBD2; and (9) GlnPQ without SBDs and without linker (GlnPQ-ΔSBD).

with the TMD, even when it is in a closed-unliganded conformation [8]. Thus, it is possible that the observed ATPase activity in the absence of ligand is due to docking of the closed-unliganded SBD, which would then initiate a pre-translocation state [11]. To further test this hypothesis, we generated nanodiscs with different truncated forms of GlnPQ, which are transporters with a single SBD (GlnPQ-SBD1, GlnPQ-SBD2) or no SBD (GlnPQ-L-ΔSBD, GlnPQ-ΔSBD); the size-exclusion chromatography and SDS-PAGE profiles of the various constructs are shown in Fig. 3a and b. We first tested whether or not the presence of the SBDs was responsible for the basal ATPase activity of GlnPQ. Indeed, when we compare GlnPQ-L-ΔSBD with full-length GlnPQ, we see a reduced ATPase activity (Fig. 3c: compare 2 with 7). When only

SBD2 or SBD1 is present, we observe a 4- to 8-fold increase in basal ATPase activity (Fig. 3c: compare 2 with 3 and 5), indicating that the SBDs are responsible for futile hydrolysis of ATP. Next, we tested several SBD mutants that in smFRET experiments displayed different dwell times for the closed-unliganded state. We find an apparent correlation between the dwell times and the basal ATPase activity (Fig. 3c: 3–6 and Table 1). This indicates that the closed-unliganded state of SBD1 and SBD2 can initiate the ATP cycle of GlnPQ and may do so in similar manner as the closed-liganded state. The observation that the activity of full-length GlnPQ is not the cumulative of GlnPQ-SBD1 and GlnPQ-SBD2 most likely reflects the competition between SBD2 and SBD1 for docking onto the TMD; this competition no longer occurs in GlnPQ-SBD1 and

Fig. 4. Arginine does not cause smFRET-observable conformational changes in isolated SBDs but inhibits asparagine binding. (a) Cartoons depicting the different scenarios of ligand binding; SBD1, green icon; arginine, yellow oval; asparagine, gray circle. (b) Apparent FRET values, E^* , for different states of SBD1; E^* values range from 0 to 1. Dashed blue lines are visual guides to indicate the shift in E^* values upon addition of ligand. (1) E^* of SBD1 is characterized by a Gauss-shaped distribution that is centered around 0.68 ($= \langle R^* \rangle$ with $\sigma = 0.06$); (2) 10 mM Arginine has no influence on the distribution, $\langle R^* \rangle \sim 0.68$ and $\sigma = 0.06$; (3) 10 mM asparagine shifts $\langle R^* \rangle$ to $E^* = 0.84$ and $\sigma = 0.05$; (4) 10 mM arginine plus 10 mM asparagine yields $\langle E^* \rangle = 0.84$ and $\sigma = 0.05$; (5) 50 μ M of asparagine gives $\langle E^* \rangle \approx 0.69$ and $\sigma \approx 0.07$, corresponding to the open conformation (minor fraction) $E^* = 0.85$ and $\sigma = 0.04$, corresponding to closed conformation (major fraction); (6) 50 μ M of asparagine plus 10 mM of arginine yields $\langle E^* \rangle \approx 0.69$ and $\sigma \approx 0.06$ (open conformation) and $\langle E^* \rangle \approx 0.84$ and $\sigma \approx 0.06$ (closed conformation).

GlnPQ-SBD2. Moreover, the spacing between SBD1 and TMD is different in wild-type GlnPQ and GlnPQ-SBD1 (Fig. 3c: 7).

We have previously shown that the mutation D417F prevents SBD2 from closing, and indeed, this mutant also displays diminished ATP hydrolysis when analyzed in the context of the full-length GlnPQ (Fig. 3c: compare 7 and 8) and single-receptor GlnPQ-SBD2 (Fig. 3c: compare 3 and 4). Similarly, the E184D V185E mutations decrease the ATPase activity in the single-receptor GlnPQ-SBD1 constructs (Fig. 3c: compare 5 and 6). Surprisingly, when we truncate the transporter by removing not only the two SBDs but also the linker region that connects SBD2 with the TMD, GlnPQ- Δ SBD, we observe an almost 30-fold increase in basal ATPase activity, indicating that the linker plays an important role in the coupling of SBD docking and ATP hydrolysis (Fig. 3c: 9, *vide infra*).

Arginine inhibition of SBD docking and substrate-dependent ATP hydrolysis

To further investigate the state of the SBD that results in ATP turnover, we used arginine as ligand.

Arginine is not transported by GlnPQ, but this amino acid inhibits the uptake of glutamine [7]. To elucidate the interaction of arginine with GlnPQ and to determine the conformation of the isolated SBDs, we performed smFRET measurements, as described previously [8]. We labeled double-Cys proteins with AlexaFluor555-maleimide (fluorescence donor) and AlexaFluor647-maleimide (acceptor) and performed so-called alternating laser-excitation experiments on a confocal microscope. Conformational changes are observed as a change in FRET efficiency (E^*) of molecules diffusing through the focal volume of the microscope. Figure 4 shows that the FRET efficiency of isolated SBD1 changes upon addition of asparagine, a high-affinity ligand that binds with a dissociation constant (K_D) of 0.3 μ M [8]. The FRET efficiency does not change in the presence of 10 mM of arginine, but arginine affects the E^* of SBD1 with 50 μ M asparagine (Fig. 4, compare panels 5 and 6). Thus, SBD1 can interact with arginine, but the binding event does not lead to closing of the protein. Hence, we refer to the arginine-bound state as the open-liganded conformation. In agreement, arginine inhibits the uptake of glutamine in proteoliposomes but is not transported itself (data not shown); the inhibition of transport is

(a)

(b)

Fig. 5. Arginine competition and evidence that the closed state of the SBDs trigger ATP hydrolysis. (a) The basal ATPase activity is shown at different concentrations of L-arginine for the wild type GlnPQ (black circles), GlnPQ-SBD2 (white squares), GlnPQ-SBD1 (black triangles) and GlnPQ-SBD2-D417F (white diamonds). (b) The ATPase activity of GlnPQ-SBD2 is shown at different concentrations of glutamine in the absence (circles) and presence of 10 mM arginine (triangles). The dotted line represents the basal ATPase activity, obtained from the arginine inhibition experiment. The basal ATPase activity has been subtracted for the calculation of the kinetic parameters.

