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Protein Linkers Provide Limits on the Domain Interactions in the ABC Importer GlnPQ and Determine the Rate of Transport

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Abstract

GlnPQ is an ATP-binding cassette importer with a unique domain organization and intricate transport behavior. The protein has two extracytoplasmic substrate-binding domains (SBDs) per membrane subunit, each with different specificity for amino acids and different spacing to the translocator domain. We determined the effect of the length and structure of the linkers, which connect the SBDs to each other and to the membrane-embedded translocator domain, on the transport by GlnPQ. We reveal that varying the linker length impacts transport in a dual manner that depends on the conformational dynamics of the SBD. Varying the linker length not only changes the time for the SBD to find the translocator (docking) but also changes the probability to release the substrate again, thus altering the transport efficiency. On the basis of the experimental data and mathematical modeling, we calculate the docking efficiency as function of linker length and lifetime of the closed conformation. Importantly, not only linker length but also features in the sequence are important for efficient delivery of substrate from SBD to the translocator. We show that the linkers provide a platform for SBD docking and are not merely flexible structures.

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Introduction

ATP-binding cassette (ABC) transporters can function as importers or exporters. Both importers and exporters consist of two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding subunits (NBDs), which power the transport through hydrolysis of ATP. The TMDs and NBDs together constitute the translocator. ABC importers are on structural and mechanistic grounds subdivided in Types I, II and III [1–3]. The mechanism of transport of Type I and II ABC importers involves the binding and release of substrate from a dedicated soluble substrate-binding protein (SBP) or membrane-tethered substrate-binding domain (SBD) and alternating access of the substrate-binding pocket in the TMD.

SBPs of Gram-negative bacteria are able to freely diffuse in the periplasm [4], whereas in Gram-positive bacteria and archaea, they are tethered to the membrane via a lipid modification or covalently linked to the translocator [5]. ABC importers with one or more SBDs fused to the TMD subunits are mostly found in the PAO and OTCN families of the ABC superfamily [5],

and these systems transport amino acids and compatible solutes. Homologous of the maltose transporter MalFGK₂ with maltose binding protein (MalE) fused to one of the TMDs have been discovered recently (<https://www.rcsb.org/structure/6ffl>). GlnPQ from *Lactococcus lactis* is a Type I ABC importer that has two SBDs (called SBD1 and SBD2) fused to the N-terminus of the TMD, leading to a total of four SBDs per translocator (Fig. 1). The N-terminal SBD1 binds asparagine with a dissociation constant K_D below 1 μ M and glutamine, histidine and arginine with K_D values of 100 μ M or higher (unpublished results), whereas SBD2 is specific for glutamine [6]. In our previous work, we established a connection between the conformational dynamics of the SBD and the transport of glutamine and asparagine [7]. We showed that SBD1 and SBD2 bind amino acids by an induced-fit mechanism, that is, substrate-triggered closing of the SBD. Interestingly, the lifetime of the closed conformation influences transport in a reciprocal manner; that is, transport is fast when the lifetime is short. It was hypothesized that the faster opening accelerates the transfer of substrate from the SBD to the translocator, thereby increasing the overall transport rate. However,

little is known about the process of SBD docking onto the translocator, how this relates to the overall translocation cycle and how the dynamic properties of SBD influence this process.

In case of soluble or lipid-anchored SBPs of Type I ABC importers, the Michaelis constant (K_m) of the TMD for substrate-loaded SBPs is at least 50 μM [8–10]. These low affinities may necessitate a high concentration of SBPs in the periplasm, which is in accordance with the observation that in the *Escherichia coli* periplasm maltose-binding protein can reach a concentration of $\sim 1\text{ mM}$ [11]. Alternatively, surface tethering of the SBD to the membrane or translocator ensures a high concentration that may saturate the transport reaction. For OpuA, it has been shown that complementation of the translocator with soluble SBD is very inefficient [12]. Even at 40 μM of soluble SBD, the rate of transport was only a fraction of wild-type OpuA, suggesting that the K_m for SBD may be in the submillimolar or even millimolar range.

Little is known about the relationship between linker length (or structure) and the interdomain interactions in

native multidomain (membrane) proteins. Most often in native proteins, only the composition of inter-domain linkers is studied. For instance, in the human protein tyrosine phosphatase non-receptor 4 the linker composition was shown to be important for communication between the two globular domains of the active complex [13]. The importance of protein linker length and structure is well documented for engineered fluorescence-based biosensors [14]. Often repeats of glycine and serine are used to generate linkers which are assumed to adopt a random coil structure and do not interfere with the protein domains of interest [15]. For luciferase, it was shown that introducing a random linker between the two proteins domains affects the substrate affinity but not the bioluminescence [16].

Here, we show that the length and composition of the domain linkers vary enormously between ABC importers. We further investigated the role of length and structure of the linkers between the SBDs and between the SBDs and translocator in GlnPQ from *L. lactis*. We reveal that varying the linker length impacts transport in a dual manner that depends

Fig. 1. Schematic representation of GlnPQ and the linkers connecting the SBDs. Single SBD transporters were designed on the basis of the sequence identity between SBD1 and SBD2. Substrates are depicted by blue (glutamine) or red balls (asparagine); black shapes in the NBDs indicate two molecules of ATP. Linker modifications between SBD1 and SBD2 were made C-terminal of Ala-251. For the SBD–TMD linker, all deletions and insertions were made C-terminal of Ser-482 (see Table 1). In the schematic on the right, we depict the space that is probed by the SBD for three different linker lengths. The apparent SBD concentration is calculated as a function of linker length, assuming that the inserted peptides behave as a random coils. A peptide in extended state has angles $\phi = -135^\circ$ and $\Psi = +135^\circ$ and spacing of 0.35 nm between amino acids. For a linker of 19 amino acids (6.7 nm) connected to a protein with $r = 2\text{ nm}$, the maximal distance of the center of mass of the protein from the anchor point is 8.7 nm, yielding an apparent SBD concentration of 1.2 mM. The concentration is 0.2 and 5.8 mM for linkers of 39 and 9 amino acids, respectively, corresponding to the insertion of 20 or deletion of 10 amino acids in the native linker.

on the conformational dynamics of the SBD. However, not only linker length but also sequence features are important for efficient delivery of substrate from SBD to the translocator.

Results

Inter-domain linkers of ABC importers

Schematics of GlnPQ and deletion constructs are presented in Fig. 1. We estimated the end and start points of the linkers between SBD1 and SBD2 and between SBD2 and the TMD of GlnPQ on the basis of the X-ray crystal structures of the SBDs [6] and the homology model of the full-length protein, taking advantage of the X-ray crystal structures of GlnPQ homologs, the ABC importers Art(QN)₂ and MetI [17,18]. To assign the linker between SBD2 and the TMD, we aligned the sequences of ArtQ, GlnP and MetI (Fig. S1a). We estimate the linker between SBD2 and TMD to be 19 amino acids long. The 19-amino-acid linker does not have obvious secondary structural elements, but the C-terminal end of the sequence is conserved among homologs (Fig. S2a). To assess the variation in the linkers, we aligned multiple homologs of the permease part of GlnPQ complex, and we find that the linkers vary from 11 to 38 residues (Fig. S1b). The linker between SBD1 and SBD2 of GlnPQ from *L. lactis* is 14 amino acids long, which is very short compared to that of the majority of homologous proteins (Fig. S2a); the length varies from 9 to 44 residues and the peak is at 20 amino acids (Fig. S2b).

Linker between SBD1 and SBD2

We designed mutants with deletions of 2-, 5- and 8-amino-acid C-terminal of SBD1 (C-terminal of Ala-251). We performed glutamine and asparagine transport assays at 100 μ M, which is well above the K_D of the SBDs for these amino acids (Fig. 2). Under these conditions, asparagine is imported via SBD1 and the majority of glutamine via SBD2 [6]. The transport of glutamine is much less affected by shortening of the SBD1–SBD2 linker (Fig. 2a) than the uptake of asparagine (Fig. 2b). In fact, deleting 8 amino acids diminishes asparagine uptake by more than 90%, whereas the uptake of glutamine is not significantly affected. The Western blot analysis shows that the proteins are expressed at comparable levels (Fig. 2c). These data suggest that the inter-SBD linker of 14 is minimally needed for SBD1 to deliver asparagine to the translocator domain, but this length is not critical for transport of glutamine via SBD2. We also inserted a 20-amino-acid (GGGS)₄AAQL sequence C-terminal of Ala-251, but increasing the linker length had little effect on the uptake of asparagine or glutamine (Fig. 2ab).

Linker between SBD2 and TMD

In wildtype GlnPQ the 19-amino-acid linker between SBD2 and TMD is long enough to allow docking of SBD2, and SBD2 can be displaced sufficiently to allow docking of SBD1. To simplify the analysis of the effect of inter-SBD–TMD linker length, we constructed GlnPQ-SBD2 (Fig. 1). Figure 3a shows that deletion of only 4 amino acids already gives a 70% reduction in activity, and deletion of 10 or 15 amino acids completely

Fig. 2. Effect of SBD1–SBD2 linker length on transport activity. (a, b) Initial rates of uptake of glutamine (a, gray) and asparagine (b, sparse) in *L. lactis* GKW9000, complemented *in trans* with the indicated constructs (expressed from the *nisin A* promoter). The final substrate concentrations were 100 μ M [³H]-glutamine or [³H]-asparagine. (c) Western blot showing the relative expression of the GlnPQ proteins. For immunostaining, both GlnP (SBD-specific antibody) and GlnQ (his-tag antibody) were detected on the same blot. Marker sizes (M) are shown on the left side of the immunoblot.

Fig. 3. Effect of SBD2-TMD linker length on transport activity. Initial rates of uptake of glutamine (gray) and asparagine (sparse) in *L. lactis* GKW9000, complemented *in trans* with the indicated constructs (expressed from the nisin A promoter as described in [Materials and Methods](#)). The final substrate concentrations were 25 μM [³H]-glutamine and 100 μM [³H]-asparagine. All deletions start after Ser-282 and are specified in [Table 1](#). (a) GlnPQ-SBD2 derivatives, (b) GlnPQ derivatives, (c): Western blot showing the relative expression levels of the mutant proteins. Top panel: crude cell lysates; bottom panel, protein expression in membrane vesicles. Marker sizes are indicated on the left side. For immunostaining, antibodies raised against the SBDs were used. Experiments for GlnPQ-SBD2 and GlnPQ were done on different days.

abolishes glutamine transport. If instead of deleting 4 or 10 amino acids, the residues were replaced by a random sequence (denoted as GlnPQ-SBD2-Δ4 + 4 and Δ10 + 10), the activity was 30%–50% of GlnPQ-SBD2. To determine if the same is true for asparagine transport via SBD1, we made the Δ10 and Δ10 + 10 truncations in wild-type GlnPQ. We find that GlnPQ-Δ10 is completely inactive in both asparagine and glutamine transport, whereas GlnPQ-Δ10 + 10 has

full asparagine transport and approximately 50% glutamine transport activity ([Fig. 3b](#)). Although the expression levels may differ slightly ([Fig. 3c](#)), the differences in transport activity cannot be explained by the variations on the immunoblots. The partial recovery of activity in the Δ10 + 10 constructs suggests that not only linker length but also features in the sequence are important for efficient delivery of glutamine from SBD2 to the translocator unit. On the other hand, asparagine

Fig. 4. Positional dependence of inserting 20-amino-acid sequences into the SBD2-TMD linker. (a) Initial rates of uptake of glutamine (gray) and asparagine (sparse) in *L. lactis* GKW9000, complemented *in trans* with the indicated constructs (expressed from the nisin A promoter as described in [Materials and Methods](#)). The final substrate concentrations were 100 μM [³H]-glutamine and 100 μM [³H]-asparagine. (b) Western blots showing the relative expression levels of the proteins. For immunostaining, both GlnP (SBD-specific antibody) and GlnQ (his-tag antibody) were detected on the same blot. Marker is indicated on left side.

transport via SBD1 is not influenced by the linker composition, only the length is important for optimal delivery to the TMD.