thus caused by the competition between arginine and glutamine binding. Similar observations were made for isolated SBD2, which binds glutamine with a K_D of 0.9 μM [8]; arginine does not elicit a change in conformation in SBD2 but competes with glutamine binding (Table 2). The apparent inhibition constants (K_i) of arginine binding to SBD1 and SBD2 are 0.65 and 0.25 mM, respectively; these are averages of the values shown in Table 2. We observe a small difference in K_i values for arginine binding to the isolated SBD1 and the corresponding tandem construct when glutamine is used as substrate, which is not seen with asparagine. Similarly, the K_i values for arginine binding differ by a factor of 3 when SBD2 and the corresponding SBD1–SBD2 construct are compared. Although we have no biochemical evidence that SBD1 and SBD2 of GlnPQ bind to each other [6,8], these data may suggest some functional interaction.

Next, we measured the basal ATPase activity of GlnPQ, GlnPQ-SBD1 and GlnPQ-SBD2 in nanodiscs and find that the rate of hydrolysis decreases with increasing arginine concentration (Fig. 5a). This further supports the hypothesis that the closed conformation of the SBDs is responsible for triggering the increase in basal ATPase activity. By fitting

the data points to a hyperbolic function, analogous to the Michaelis–Menten equation, we obtain binding constants for arginine of 1.3 mM (GlnPQ-SBD1) and 0.8 mM (GlnPQ-SBD2), which are a little higher than the K_i values found in the smFRET experiments (Table 2). The activity in the presence of saturating amounts of arginine indicates the SBD-independent basal ATPase activity, which is subtracted in further experiments to obtain more accurate kinetic parameters.

In Fig. 5b, we show the ATPase activity of GlnPQ-SBD2 as a function of glutamine concentration, a substrate that is actively transported. In the presence of 10 mM arginine, the apparent K_M increases but the V_{max} stays the same, which is a classical case of competitive inhibition (Fig. 5b). The K_M for glutamine transport is 2.3 μM , and the K_i for arginine is 0.6 mM. A similar experiment was performed with GlnPQ-SBD1 and asparagine as substrate, and similar competitive inhibition was observed (Table 3). Taken together, these results show that arginine is a competitive inhibitor for the import of glutamine and asparagine via SBD2 and SBD1, respectively, and arginine traps the SBDs in the open state, preventing transport and futile hydrolysis of ATP.

The effect of KCl on SBD docking and tight coupling of ATP hydrolysis and substrate transport

In our initial optimization of the ATPase activity assay, we observed a lower basal activity when the KCl concentration was relatively high (data not shown). This might indicate that electrostatic interactions play a role in the docking of the SBDs, which warranted further investigation. When we performed the arginine inhibition experiment with full-length GlnPQ, we observed a significant difference with and without 300 mM KCl (Fig. 6a). The basal ATPase activity is twofold higher at the lower salt concentration, indicating that the SBDs dock more frequently onto the TMD and presumably initiate the ATP cycle. Probing the binding of asparagine to SBD1 in the presence of various KCl concentrations gives almost no change in K_D , except when the concentration of KCl is in molar range (Table 4). This either indicates that both opening and closing rates are not affected by KCl concentrations, or that they are affected to the same extent; the latter seems less likely. Thus, we propose that at physiological ionic

strength, docking rather than closing or opening of the SBDs is partly governed by electrostatic interactions. At the level of the kinetics of glutamine-induced ATP hydrolysis in full-length GlnPQ, we find that the K_M for glutamine is not affected but the turnover number is increased at low salt concentration (Fig. 6b: top 2 curves).

Next, we determined whether or not glutamine-induced hydrolysis of ATP is tightly coupled to transport. We reconstituted GlnPQ in proteoliposomes with the same lipid mixture as used for the nanodiscs and analyzed glutamine uptake without additional KCl. When we compare the kinetics of glutamine transport in proteoliposomes with the hydrolysis of ATP in nanodiscs in the absence of KCl, we observe a similar K_M and an almost twofold difference in V_{max} (Fig. 6b: compare top and bottom curve; Table 5). This indicates that there is a tight coupling of glutamine transport and ATP hydrolysis and suggests that the mechanistic stoichiometry of ATP per glutamine is 2, that is, if we assume 50% of the GlnPQ molecules in the proteoliposomes is oriented inside-in and 50% is oriented inside-out.

(a)

(b)

Fig. 6. Effect of KCL on docking of the SBDs and coupling of ATP hydrolysis and glutamine transport. (a) The basal ATPase activity is shown at different concentrations of L-arginine for the wild-type GlnPQ in the absence (white circles) and presence of 300 mM KCl (black circles); the basal buffer is composed of 50 mM potassium phosphate (pH 7.0). (b) The ATPase activity of wild-type GlnPQ is shown at different concentrations of glutamine in the absence (white circles) and presence of 300 mM KCl (black circles). The basal ATPase, obtained from the arginine inhibition experiment (V_{max} in Table 3), was subtracted. The uptake of [14 C]glutamine in proteoliposomes containing wild-type GlnPQ at different glutamine concentrations is shown in black triangles. The uptake experiments were done in the absence of KCl in 50 mM potassium phosphate (pH 7.0).

Discussion

The combination of the nanodisc reconstitution technology and online activity assays allowed us to investigate the basal and substrate-induced ATPase activity of GlnPQ. We find that conditions that lock the SBDs in the open conformation (arginine as ligand, or D417F mutation) decrease the basal ATP hydrolysis relative to the condition that no ligand is present; under the latter condition, the SBDs can occasionally visit the closed conformation. We show by interpolation that the closed-unliganded state of the SBDs of GlnPQ is responsible for a considerable part of the basal ATPase activity (and thus sets up the system), which is in line with work done on the Type I ABC maltose importer MalEFGK₂ but differs from studies on the Type II ABC vitamin B₁₂ importer. The basal activity of GlnPQ is low but significant and may serve to prime the system for rapid uptake when nutrient availability varies over time.