To determine if the composition of the 19-amino-acid inter-SBD2–TMD linker is important for the interactions between the SBDs and the TMD, we designed mutants in wild-type GlnPQ with a 20-amino-acid sequence, (GGGS)₄AAQL, inserted at different positions (C-terminal of Ser-482, Lys-492 and Leu-512) in the linker. We find that transport of both asparagine (via SBD1) and glutamine (via SBD1 and SBD2) is abolished if we move the insertion to the middle position (Lys-492) or after the C-terminal end (Leu-512) of the native linker (Fig. 4a). Western blot analysis showed that expression of each of the insertion mutants is comparable to wild-type GlnPQ except for GlnPQ (+20 at Lys-492) (Fig. 4b). The band below GlnP is a breakdown product (denoted as GlnP* in Fig. 4b). Especially the 20-amino-acid insertion in the middle of the linker region induced breakdown. Overall, the data indicate that a 19-amino-acid flexible linker is not sufficient for the functional interaction of SBD2 with the TMD: the integrity of the native linker is important for transport.

In the ProU system of *E. coli*, the SBD (ProX) is a soluble protein present in the periplasm. Previously ProX was expressed in the Gram-positive bacterium

Fig. 5. *In vivo* complementation of GlnPQΔSBD constructs with lipid-anchored SBD2. Transport of glutamine was measured in cells expressing lipid-anchored SBD2 without GlnPQ (black squares), with GlnPQ+L19-TMD (open squares), GlnPQ+L1-TMD (open circles) or GlnPQ-L5TMD (open triangles). The final [³H]-glutamine concentration was 100 μM.

L. lactis as a lipid-anchored protein tethered to the outer surface of the plasma membrane [19], where it complemented the ProVW proteins. We now used the same strategy to determine if we could complement GlnPQ-ΔSBD constructs (Fig. 1) with lipid-anchored

Table 1. Mutants used in this study

Linker modifications in GlnPQ-SBD1 and GlnPQ-SBD2	
Native linker SBD2–TMD #483–501	DAKTIQSSAKENTFFGILQ
Δ4	—IQSSAKENTFFGILQ
Δ10	—ENTFFGILQ
Δ4+4	gggsIQSSAKENTFFGILQ
Δ10+10	ggsggggsaaENTFFGILQ
+5	gggsaDAKTIQSSAKENTFFGILQ
+10	ggsggggsaaDAKTIQSSAKENTFFGILQ
+20	ggsgggsgggsggggsaaqDAKTIQSSAKENTFFGILQ
+40	ggsgggsgggsggggsaaqggsgggsgggsggggsaaqDAKTIQSSAKENTFFGILQ
Linker modifications in wildtype GlnPQ	
GlnPQ-Δ10	—ENTFFGILQ
GlnPQ-Δ10+10	ggsggggsaaENTFFGILQ
GlnPQ after #482+20	ggsgggsgggsggggsaaqDAKTIQSSAKENTFFGILQ
GlnPQ after #492+20	DAKTIQSSAKggsgggsgggsggggsaaqENTFFGILQ
GlnPQ after #512+20	DAKTIQSSAKENTFFGILQNNWEQIGRGLLggsgggsgggsggggsaaq
Modifications in linker between SBD1 and SBD2	
Native linker SBD1–SBD2#248–261	GITATKKATPKKDV
GlnPQ-SBD12-Δ2	GITA-KATPKKDV
GlnPQ-SBD12-Δ5	GITA—PKKDV
GlnPQ-SBD12-Δ8	GITA—DV
GlnPQ-SBD12#251+20	GITAggsgggsgggsggggsaaqTKKATPKKDV
Truncations to create SBDless constructs	
+L19-TMD	mgDAKTIQSSAKENTFFGILQnnweqigrll
+L1-TMD	mgENTFFGILQnnweqigrll
-L5-TMD	mgigrll
Lipid anchored SBD2 #255–484	Lipid-CGSNQSSSgggsaggggs-SBD2

The original linker of GlnPQ is indicated in capital letters; underlined sequences are not part of the linker; and inserted linkers are indicated in italics. GlnP has a signal sequence, which is cleaved off after insertion of the protein in the membrane. The numbering used is for the protein with signal sequence, starting from Met-1.

SBD2. We made GlnPQ- Δ SBD constructs with different portions of the predicted linker (or linker with extension into the C-terminus of SBD2) connected to the TMD. In this way, we probed if the linker itself has features that are needed for docking of SBD2 onto the translocator domain. We measured uptake of glutamine in cells constitutively expressing lipid-anchored SBD2 and nisin A-induced GlnPQ- Δ SBD with -5 , $+1$ or $+19$ amino acids of the predicted linker region (see Table 1). We only find significant complementation with GlnPQ Δ SBD+L19, that is, the complete native linker between TMD and SBD2 (Fig. 5). The rate of glutamine uptake by GlnPQ Δ SBD+L19/lipid-anchored SBD2 is much lower than that of GlnPQ-SBD2, which we attribute to the much lower effective SBD2 concentration (difference between membrane-lipid tethering and protein tethering). Next, we determined the expression levels of the GlnPQ Δ SBD constructs by purifying the proteins via a His-tag on GlnQ subunit; we

could not use the GlnP antibodies because they are directed against the SBDs. From the purification of the proteins, we conclude that GlnPQ-L5-TMD and GlnPQ+L19-TMD are well expressed but GlnPQ+L1-TMD is not (data not shown). Taken together, the data indicate that the predicted linker with extension provides structural context needed for docking of SBD2.

Effect of linker length on transport kinetics

To get further insight into the docking process and its contribution to the translocation cycle, we investigated the transport kinetics of GlnPQ with varying linkers. The linker length was varied by inserting GGGs repeat sequences at a non-critical position in the native SBD2-TMD linker, thereby varying the available volume that is accessible to the SBD and thus varying the effective concentration of the receptor. We used the

Fig. 6. Kinetic parameters of amino acid transport by GlnPQ-SBD1 and GlnPQ-SBD2 with different linker insertions. (a) Michaelis-Menten kinetics of [¹⁴C]-glutamine transport by GlnPQ-SBD2. Squares, GlnPQ-SBD2; open circles, GlnPQ-SBD2 + 20; triangles, GlnPQ-SBD2 + 40. (b) Kinetic parameters as a function of linker length for glutamine transport by GlnPQ-SBD2. (c) Asparagine transport by GlnPQ-SBD1. (d) Glutamine transport by GlnPQ-SBD1. K_M (solid line) and V_{max} values (dashed line) are plotted. The lengths of the insertions in the SBD-TMD linkers are indicated on the x-axis by the number of amino acids. The error bars indicate SD; *n* = 2 or 3 (see Table 2). We previously reported a K_m for asparagine transport by GlnPQ-SBD1 of 24.4 ± 0.9 μM [7] and now find a value of 1.2 ± 0.8 μM. We discovered by TLC analysis that previous batches of [³H]-asparagine did not meet the specifications given by ARC, which led to an overestimation of the apparent Michaelis constant; the V_{max} changed from 21.0 ± 3.0 to 15.5 ± 1.8 nmol min⁻¹ mg protein⁻¹. ARC Inc. has confirmed that previous batches of [³H]-asparagine had a higher concentration of asparagine than indicated on the datasheet and thus a lower specific activity.

Table 2. Kinetic parameters of asparagine and glutamine transport

Constructs	Asparagine transport			Glutamine transport				
	Number of experiments	K_M (μM)	V_{max} (nmol/min mg)	Efficiency of docking Θ	Number of experiments	K_M (μM)	V_{max} (nmol/min mg)	Efficiency of docking Θ
GlnPQ-SBD1	3	1.2 ± 0.8	15.5 ± 1.8	0.43 ± 0.29	3	16.2 ± 8.2	46.3 ± 5.1	0.10 ± 0.05
GlnPQ-SBD1 + 5	3	1.6 ± 0.9	11.3 ± 3.1	0.24 ± 0.15	2	12.8 ± 3.4	54.1 ± 7.6	0.14 ± 0.04
GlnPQ-SBD1 + 10	3	1.2 ± 0.7	10.8 ± 4.5	0.30 ± 0.21	2	14.2 ± 2.8	31.4 ± 2.1	0.07 ± 0.02
GlnPQ-SBD1 + 20	2	2.3 ± 1.1	8.3 ± 5.0	0.12 ± 0.9	3	35.3 ± 2.8	19.1 ± 7.3	0.02 ± 0.01
GlnPQ-SBD1 + 40	2	2.4 ± 0.9	4.0 ± 1.6	0.06 ± 0.03	2	58.8 ± 5.7	25.7 ± 4.2	$0.01 \pm <0.01$
GlnPQ-SBD1 (E184D V185E)	4	2.2 ± 1.2	12.7 ± 3.7	0.19 ± 0.12	2	0.5 ± 0.2	18.7 ± 2.6	1.24 ± 0.53
GlnPQ-SBD1 + 10 (E184D V185E)	2	1.6 ± 0.5	6.1 ± 3.1	0.13 ± 0.08	2	0.7 ± 0.1	20.5 ± 0.2	0.98 ± 0.14
GlnPQ-SBD1 + 20 (E184D V185E)	2	2.2 ± 0.9	6.5 ± 0.8	0.10 ± 0.04	2	1.7 ± 1.6	12.8 ± 6.8	0.25 ± 0.27
GlnPQ-SBD2	nd	nd	nd	nd	4	2.3 ± 0.3	71.5 ± 17.5	1.04 ± 0.29
GlnPQ-SBD2 + 20	nd	nd	nd	nd	5	4.2 ± 1.6	33.9 ± 7.3	0.27 ± 0.12
GlnPQ-SBD2 + 40	nd	nd	nd	nd	3	2.2 ± 1.0	14.2 ± 6.8	0.22 ± 0.14

The initial rates of transport were obtained from progress curves as shown in Fig. 5; experiments were performed at substrate concentrations ranging from 0.20 to 100 μM for asparagine and from 0.10 to 100 μM for glutamine. The data were analyzed with the Michaelis-Menten equation, and K_M and V_{max} values were extracted. Errors are SD over n number of experiments.

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single SBD constructs GlnPQ-SBD1 and GlnPQ-SBD2. For GlnPQ-SBD1, we inserted 5, 10, 20 or 40 amino acids; and for GlnPQ-SBD2, 20 or 40 amino acids. Transport experiments showed Michaelis–Menten behavior (Fig. 6a) and were analyzed to yield V_{max} and K_m values (Table 2).

In Fig. 6b, c, d, the V_{max} and K_m values are shown as a function of the inserted linker length. Independently of the involved SBD or substrate, the V_{max} decreased when the linker length was increased (dashed lines). Interestingly, the K_m was little affected by variations in linker length for high-affinity glutamine ($K_D = 0.9$ μ M; Fig. 6b) and asparagine ($K_D = 0.2$ μ M; Fig. 6c) transport via SBD2 and SBD1, respectively. However, the K_m significantly increased when the linker length was increased for low-affinity ($K_D = 92$ μ M) glutamine transport via SBD1 (Fig. 6d). These observations indicate that (i) docking of SBD onto the translocator is one of the rate-determining steps of the translocation cycle of GlnPQ, and (ii) the SBD substrate affinity influences the translocation cycle.