We observe that SBD1 and SBD2 in the closed conformation (with/without glutamine or asparagine) initiate the ATP cycle, presumably by stabilizing a pre-translocation state of GlnPQ (see Introduction and Fig. 6 in Ref. [8]). The docking of SBDs likely triggers further conformational changes of the ATP-bound state of the transporter, which leads to a transition to the outward-facing conformation of the transport domain. Although our results are in line with the broadly accepted Switch Model [24–26], other views or mechanistic models cannot be ruled out entirely. In brief, the group of Duong has proposed that open-unliganded MalE binds with high affinity to MALFGK₂ in the outward-facing conformation, which is triggered by ATP alone. Maltose then binds to the MalE–MALFGK₂ assembly. Upon ATP hydrolysis, the transporter isomerizes to the inward-facing conformation, and maltose is released in the cytosol [14]. We have no indications that SBDs in the open conformation bind to the TMDs of GlnPQ. The Reciprocating Model of the group of Jones and George [16] is compatible with neither an ATP/substrate stoichiometry of 2 nor the crystal structures of whole ABC importers. Finally, we are not aware of two distinct substrate translocation pathways within ABC importers.

We made the surprising observation that deletion of the linker between SBD2 and TMD induces a very high basal ATPase activity (Fig. 3c: lane 9). Furthermore, we find that GlnPQ-ΔSBD can be complemented by soluble SBD2 provided *in trans*, whereas GlnPQ-ΔSBD cannot. Both observations point toward a role of the linker in the coupling mechanism, possibly by acting as a gate for transport or and serving a restraining role in the conformation dynamics of the system. In follow-up work, we have varied the length and amino acid composition of the linkers connecting SBD1–SBD2 and SBD2–TMD.

We find that part of the SBD2–TMD interdomain sequence is more than a flexible linker and may provide part of the platform for docking of the SBDs onto the TMD (Schoorman-Wolters, de Boer and Poolman, unpublished results).

The role of the linker between SBD2 and TMD of GlnPQ is reminiscent of the role of the periplasmic P2 domain in the maltose transporter, although the linker is only 30 residues long, whereas P2 is a domain of 180 amino acids [27]. The maltose-binding protein MalE has been shown to interact with the P2 domain of the TMD MalF, and thiol-mediated cross-linking of MalE to the TMD domain via a 25 Å linker did not affect transport efficiency. It has been suggested that the P2 domain is responsible for the continuous docking of MalE onto MalFG, and therefore, the tethering of MalE to the TMD is without effect [28]. The absence of such a domain in GlnPQ might indicate the necessity of covalent linkage of the SBDs and thereby keep the local receptor concentration high- to allow low-affinity binding (docking) of the receptors.

The low basal ATPase activity in the absence of SBDs in GlnPQ-ΔSBD (Fig. 3c, lane 2) is still higher than the ATPase activity of nanodiscs without a membrane protein present. Although the prepared nanodiscs are relatively pure by SDS-PAGE criteria and different preparations show a similar basal ATPase activity, we cannot exclude that the low ATPase activity is caused by a contaminating protein, which is reconstituted in a fraction of the nanodiscs. Alternatively, a fraction of the GlnPQ nanodiscs may not be correctly folded and show SBD- and ligand-independent ATPase activity. We therefore subtracted this basal activity to dissect the effects of open and closed states of the SBDs and the presence of substrates on the ATPase activity of GlnPQ derivatives.

Experiments with the maltose transporter in nanodiscs show low basal ATPase activity without MalE and a turnover number for ATP hydrolysis after the addition of MalE of 15–30 per minute [14,29]. This is comparable to what we find in our analysis (Fig. 2) and may indicate that the intrinsic closing of MalE is also responsible for the basal ATPase activity in the maltose transporter. Although NMR studies showed that ~95% of MalE is in an open state and ~5% in a closed state [30], smFRET experiments did not reveal intrinsic closure in the wild-type MalE, which might suggest very fast closing and opening dynamics, beyond the time resolution of smFRET [31,32]. It is not known if and how the P2 domain of MalF affects the intrinsic closing of MalE [27,33]. The effect on the basal ATPase activity was also analyzed for MalE mutants that were shown by smFRET to be in the closed state for ~40% of the time, and these studies showed that basal ATPase activity with the MalE mutants is less than with the wild-type protein [32,34]. Although these observations are not consistent with a mechanism in

which the closed state of the SBP triggers the activation of the transporter, we note that these authors [34] report a very high basal ATPase activity (e.g., as compared to work of others [29], which is indicative of issues with the sample preparation.

For GlnPQ, smFRET analysis shows that SBD1 and SBD2 are in the closed state a few percent of the time with dwell times of 236 and 61 ms, respectively [8]. These dwell times are relatively long and the SBDs thus have a high probability to dock onto the TMD and initiate the ATP cycle. Only in the presence of saturating concentrations of amino acids will this futile ATP hydrolysis be outcompeted.

Finally, by comparing the ATPase activity in nanodiscs with the transport activity in proteoliposomes and assuming a random orientation of GlnPQ in the vesicle membrane, we obtain an ATP/glutamine stoichiometry of about 2, that is, when the $V_{\max}$ of glutamine-stimulated ATP hydrolysis is divided by the $V_{\max}$ of glutamine transport (Table 5). This value is similar to what we previously reported for the Type I glycine betaine transporter OpuA [17].

In conclusion, the basal ATPase activity of the Type I ABC importer GlnPQ is caused by the intrinsic closing and the relatively long life times of the closed state of the SBDs. In the closed state (with/without ligand), the SBDs dock onto the TMD of GlnPQ and trigger the hydrolysis of ATP. We also show that relatively little futile hydrolysis of ATP takes place when saturating amounts of amino acids are present, and we suggest an ATP to amino acid stoichiometry of close to 2.

Materials and Methods

Materials

M17 broth was obtained from Oxoid and Gistex LS from Brenntag. Ni-Sepharose 6 fast flow came from GE Healthcare; *N*-dodecyl- β -D-maltoside from Glycon; Triton X-100 from Sigma-Aldrich; Biobeads SM-2 from Bio-Rad; and total *E. coli* lipid extracts, L- α -phosphatidylcholine from egg yolk, and synthetic lipids (dioleoylphosphatidyl derivatives: DOPE, DOPG and DOPC) from Avanti Polar lipids. Radiolabeled L-[^{14}C] glutamine was obtained from ARC radiochemicals (240 $\mu\text{Ci}/\text{mmol}$) and L-[^3H]asparagine (5 Ci/mmol) from PerkinElmer. Creatine-phosphate was obtained from Sigma. Creatine kinase and ATP were obtained from Roche Applied Science. All other chemicals were of analytical grade and obtained from varying commercial companies. The strains and plasmids used in this study are shown in Table 6.

Expression and purification of MSP1D1

Expression and purification of MSP1D1 was performed as described previously [37].

Table 1. Basal ATP hydrolysis and fraction of time the SBD spends in the closed state when no ligand is present

Probed variant	Basal ATP hydrolysis (min^{-1})	Fraction of time in closed state
GlnPQ-SBD2	3.9	0.02
GlnPQ-SBD2(D417F)	2.2	<0.001
GlnPQ-SBD1	8.1	0.086
GlnPQ-SBD1 (E184D,V185E)	3.5	0.036

The fraction of time the SBDs spend in the closed state is taken from previous work [8] and this study. In short, the validated traces are concatenated and fitted with a two-state model. The fraction of time in the closed state is defined as the ratio of combined time in closed state divided by the total duration of the analyzed traces.

Expression of GlnPQ and derivatives

The *glnPQ* genes used in this study are from *L. lactis* IL1403 and they are expressed in *L. lactis* GKW9000, a derivative of the MG1363 strain [17]. *L. lactis* GKW9000 was cultivated semi-anaerobically at 30 °C in a medium containing 2% (w/v) Gistex (Brenntag, Groningen) and 65 mM sodium-phosphate, pH 7, supplemented with 1% (w/v) glucose and 5 $\mu\text{g}/\text{ml}$ chloramphenicol when carrying pNZglnPQhis or other derivatives. For the preparation of membrane vesicles, cells were grown in a 3-l pH-regulated bioreactor at pH 6.5 to an A_{600} of 2, after which the transcription from the *nisA* promoter was switched on by the addition of 0.01% (v/v) culture supernatant of the Nisin A-producing strain NZ9700 and an additional 1% (w/v) glucose. The cells were grown for an additional 2 h, harvested by centrifugation (15 min, 6000g) and washed once with 100 mM potassium phosphate (pH 7.5). Membrane vesicles were prepared according to standard procedures [5].

Purification of GlnPQ and derivatives

Typically, 150 mg of total protein in membrane vesicles was solubilized in 50 mM potassium phosphate (pH 8.0), 200 mM NaCl, 20% glycerol and 0.5%

Table 2. Inhibition coefficients (K_i) for arginine determined from asparagine and glutamine binding to isolated SBD1 and SBD2

SBD variants	Ligand	K_i (mM)
SBD1(T159C/G87C)	Asparagine	0.71
SBD1(T159C/G87C)	Glutamine	0.87
SBD1(T159C/G87C) + SBD2	Asparagine	0.67
SBD1(T159C/G87C) + SBD2	Glutamine	0.34
SBD2(T369C/S451C)	Glutamine	0.13
SBD2(T369C/S451C) + SBD1	Glutamine	0.38

Isolated AlexaFluor-labeled SBD1, SBD2 and tandem SBD1–SBD2 variants were titrated with asparagine or glutamine, while the arginine concentration was kept at 10 mM. The observed FRET changes were used to calculate the K_D for asparagine or glutamine binding, and the K_i for competitive binding of arginine.

Table 3. Kinetic parameters of ATP hydrolysis by GlnPQ in nanodiscs and arginine inhibition

GlnPQ variant (amino acid probed)	Basal ATP hydrolysis titrated with arginine		Glutamine- stimulated ATP hydrolysis		
	K_M (mM)	V_{max} (min^{-1})	K_M (μM) ^a	$K_{M,app}$ (μM) ^b	K_i (mM)
GlnPQ (Gln)	0.3	3.6	0.7	ND	ND
GlnPQ-SBD2 (Gln)	0.8	3.0	2.3	40.2	0.6
GlnPQ-SBD1 (Asn)	1.3	5.4	1.0	11.9	0.9

The basal ATP hydrolysis was measured for GlnPQ variants in nanodiscs at increasing concentrations of arginine. The glutamine-stimulated ATP hydrolysis was measured in the absence and presence of 10 mM arginine; from these experiments, the K_i for arginine inhibition was calculated.

^a Without arginine.

^b With arginine.

(w/v) DDM for 30 min at 4 °C. The sample was centrifuged (12 min, 300,000g) and the supernatant was collected. Subsequently, GlnPQ or its derivatives were allowed to bind to Ni-Sepharose (1.5-ml bed volume) for 1 h at 4 °C after the addition of 10 mM imidazole. The resin was rinsed with 20 column volumes of wash buffer [50 mM potassium phosphate (pH 8.0), 200 mM NaCl, 20% (v/v) glycerol, 50 mM imidazole plus 0.02% (w/v) DDM]. The protein was eluted with 5 column volumes of elution buffer [50 mM potassium phosphate (pH 8.0), 200 mM NaCl, 10% (w/v) glycerol, 500 mM imidazole plus 0.02% (w/v) DDM]. The purified GlnPQ was either reconstituted in nanodiscs or in proteoliposomes as described below.

Reconstitution of purified GlnPQ or derivatives in nanodiscs

The required amount of preformed liposomes, prepared as described previously [38], composed of

Table 5. Kinetic parameters of glutamine-stimulated ATP hydrolysis in nanodiscs and glutamine transport in proteoliposomes, using full-length GlnPQ