We tested these hypotheses further by studying a GlnPQ derivative with an altered substrate-affinity in SBD1. Previously, we showed that two mutations in the active site of SBD1, SBD1(E184D–V185E) [7], to mimic the binding pocket of SBD2, resulted in a 70-fold higher affinity for glutamine (K_D from 92 to 1.3 μ M) but the protein kept a relatively low K_D for asparagine (K_D increased from 0.2 to 2.4 μ M). We now introduced these active site mutations in GlnPQ-SBD1 and probed the effect of linker length on the kinetic parameters of transport. We find that, when the linker length is increased, the V_{max} of asparagine (Fig. 7a) and glutamine (Fig. 7b) transport via GlnPQ-SBD1(E184D V185E) decreases, whereas the effect on K_m is not significant.

In conclusion, increasing the linker length impedes transport in GlnPQ, irrespective of the SBD or the substrate transported, and the effect is exerted primarily at the level of the V_{max} . When the K_D of the SBD for substrate is (relatively) high, an additional decrease in transport can be observed that is exerted at the level of the K_m of transport.

Dual role of docking on the transport kinetics

To understand the relationship between substrate affinity and docking, we performed mathematical modeling to describe the transport kinetics of GlnPQ at steady state (details in the Materials and Methods section). For this, we constructed a reaction chain that includes five states, which builds on data available for Type I importers and uses data specific for GlnPQ (Fig. 8). In brief, the translocation cycle is initiated by substrate binding by the SBD ($S_I \rightarrow S_{II}$ in the scheme). Once the SBD is in the closed conformation (S_{II}), it can dock onto the TMD ($S_{II} \rightarrow S_{III}$) and transfer the substrate to the translocator ($S_{III} \rightarrow S_{IV}$). In this step, the SBD has

Fig. 7. Kinetic parameters of amino acid transport by GlnPQ-SBD1(E184D_V185E) with different linker insertions. Transport of [3 H]-asparagine (a) and [3 H]-glutamine (b) was assayed in whole cells of *L. lactis* GKW9000 complemented *in trans* with GlnPQ-SBD1(E184D_V185E), GlnPQ-SBD1(E184D_V185E) + 10 and GlnPQ-SBD1(E184D_V185E) + 20. K_m (solid line) and V_{max} values (dashed line) are plotted. The lengths of the insertions in the SBD-TMD linkers are indicated on the x-axis by the number of amino acids. The error bars indicate SD; $n = 2$ (Table 2).

to open to transfer the substrate to the TMD, which undergoes a large conformational change from inward- to outward-facing. As shown in our previous work [6], this step depends on the lifetime of the closed conformation. The final steps of translocation ($S_{IV} \rightarrow S_V$ and $S_V \rightarrow S_I$) are for simplicity treated as a single step and includes substrate transfer from the TMD to the cell interior accompanied by ATP hydrolysis and release of Pi and ADP. Finally, binding of ATP will complete the transport cycle and bring the translocator back to the initial state (S_I).

We find that the transport rate of the model follows Michaelis-Menten behavior (derivation of model and Eqs. (1)–(6) are described in the Materials and Methods section), with a Michaelis-Menten constant equal to

$$K_m = K_D \frac{t_2 + t_{-1}}{t_2 + t_3 + t_4}, \quad (7)$$

and a maximal rate of transport of

$$V_{max} = \frac{E}{t_2 + t_3 + t_4} \quad (8)$$

where t_i is the expected time needed to make a transition between the connecting states of the chain as indicated in Fig. 8 and E is the transporter concentration. The transport data are well described by Michaelis-Menten behavior and thus support the model (Fig. 6a). In this model, the V_{max} depends only on the steps after substrate binding (S_{II} and onward) and the K_m on all the steps involved. The dissociation constant of the SBD for substrate is given by

$K_D = t_1/t_{-1}$, where t_{-1} is the lifetime of the closed conformation (*holo* state) and t_1/L_{OUT} the lifetime of the open conformation (*apo* state), where L_{OUT} is the substrate concentration in the external environment.

The model is used to qualitatively explain the transport kinetics of GlnPQ. We find that independent of the SBD or substrate, the V_{max} decreases when the linker length increases (Figs. 6 and 7). This can be explained by the additional time needed for the closed conformation of the SBD to find and dock onto the translocator (if t_2 is increased by Δt then $V_{max}(t_2 + \Delta t) < V_{max}(t_2)$). In addition, a small perturbation on t_2 only has an effect on V_{max} if $t_2 \sim t_3 + t_4$; hence, docking is one of the rate-determining steps of the GlnPQ translocation cycle.

The K_m is unaffected by an increase in linker length when the SBDs bind ligand with high affinity (Figs. 6 and 7), from which we conclude that $t_{-1} \sim t_2$, since we already concluded from the V_{max} data that $t_2 \sim t_3 + t_4$. This holds true for SBD1 and SBD1(E184D/V185E) binding asparagine and SBD2 or SBD1(E184D/V185E) binding glutamine, which suggests that in these cases the lifetime of the closed conformation (t_{-1} is between 60 and 280 ms [7]) is similar to the time needed to find and dock onto the translocator (t_2). However, in case the K_m increases with increasing linker length, as observed for glutamine transport via SBD1, we conclude that $t_{-1} < t_3 + t_4$ and thus $t_{-1} < t_2$, since $t_2 \sim t_3 + t_4$. This implies that SBD docking is slow compared to opening of the SBD and release of substrate back into the external environment, which is in agreement with the extremely short lifetime of the closed conformation of SBD1 occupying glutamine ($t_{-1} < 1$ ms [7], compared to the other SBDs and substrates ($t_{-1} > 60$ ms [7])).

Fig. 8. Model of the translocation cycle of GlnPQ. SBD1 and SBD2 are indicated in green and brown, respectively, and asparagine and glutamine are depicted as red and blue circles, respectively. TMDs and NBDs are labeled in gray and orange, respectively. A time constant t_i is assigned to each step as indicated and is used in the mathematical model as described in the text. In the schematic, the translocation cycle starts with the binding of glutamine (blue circle) to one of the SBD1s (top right); the other SBD1 has asparagine bound (red circle), and one of the SBD2s has glutamine bound and the other is empty.

From the experimental data and our model, an intricate view of the docking process emerges. Once an SBD has acquired a substrate, it has to dock onto the translocator before the SBD opens and releases the substrate back into the external environment. This process impedes transport when it is inefficient; that is, opening of the SBD is faster than docking onto the translocator. The efficiency of the processes is $\Theta = t_1/(t_2 + t_1)$ and is termed the efficiency of docking Θ (for details, see the [Materials and Methods](#) section). We calculated Θ by noting that $\Theta = t_1 k_{\text{cat}}/K_m$, where the k_{cat} is the maximal rate of transport of a single transporter. GlnPQ constitutes 0.2–0.5% of total cell protein in *L. lactis* (under the ambient experimental conditions) from which calculated the concentration of the transporter (E) in our assay; k_{cat} is obtained

from V_{max}/E . In addition, we showed in our previous work that t_1^{-1} is around $20 \mu\text{M}^{-1} \text{s}^{-1}$ [7], which allows us to estimate Θ as function of linker length (Table 2). We estimate that Θ is 1.04 ± 0.29 for glutamine transport via GlnPQ-SBD2 (Table 2). This means that $104\% \pm 29\%$ of the initial binding events by SBD2 are successfully followed by actual translocation. On the contrary, only a small fraction ($\Theta = 10\% \pm 5\%$) of the binding events is successfully followed by actual translocation when glutamine is transported via SBD1 instead (Table 2). In Fig. 9 we show Θ as function of linker length and opening rate of SBD1. We conclude that Θ decreases when (i) the linker length increases or (ii) the opening of the SBD is faster (Fig. 9). Hence, docking is most inefficient for glutamine transport via SBD1 ($\Theta = 10\% \pm 5\%$; shortest dwell time) and is

Fig. 9. Docking efficiency of SBD1 in the constructs GlnPQ-SBD1 and GlnPQ-SBD1(E184D_V185E) with different linker insertions. Docking efficiency is calculated with Eq. (6) and related to the openings rate of the SBD as determined in our previous work [7]. GlnPQ-SBD1 (black), GlnPQ-SBD1 + 10 (red) and GlnPQ-SBD1 + 20 (blue).

most efficient when glutamine is transported via SBD1 (E184D V185E) ($\Theta = 124\% \pm 53\%$; longest dwell time).

In conclusion, when the linker length is increased, it takes more time to form the SBD-translocator complex, and hence, the $V_{\max}$ decreases. When complex formation is slower than opening of the SBD, docking is inefficient and will additionally impact the K_m . An SBD occupying a substrate will only manage to dock efficiently onto the translocator when the affinity is sufficiently high to prevent premature release of the substrate back to the environment. However, when the affinity is too high (long lifetime of the closed conformation), it can impact and diminish the efficiency of other steps in the translocation cycle ($S_{III} \rightarrow S_{IV}$ in the scheme as shown previously [7]).

Discussion

All known structures of ABC importers are from systems with soluble periplasmic SBP and their properties are rather well documented. In brief, these studies indicate different modes of interactions between TMD and SBP for Type I and Type II importers [20–24]. For Type I importers, the affinity of TMD for the SBP is low when inferred from transport measurements (K_m in the high micromolar range), and the K_m is not changing much in the presence of ligand [25–29]. Type II importers show nanomolar affinities (K_D) for the binding of SBP to the TMD, and the K_D values become higher in the presence of ligand as shown by surface plasmon resonance for the molybdate/tungstate transporter hiMolBC-A [27] and the vitamin B₁₂ importer BtuCD-F [28]. *In vivo* the concentration of periplasmic binding proteins can reach 1 mM as shown specifically for maltose (MBP) [30] and histidine binding proteins

(HisJ) [26]. The relative concentration of several periplasmic SBPs has been determined in a large proteomic study in *E. coli* [31], which shows up to 5- to 10-fold differences in SBP expression depending on the growth condition (growth rate, absence or presence of inducer).

We study ABC transporters in Gram-positive bacteria that have one, two or three substrate receptors per transmembrane unit and thus have two, four or six SBDs in the functional dimeric complex [4–6,11,31]. Little is known about these receptors, whether they compete with each other to form the SBD-translocator complex and what the role is of the linkers between the SBDs and the SBD and TMD.

Alignment of the linkers of GlnPQ homologs and structure prediction methods do not give conclusive information about the nature of the linkers, whether they contain structural elements or are fully flexible. We now show that the 19 amino acids bridging SBD2–TMD and the 14 amino acids linking SBD1–SBD2 are close to the minimum number needed for the transporters to function. Furthermore, we show that a part of the 19-amino-acid linker between SBD2 and TMD may also serve a structural role, which we infer from measurements where GlnPQΔSBD is complemented with lipid-anchored SBD2. Thus, the linker region may form part of the docking platform for the SBD in the interaction with the TMD. Furthermore, we recently showed that removal of the linker region from GlnPQΔSBD increased the futile hydrolysis of ATP by a factor of 4–5 [33], confirming its importance in the transmembrane signaling by the TMD.