	Glutamine-stimulated ATP hydrolysis in nanodiscs		Glutamine uptake in proteoliposomes	
	K_M (μM)	V_{max} (min^{-1})	K_M (μM)	V_{max} (min^{-1})
300 mM KCl	0.7	12.4	ND	ND
No KCl	0.3	15.1	0.8	8.5

various mol% of DOPC, DOPE, and DOPG, was thawed and extruded through a polycarbonate filter (400-nm pore size). After extrusion, 0.5% (w/v) DDM was added to the liposomes, and the solution was vortexed for 30 s. The solubilized lipids, purified MSP1D1 and purified GlnPQ were mixed in a final volume of 700 μl , giving rise to the following composition: 50 mM potassium phosphate (pH 7.0), 12 mM DDM, 10% (w/v) glycerol, 2.86 μM GlnPQ, 14.3 μM MSP1D1 and 1.43 mM lipid. This corresponds to a molar ratio of GlnPQ/MSP1D1/lipid of 1:5:500 for full-length GlnPQ. The weight amount of protein for the derivatives of GlnPQ with one SBD or no SBD was adjusted to obtain molar ratios of 1:2.5:250 and 1:1:100, respectively. Noteworthy is that optimal reconstitution was obtained when the purified GlnPQ was diluted with elution buffer to 400 μl (2 nmol GlnPQ) after which DDM, MSP1D1, lipids and glycerol in 50 mM potassium phosphate (pH 7.0) were added from a mastermix to a total of 700 μl . The reconstitution mixture was incubated for 60 min at 4 °C with gentle agitation, after which 500 mg of SM-2 Biobeads (Biorad) was added. The mixture was incubated overnight at 4 °C with gentle agitation. The mixture was separated from the beads by transferring the mixture to a clean Eppendorf tube using a syringe, and aggregated protein was removed by subsequent centrifugation (20,000g, 10 min, 4 °C). The GlnPQ reconstituted nanodiscs were fractionated

Table 4. Influence of salt (KCl) on K_D of asparagine binding to SBD1(T159C/G87C)

KCl (mM)	K_D for asparagine (μM)
150	0.34
350	0.37
550	0.40
750	0.47
950	0.44
1150	0.61

Fluorescently labeled SBD1 was probed with varying concentrations of KCl, while the asparagine concentration was kept constant 1 μM . The observed change in FRET was used to calculate K_D as described before. The standard amount of KCl used in fluorescence experiments is 150 mM. Only extreme concentrations of salt (>1 M) lead to significant change in the K_D of SBD1 for asparagine.

Table 6. Strains and plasmids

Strains/plasmids	Reference
<i>L. lactis</i> GKW9000	[5]
pNZglnPQhis	
pNZglnPQhis-SBD1	
pNZglnPQhis-SBD2	
pNZglnPQhis-SBD1(T159C,G87C)	[8]
pNZglnPQhis-SBD2(T369C,S451C)	
pNZglnPQhis-SBD1(T159C,G87C) + SBD2	
pNZglnPQhis-SBD2(T369C,S451C) + SBD1	
pNZglnPQhis(D417F)	
pNZglnPQhis-SBD2(D417F)	
pNZglnPQhis-SBD1(E184D,V185E)	
pNZglnPQhis-LTMD	
pNZglnPQhis-TMD	
pMSP1D1	This paper [35]
<i>E. coli</i> BL21(DE3)	[36]

by size-exclusion chromatography, using a Superdex 200 10/300 GL column (GE Healthcare) and 50 mM potassium phosphate (pH 7.0), 200 mM NaCl and 4% (w/v) glycerol as buffer. The fractions containing nanodiscs with GlnPQ or derivatives were concentrated in 0.5 ml 100 MWCO concentrators (Vivaspin) to a final protein concentration of 4 μ M; protein concentrations were determined from the absorbance at 280 (using the molar extinction coefficients of the proteins) and SDS-PAGE analysis. Gels were imaged on a Fujifilm LAS 3000 Imager and, if necessary, gel band quantification of the GlnQ subunit was used to adjust the concentration of GlnPQ and derivatives. Nanodiscs were stored in -80°C before further analysis.

ATPase activity Assay with GlnPQ reconstituted in nanodiscs

The activity of GlnPQ (or derivatives) was determined by measuring the ATPase activity of the transporter using a coupled enzyme assay. The measurements were performed at 30°C in a 96-well plate using a Spark 10 M 96-well plate reader (Tecan). A standard measurement solution of 200 μ l/well was composed of 50 mM potassium phosphate (pH 7.0), 100 nM GlnPQ (or derivatives) incorporated in nanodiscs, 4 mM sodium phosphoenolpyruvate, 0.3 mM NADH, 300 mM KCl and 3.5 μ l of pyruvate kinase/lactic dehydrogenase enzyme mixture from rabbit muscle (Sigma-Aldrich) in 50% glycerol, with different concentrations of amino acids as indicated in the figure legends. After incubation of the assay mixture for 3 min at 30°C , 10 mM MgATP (pH 7.0) was added to each well, and the absorbance of NADH at 340 nm was followed for 20 min. The oxidation of NADH is stoichiometric with the amount of ATP consumed, and the ATPase activity was expressed as turnover number. For analysis, the background activity, measured when no pyruvate kinase/lactic dehydrogenase were added, was subtracted from the activity measured in the presence of these enzymes. Using OriginPro8 software, the data were fitted to the appropriate biophysical models.

Reconstitution of GlnPQ (or derivatives) in liposomes and transport assay

Purified GlnPQ (or derivatives) was reconstituted in liposomes composed of synthetic lipids with a molar ratio of DOPE:DOPC:DOPG of 50:12:38 as described before [38] with a final protein/lipid ratio of 1:100 (w/w). An ATP regenerating system, consisting of 50 mM potassium phosphate (pH 7.0), creatine kinase (2.4 mg/ml), $\text{Na}_2\text{-ATP}$ (10 mM), MgSO_4 (10 mM) and $\text{Na}_2\text{-creatine-phosphate}$ (24 mM) was enclosed in the proteoliposomes by two freeze/thaw cycles, after which the vesicles were stored at -80°C . On the day of the uptake experiment, the proteoliposomes

were extruded (13 passes) through a polycarbonate filter (200-nm pore size), diluted to 3 ml with 100 mM potassium phosphate (pH 7.0), centrifuged (265,000g for 20 min) and washed twice and resuspended in 100 mM potassium phosphate (pH 7.0) to a concentration of 50 mg of lipid/ml. The reconstitution efficiency was quantified by comparing gel band intensity of GlnP and GlnQ to a series of standard concentrations of purified GlnPQ, using SDS-PAGE analysis. To calculate the turnover number, we assumed a 50/50 orientation of GlnPQ in the proteoliposomes. Uptake was measured in 100 mM potassium phosphate (pH 7.0) supplemented with different concentrations of radioactive glutamine or asparagine. This medium, supplemented with the appropriate substrate, was incubated at 30°C for 2 min prior to adding proteoliposomes (kept on ice) to a final concentration of 1–5 mg of lipid/ml. At given time intervals, 40 μ l samples were taken and diluted with 2 ml of ice-cold isotonic buffer (100 mM potassium phosphate, pH 7.0). The samples were collected on 0.45- μ m pore size cellulose nitrate filters and washed twice as described above. After the addition of 2 ml Ultima Gold scintillation liquid (PerkinElmer), radioactivity was measured on a Tri-Carb 2800TR (PerkinElmer). Using OriginPro8 software, the data were fitted to the appropriate biophysical models.