Soluble SBPs of ABC importers, such as MBP of the maltose transporter from *E. coli*, compete with each other for the translocator, and the transport rate saturates at high substrate and SBP concentration [26]. At low substrate concentrations, the transport rate plummets by the competition between unliganded and closed liganded SBP for docking onto the translocator domain, as shown for the maltose, histidine and oligopeptide transporters [26,29,30]. The relatively short linkers between the SBDs and the translocator in GlnPQ and related transporters allow the proteins to probe a small volume around the translocator, leading to an effective concentration of the SBDs in the (sub) millimolar range (Fig. 1). In contrast to soluble SBPs, we do not see any saturation in the transport rate when the linker length is varied, and thus, the effective SBD concentration is altered, which could indicate that the affinity of the TMDs for SBDs in GlnPQ is even lower than reported for ABC importers with soluble or lipid-anchored SBPs. However, it must be noted that mechanistic differences may exist between these transporters, which could give rise to different transport kinetics. For example, every soluble SBP can interact with every available translocator, whereas the SBDs of GlnPQ and related transporters can only interact with the translocator to which they are fused.

In previous work, we used single-molecule Förster resonance energy transfer to probe the conformational dynamics of SBD1 and SBD2 and correlated this to transport. We now analyzed the transport cycle of GlnPQ further by perturbing the translocation paths by engineering the linkers between the SBDs and between SBD2 and the TMD. We find that increasing the linker length decreases the $V_{\max}$, suggesting that the time needed for the substrate-loaded SBD to find and dock onto the translocator has increased. For the first time, we were able to calculate the docking efficiency as function of linker length and lifetime of the closed conformation. Based on our mathematical model, we conclude that the docking process takes around 60–280 ms or longer if the efficiency of the docking process is low (e.g., in case of lipid-anchored SBD2 when the effective SBD2 concentration is low). The $V_{\max}$ of glutamine transport by GlnPQ *in vivo* is about $10 \text{ nmol min}^{-1} (\text{mg of total protein})^{-1}$, that is, at an estimated GlnPQ expression of 0.2%–0.5% of total cell protein; hence, we infer a turnover number for glutamine transport of $7\text{--}17 \text{ s}^{-1}$. On the basis of these numbers and the fact that the $V_{\max}$ varies with linker length, we conclude that the docking process is one of the rate-determining steps of the translocation cycle of GlnPQ.

At high substrate concentrations, transport does not depend on the docking efficiency, as any substrate molecule released from the SBD is quickly replaced by a new one (see also Eq. (8)). Under these conditions, the decrease in the transport rate with increasing linker length is due to the longer time that is needed for the substrate-loaded SBD to find the translocator. At low substrate concentrations, an additional effect influences the transport process. Now a new molecule not rapidly replaces a substrate released from the SBD. Hence, depending on the timescales of substrate release (lifetime of closed conformation) and the finding of the translocator (length of linker), this will additionally impact transport as is observed for glutamine transport via SBD1. When substrate availability is limiting, the reduction in glutamine transport via SBD1 is due to the increase in K_m and decrease in $V_{\max}$, as the transport rate at low substrate concentrations is directly proportional to $V_{\max}/K_m$. Finally, a dominant effect of docking of the *apo* SBD state would have an effect on the K_m and not on the $V_{\max}$ of transport, which is not what we observe. We therefore did include docking of the *apo* SBDs in our model.

In conclusion, we show that the linkers of GlnPQ that connect the SBDs to the TMDs provide a platform for docking and are not merely flexible structures. The linker length determines the effective receptor concentration and impacts transport in a dual manner ($V_{\max}$ and K_m) that depends on the conformational dynamics of the SBD. Our results show how nature might fine-tune the transport of essential nutrients into the cell by varying the dynamic properties of the SBDs and the linkers that connect them to the translocator.

Materials and Methods

Strain construction

L. lactis GKW9000 carrying pNZglnPQhis or derivatives was cultivated semi-anaerobically at 30 °C in M17 (Oxoid) medium supplemented with 1% (w/v) glucose and 5 µg/ml chloramphenicol, unless indicated otherwise. Strain GKW9000 is a derivative of NZ9000 with the *glnPQ* genes deleted. Mutants of GlnPQ were made by uracil excision-based cloning, using pfuX7 polymerase [32], or by inserting gene fragments at unique restriction sites using Phusion polymerase (Fermentas). For GlnPQ-SBD, an NcoI site was introduced at different positions in the linker region to find the optimal constructs for *in trans* complementation by lipid-anchored SBD2 (see Table 1 and the Results section).

The lipid-anchored SBD2 was created in the same way as previously done for ProX [19] by fusing the coding sequence of SBD2 to the signal sequence of OppA. The pAMP31 vector carries the signal sequence of *oppA*, which is cleaved at position 23, and the N-terminal cysteine is then lipid-modified by the host cell. In the final construct (pAmp31_SBD2), a 17-amino-acid linker connects the lipid-modified Cys with the N-terminus of SBD2 (position A255–A484 of full-length GlnPQ). The lipid-anchored SBD2 is constitutively expressed from the constitutive lactococcal P32 promoter in pAmp31_SBD2; vector selection is done with 5 µg/ml erythromycin.

All mutants are described in Table 1.

Uptake experiments in whole cells

Cells were grown in GM17 to an OD_{600} of 0.4, induced for 1 h with 0.01% of culture supernatant of the nisin A-producing strain NZ9700 and harvested by centrifugation for 10 min at 4000g; the final nisin A concentration is ~1 ng/ml. After washing twice with 10 mM Pipes–KOH and 80 mM KCl (pH 6.0), the cells were resuspended to $\text{OD}_{600} = 50$ in the same buffer. Uptake experiments were performed at 0.1–0.5 mg/ml total protein in 30 mM Pipes–KOH, 30 mM Mes–KOH and 30 mM Hepes–KOH (pH 6.0). Before starting the transport assays, the cells were equilibrated and energized at 30°C for 3 min in the presence of 10 mM glucose plus 5 mM MgCl_2 . After 3 min, the uptake reaction was started by the addition of either [^{14}C]-glutamine (PerkinElmer), [^3H]-glutamine (Perkin Elmer) or [^3H]-asparagine [American Radiolabeled Company Inc. (ARC)]; the specific radioactivity was adjusted in the different experiments to have the radioactive counts at least 10-fold above the background; the final amino acid concentrations are indicated in the figures, tables and/or figure legends. At given time intervals, samples were taken and diluted into 2 ml ice-cold 100 mM LiCl. The samples were

rapidly filtered through 0.45- μm pore-size cellulose nitrate filters (Amersham) and the filter was washed once with ice-cold 100 mM LiCl. The radioactivity on the filters was determined by liquid scintillation counting.