Purification of cysteine-containing SBD mutants

E. coli cells expressing SBD1, SBD2 or tandem SBD1–SBD2 (with double Cys for fluorophore labeling, see Table 2) were grown as described [8] and harvested by centrifugation (15 min, 6000 $\times$ g) and washed once with 100 mM KPi (pH 7.5). After resuspension in 50 mM KPi (pH 7.5), 20% glycerol and the addition of 0.1 mg/ml DNase and 1 mM PMSF, 1 mM DTT the cells were disrupted by sonication. The supernatant (after disruption of the cells) was collected after ultracentrifugation (90 min, 150,000 $\times$ g) and stored at -80°C after flash freezing in liquid nitrogen. For purification of SBD1, SBD2 and SBD1–SBD2 double-Cys derivatives, the cell lysate was thawed and resuspended in 50 mM KPi (pH 7.5), 200 mM KCl, 20% (v/v) glycerol and 20 mM imidazole. The lysate was incubated for 1 h with Ni^{2+} -Sepharose resin at 4°C . To remove unbound proteins, the resin was washed with 20 column volumes of the same buffer supplemented with 50 mM imidazole. Bound protein was eluted with 3 column volumes of buffer [50 mM KPi (pH 7.5), 200 mM KCl, 20% (v/v) glycerol, 500 mM imidazole]. The concentration of the protein was determined from the absorption at 280 nm, using extinction coefficients of 34,380, 28,880 and 63,260 $\text{M}^{-1}\text{cm}^{-1}$ for SBD1, SBD2 and SBD1–SBD2 derivatives, respectively. Subsequently, all SBDs were concentrated in 0.5 ml 30,000 MWCO concentrators (Vivaspin) to a final concentration of 5 mg/ml, dialyzed against 50 mM KPi

(pH 7.5), 200 mM KCl and 50% (v/v) glycerol plus 1 mM DTT, and aliquoted and stored at -80°C .

Protein labeling

Stochastic labeling with maleimide derivatives of donor and acceptor fluorophores was carried out on ~ 5 nmol of purified protein. SBD derivatives were labeled with AlexaFluor555 and AlexaFluor647–maleimide in a ratio of protein:AF555:AF647 of 1:5:5, essentially as described in Ref. [8]. Briefly, unlabeled, cysteine-containing SBD1, SBD2 and tandem SBD1–SBD2 were stored with 1 mM DTT to prevent oxidization of the thiols. Upon thawing of the protein samples, the purified proteins were treated with an additional 10 mM DTT for 30 min. Next, the proteins were diluted 10 times with 50 mM KPi (pH 7.4) and 50 mM KCl and incubated with 100 μl Ni^{2+} -Sephacrose resin (Fast Flow; GE Healthcare) and incubated for 5 min. To remove unbound protein, the resin was washed with 10 column volumes (1 ml) of 50 mM KPi (pH 7.4) and 50 mM KCl. Meanwhile, fluorophore aliquots were dissolved in 10 μl of water-free DMSO and mixed with the resin and poured in the column. The column was sealed with parafilm and incubated for 4 h (or overnight) in the dark at 4°C . Afterward, to remove unreacted dyes, the resin was washed with 10 column volumes of 50 mM KPi (pH 7.4) and 50 mM KCl with additional 10% glycerol. This was followed by 100 column volumes of 50 mM KPi (pH 7.4), 50 mM KCl and 50% glycerol, and finally with 10 column volumes of 50 mM KPi (pH 7.4) and 50 mM KCl. The protein was stepwise eluted in 0.5 ml fractions of 50 mM KPi (pH 7.4), 50 mM KCl, 5% glycerol and 500 mM imidazole. To further purify the AlexaFluor555/647-labeled proteins, size-exclusion chromatography was used (Superdex-200); the protein fractions after size-exclusion chromatography were used immediately for smFRET studies.

smFRET and data analysis

Single-molecule alternating-laser excitation (ALEX) experiments were performed on a custom-built confocal microscope, described in detail in Ref. [39]. A laser beam from a supercontinuum laser (SuperK Extreme, NKT Photonics, Denmark) was filtered with an acousto-optic tunable filter, resulting in excitation at 532 and 640 nm. Alternation between excitation wavelengths was achieved by modulation of acousto-optic tunable filter in 50- μs intervals. Average laser powers used were 55–60 μW at 532 nm ($\sim 27\text{--}30\text{ kW/cm}^2$) and 20–25 μW at 640 nm ($\sim 15\text{--}25\text{ kW/cm}^2$). In all cases, custom-developed software was used for data collection and further analysis [39].

ALEX experiments were carried out at 25–50 pM of AlexaFluor555/647-labeled protein in 50 mM KPi (pH 7.4) and 150 mM KCl. For photostabilization, 1 mM Trolox and 10 mM β -mercaptoethylamine

were added to the buffer. AlexaFluor555/647-labeled SBDs were titrated with varying ligand concentrations (from low μM to mM) in one continuous ALEX experiment. To monitor and account for possible changes in the experimental settings, every set of ALEX experiments on SBDs was benchmarked against dsDNA labeled with Cy3B and ATTO647N derivatives (IBA, Germany), as described in Ref. [40].

The fluorescence of freely diffusing molecules was analyzed according to Kapanidis *et al.* [39]. In short, fluorescence was recorded via the donor and acceptor detection channels (D_{em} and A_{em} , respectively). Based on difference in arrival times, fluorescence photons were attributed to either donor- or acceptor-based excitation. On the basis of that, donor-based donor emission $F(\text{DD})$, donor-based acceptor emission $F(\text{DA})$ and acceptor-based acceptor emission $F(\text{AA})$ were measured. The apparent FRET efficiency, E^* , which monitors the proximity between the two fluorophores, and fluorophore stoichiometry, S , which reflects the ratio of donor to acceptor labeling for a particular sample, were calculated according to:

$$E^* = \frac{F(\text{DA})}{F(\text{DA}) + F(\text{DD})}$$

$$S = \frac{F(\text{DD}) + F(\text{DA})}{F(\text{DD}) + F(\text{DA}) + F(\text{AA})}$$

Binning of the detected bursts into a 2D E^*/S histogram yielded sub-populations that can be separated according to their S -values. One-dimensional E^* - and S -distributions were fitted using a Gaussian function, yielding the mean values of the distribution and associated standard deviation σ .

E^* - S histograms of the *apo*-protein and fully saturated with ligand (10–50 times K_D) were used to determine apparent E^* distributions for the open and closed conformation, respectively. Subsequently, these distributions were used to fit the titration data at intermediate substrate concentrations. The fractional occupancy of the high-FRET Gaussian as a function of substrate concentration was fitted afterward with a one-side-binding model, which yielded B_{max} (maximal fraction of closed state) and K_D , dissociation constant for ligand binding.

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SBD, substrate-binding domain; ABC, ATP binding cassette; TMD, transmembrane domain; NBD, nucleotide-binding domain; SBP, substrate-binding protein; MSP, membrane scaffold protein.

References

- [1] C.F. Higgins, ABC transporters: from microorganisms to man, *Annu. Rev. Cell. Biol.* (1992) 67–113.
- [2] E. Biemans-Oldehinkel, M.K. Doeven, B. Poolman, ABC transporter architecture and regulatory roles of accessory domains, *FEBS Lett.* 580 (2006) 1023–1035, <https://doi.org/10.1016/j.febslet.2005.11.079>.
- [3] G.H. Scheepers, J.A. Lycklama a Nijeholt, B. Poolman, An updated structural classification of substrate-binding proteins, *FEBS Lett.* 590 (2016) 4393–4401, <https://doi.org/10.1002/1873-3468.12445>.
- [4] L.J.Y.M. Swier, D. Slotboom, B. Poolman, ABC transporters: a structure–function perspective, in: G.M. Anthony (Ed.), *ABC Transp.—40 Years*, 1st ed. Springer Verlag 2015, pp. 3–36.
- [5] G.K. Schuurman-Wolters, B. Poolman, Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*: an ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains, *J. Biol. Chem.* 280 (2005) 23785–23790, <https://doi.org/10.1074/jbc.M500522200>.
- [6] F. Fulyani, G.K. Schuurman-Wolters, A.V. Žagar, A. Guskov, D.J. Slotboom, B. Poolman, Functional diversity of tandem substrate-binding domains in ABC transporters from pathogenic bacteria, *Structure* 21 (2013) 1879–1888, <https://doi.org/10.1016/j.str.2013.07.020>.
- [7] B. Poolman, E.J. Smid, W.N. Konings, Kinetic properties of a phosphate-bond-driven glutamate–glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*, *J. Bacteriol.* 169 (1987) 2755–2761, <https://doi.org/10.1128/jb.169.6.2755-2761.1987>.
- [8] G. Gouridis, G.K. Schuurman-Wolters, E. Ploetz, F. Husada, R. Vietrov, M. de Boer, T. Cordes, B. Poolman, Conformational dynamics in substrate-binding domains influences transport in the ABC importer GlnPQ, *Nat. Struct. Mol. Biol.* 22 (2015) 57–64, <https://doi.org/10.1038/nsmb.2929>.
- [9] R. Larsen, T.G. Kloosterman, J. Kok, O.P. Kuipers, GlnR-mediated regulation of nitrogen metabolism in *Lactococcus lactis*, *J. Bacteriol.* 188 (2006) 4978–4982, <https://doi.org/10.1128/JB.00025-06>.
- [10] M.L. Oldham, D. Khare, F.A. Quiocho, A.L. Davidson, J. Chen, Crystal structure of a catalytic intermediate of the maltose transporter, *Nature* 450 (2007) 515–521.
- [11] M.L. Oldham, J. Chen, Crystal structure of the maltose transporter in a pretranslocation intermediate state, *Science* 332 (2011) 1202–1205.
- [12] D. Khare, M.L. Oldham, C. Orelle, A.L. Davidson, J. Chen, Alternating access in maltose transporter mediated by rigid-body rotations, *Mol. Cell* 33 (2009) 528–536, <https://doi.org/10.1016/j.molcel.2009.01.035>.
- [13] M.L. Oldham, J. Chen, Snapshots of the maltose transporter during ATP hydrolysis, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 15152–15156, <https://doi.org/10.1073/pnas.1108858108>.
- [14] H. Bao, K. Dalal, E. Cytrynbaum, F. Duong, Sequential action of male and maltose allows coupling ATP hydrolysis to translocation in the MalFGK2 transporter, *J. Biol. Chem.* 290 (2015) 25452–25460, <https://doi.org/10.1074/jbc.M115.671826>.
- [15] L. Fabre, H. Bao, J. Innes, F. Duong, I. Rouiller, Negative stain single-particle em of the maltose transporter in nanodiscs reveals asymmetric closure of MalK2 and catalytic roles of ATP, MalE, and maltose, *J. Biol. Chem.* 292 (2017) 5457–5464, <https://doi.org/10.1074/jbc.M116.757898>.
- [16] P.M. Jones, A.M. George, A reciprocating twin-channel model for ABC transporters, *Q. Rev. Biophys.* 47 (2014) 189–220, <https://doi.org/10.1017/S0033583514000031>.
- [17] J.S. Patzlaff, T. van der Heide, B. Poolman, The ATP/substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA, *J. Biol. Chem.* 278 (2003) 29546–29551, <https://doi.org/10.1074/jbc.M304796200>.
- [18] R. Ernst, P. Kueppers, C.M. Klein, T. Schwarzmueller, K. Kuchler, L. Schmitt, A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 5069–5074, <https://doi.org/10.1073/pnas.0800191105>.
- [19] E. Vigonsky, E. Ovcharenko, O. Lewinson, Two molybdate/tungstate ABC transporters that interact very differently with their substrate binding proteins, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 5440–5445, <https://doi.org/10.1073/pnas.1213598110>.
- [20] N. Tal, E. Ovcharenko, O. Lewinson, A single intact ATPase site of the ABC transporter BtuCD drives 5% transport activity yet supports full in vivo vitamin B(12) utilization, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 5434–5439, <https://doi.org/10.1073/pnas.1209644110>.
- [21] N. Livnat-Levanon, A.I. Gilson, N. Ben-Tal, O. Lewinson, The uncoupled ATPase activity of the ABC transporter BtuC(2)D(2) leads to a hysteretic conformational change, conformational memory, and improved activity, *Sci. Rep.* 6 (2016) 21696, <https://doi.org/10.1038/srep21696>.
- [22] T.K. Ritchie, Y.V. Grinkova, T.H. Bayburt, I.G. Denisov, J.K. Zolnerchiks, W.M. Atkins, S.G. Sligar, Chapter 11—reconstitution of membrane proteins in phospholipid bilayer nanodiscs, *Methods Enzymol.* 464 (2009) 211–231, [https://doi.org/10.1016/S0076-6879\(09\)64011-8](https://doi.org/10.1016/S0076-6879(09)64011-8).
- [23] T.H. Bayburt, S.G. Sligar, Membrane protein assembly into nanodiscs, *FEBS Lett.* 584 (2010) 1721–1727, <https://doi.org/10.1016/j.febslet.2009.10.024>. Membrane.
- [24] C. van der Does, R. Tampé, How do ABC transporters drive transport? *Biol. Chem.* 385 (2004) 927–933, <https://doi.org/10.1515/BC.2004.121>.
- [25] C.F. Higgins, K.J. Linton, The ATP switch model for ABC transporters, *Nat. Struct. Mol. Biol.* 11 (2004) 918–926, <https://doi.org/10.1038/nsmb836>.