Western blotting analysis

Parallel to the transport assays, cell samples were frozen for subsequent analysis of protein expression by immunoblotting or protein purification when deletion mutants (in case of GlnPQ Δ SBD) prohibited the use of antibodies for Western blotting; we note that the antibodies are directed against the SBDs rather than TMD of the GlnP subunit. Cells were diluted to $\text{OD}_{600} = 15$ and combined with glass beads (0.1 mm VWR) and broken using a tissue lyser LT (Qiagen) at 50 Hz for 5 min. This step was repeated once, after cooling the samples on ice for 5 min. After waiting for the glass beads to settle, the crude cell lysate was separated by SDS–12.5% PAA gel electrophoresis, followed by Western blotting. Typically, 24 μg of total protein was applied to the gel; mouse anti-Histag antibodies (5-prime/Qiagen), and/or anti-SDB1 or anti-SBD2 from rabbit (both custom made by Eurogentec) were used for immune detection using Chemoluminescence (Tropix). To determine the expression levels in membrane fractions, the crude lysate was centrifuged for 10 min at 15,000g to remove remaining cell debris. Subsequently, the membrane vesicles were collected by centrifugation at 227,000g for 20 min. The pellet was resuspended in SDS-PAGE loading buffer and analyzed by Western blotting. We used a pre-stained molecular weight marker (Fermentas) to indicate the molecular weight on the blots. GlnPQ Δ SBD expression was estimated on the basis of the amount of purified protein obtained by Ni-Sepharose chromatography, using membrane vesicles as starting material and the His-tag on GlnQ as affinity tag for purification (Lycklama *et al.*, 2018, *J Mol Biol*, in press).

Mathematical modeling of the transport cycle of GlnPQ

We describe the translocation cycle of GlnPQ by a continuous-time Markov chain that includes five states (Fig. 8). We furthermore assume that the time evolution of the system satisfies the Markov property. This assumption is implicitly assumed when chemical reactions are modeled by a set of first-order coupled differential equations to describe the concentrations of states, as was done in our previous work [6].

Since, the transport of substrates across the membrane is a random process, the occupation of a particular state i at time t is a random variable $S_i(t)$, with $S_i(t) = \{0, 1\}$ and $i = \{I, II, III, IV, V\}$. Then, $P(S_i(t) = 1)$ and $P(S_i(t) = 0)$ are the probabilities that the transporter is in or is not in state i at time t , respectively.

We set the transition probability equal to $P(S_{II}(t + \Delta t) = 1 | S_I(t) = 1) = t_1^{-1} L_{\text{OUT}}(t) \Delta t$, where $L_{\text{OUT}}(t)$

is the substrate concentration in the external environment at time t and Δt is small unit of time. We assume that $L_{\text{OUT}}(t)$ is large enough such that $L_{\text{OUT}}(t) = L_{\text{OUT}}(0) = L_{\text{OUT}}$. For any other transition, $P(S_i(t + \Delta t) = 1 | S_j(t) = 1) = t_i^{-1} \Delta t$, where t_i is the expected time needed to make a transition between the connecting states of the chain as indicated in Fig. 8.

The steady-state behavior is then determined from the null-space of the generating matrix Q . The generating matrix Q for this model is given by

$$Q = \begin{bmatrix} -t_1^{-1} L_{\text{OUT}} & t_1^{-1} & 0 & t_4^{-1} \\ t_1^{-1} L_{\text{OUT}} & -t_2^{-1} - t_1^{-1} & 0 & 0 \\ 0 & t_2^{-1} & -t_3^{-1} & 0 \\ 0 & 0 & t_3^{-1} & -t_4^{-1} \end{bmatrix} \quad (1)$$

We are interested in $P(S_{IV}(t) = 1)$ at steady-state, since the amount of transported substrate is $v(t) = E t_4^{-1} P(S_{IV}(t) = 1)$, where E is the transporter concentration. We set $P(S_V(t) = 1) = 0$, because the high internal ATP concentration will quickly reset the system back to S_I and relaxing this assumption will not change any of our conclusions, since our work focuses primarily on the initial steps of the translocation cycle.

The transport rate of the model at steady-state follows Michaelis–Menten behavior

$$v(t) = V_{\text{max}} \frac{L_{\text{OUT}}}{K_m + L_{\text{OUT}}} \quad (2)$$

with

$$K_m = K_D \frac{t_2 + t_{-1}}{t_2 + t_3 + t_4} \quad (3)$$

and

$$V_{\text{max}} = \frac{E}{t_2 + t_3 + t_4} \quad (4)$$

Equations (2), (3) and (4) are used to analyze and interpret the experimental observations.

The efficiency of docking Θ is

$$\Theta = \frac{t_{-1}}{t_2 + t_{-1}} \quad (5)$$

and is maximal efficient when $\Theta = 1$ and most inefficient when $\Theta = 0$. Using Eqs. (3), (4) and (5), it can be shown that

$$\Theta = t_1 \frac{k_{\text{cat}}}{K_m} \quad (6)$$

where $k_{\text{cat}} = V_{\text{max}}/E$.

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Appendix A. Supplementary data

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

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