- [26] J. Chen, G. Lu, J. Lin, A.L. Davidson, F.A. Quirocho, A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle, *Mol. Cell* 12 (2003) 651–661, <https://doi.org/10.1016/j.molcel.2003.08.004>.
- [27] T. Jacso, E. Schneider, B. Rupp, B. Reif, Substrate transport activation is mediated through second periplasmic loop of transmembrane protein MalF in maltose transport complex of *Escherichia coli*, *J. Biol. Chem.* 287 (2012) 17040–17049, <https://doi.org/10.1074/jbc.M112.340679>.
- [28] S. Bohm, A. Licht, S. Wuttge, E. Schneider, E. Bordignon, Conformational plasticity of the type I maltose ABC importer, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 5492–5497, <https://doi.org/10.1073/pnas.1217745110>.
- [29] F.J.D. Alvarez, C. Orelle, Y. Huang, R. Bajaj, R.M. Everly, C.S. Klug, A.L. Davidson, Full engagement of liganded maltose-binding protein stabilizes a semi-open ATP-binding cassette dimer in the maltose transporter, *Mol. Microbiol.* 98 (2015) 878–894, <https://doi.org/10.1111/mmi.13165>.
- [30] C. Tang, C.D. Schwieters, G.M. Clore, Open-to-closed transition in apo maltose-binding protein observed by paramagnetic NMR, *Nature* 449 (2007) 1078–1082, <https://doi.org/10.1038/nature06232>.
- [31] M.-H. Seo, J. Park, E. Kim, S. Hohng, H.-S. Kim, Protein conformational dynamics dictate the binding affinity for a ligand, *Nat. Commun.* 5 (2014) 3724.
- [32] E. Kim, S. Lee, A. Jeon, J.M. Choi, H.-S. Lee, S. Hohng, H.-S. Kim, A single-molecule dissection of ligand binding to a protein with intrinsic dynamics, *Nat. Chem. Biol.* 9 (2013) 313–318, <https://doi.org/10.1038/nchembio.1213>.
- [33] T. Jacso, M. Grote, M.L. Daus, P. Schmieder, S. Keller, E. Schneider, B. Reif, Periplasmic loop P2 of the MalF subunit of the maltose ATP binding cassette transporter is sufficient to bind the maltose binding protein MalE, *Biochemistry* 48 (2009) 2216–2225, <https://doi.org/10.1021/bi801376m>.
- [34] H. Bao, F. Duong, Discovery of an auto-regulation mechanism for the maltose ABC transporter MalFGK2, *PLoS One* 7 (2012), e34836, <https://doi.org/10.1371/journal.pone.0034836>.
- [35] I.G. Denisov, Y.V. Grinkova, A.A. Lazarides, S.G. Sligar, Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size, *J. Am. Chem. Soc.* 126 (2004) 3477–3487, <https://doi.org/10.1021/ja0393574>.
- [36] F.W. Studier, B.A. Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, *J. Mol. Biol.* 189 (1986) 113–130.
- [37] A. Karasawa, L.J.Y.M. Swier, M.C.A. Stuart, J. Brouwers, B. Helms, B. Poolman, Physicochemical factors controlling the activity and energy coupling of an ionic strength-gated ATP-binding cassette (ABC) transporter, *J. Biol. Chem.* 288 (2013) 29862–29871, <https://doi.org/10.1074/jbc.M113.499327>.
- [38] E.R. Geertsma, N.A.B. Nik Mahmood, G.K. Schuurman-Wolters, B. Poolman, Membrane reconstitution of ABC transporters and assays of translocator function, *Nat. Protoc.* 3 (2008) 256–266, <https://doi.org/10.1038/nprot.2007.519>.
- [39] A.N. Kapanidis, N.K. Lee, T.A. Laurence, S. Doose, E. Margeat, S. Weiss, Fluorescence-aided molecule sorting: Analysis of structure and interactions by alternating-laser excitation of single molecules, *Proc. Natl. Acad. Sci.* 101 (2004) 8936–8941, <https://doi.org/10.1073/pnas.0401690101>.
- [40] S.J. Holden, S. Uphoff, J. Hohlbein, D. Yadin, L. Le Reste, O.J. Britton, A.N. Kapanidis, Defining the limits of single-molecule FRET resolution in TIRF microscopy, *Biophys. J.* 99 (2010) 3102–3111, <https://doi.org/10.1016/j.bpj.2010.09.005>.