Substrate Specificity and Ionic Regulation of GlnPQ from Lactococcus lactis

AN ATP-BINDING CASSETTE TRANSPORTER WITH FOUR EXTRACYTOPLASMIC SUBSTRATE-BINDING DOMAINS*

Received for publication, January 14, 2005, and in revised form, March 23, 2005
Published, JBC Papers in Press, March 28, 2005, DOI 10.1074/jbc.M500522200

Gea K. Schuurman-Wolters and Bert Poolman‡

From the Department of Biochemistry, Groningen Biomolecular Science and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands

We report on the functional characterization of GlnPQ, an ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. The first predicted transmembrane helix of GlnP was cleaved off in the mature protein and most likely serves as the signal sequence for the extracytoplasmic substrate-binding domains. Deletion analysis showed that the substrate-binding domain, in the primary sequence of GlnP nearest to the translocator domain, is used as the receptor that delivers the substrate to the translocator. Membrane reconstitution of the detergent-solubilized and purified GlnPQ complex yielded proteoliposomes that transported glutamine and glutamic acid at the expense of ATP. The transport activity of GlnPQ increased with luminal salt concentration and inter-

The basic unit of ATP-binding cassette (ABC)1 transporters consists of two translocator domains and two ATP-binding cassettes, together referred to as the translocator (1). The domains of the translocator can be present as separate subunits or fused to each other in any possible combination. In addition, prokary-

The abbreviations used are: ABC, ATP-binding cassette; SBP, substrate-binding protein; SBD, substrate-binding domain; DDM, dodecyl-

Copyright © 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Materials—M17 broth was obtained from Difco, MRS broth from Merck, nickel-nitritolactate acid resin from Qiagen Inc., N-dodecyl-

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Membrane Vesicle Preparation—L. lactis strain NZ9000 was cultivated semi-anaerobically at 30 °C in a medium containing 5% (w/v) whey permeate, 0.5% (w/v) yeast extract plus 65 mM sodium-phosphate, pH 7, supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol when carrying pNZglnPQhis or other derivatives. For the preparation of membrane vesicles, cells were grown in a 15-liter pH-regulated fermentor at pH 6.5 to an A600 of 1–2, 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. The cells were harvested, and the mem-

This paper is available on line at http://www.jbc.org

23785
bran vesicles were prepared according to standard procedures (13).
When appropriate, a chemically defined medium was used to cultivate the cells, as described previously (18).

Disruption of Chromosomal glnP—The glnP gene of L. lactis JIM7049, a derivative of IL1403, was disrupted by plasmid integration via homologous recombination using an 886-bp EcoRI fragment of the glnP gene (bp 824–1710) ligated in the integration vector pOrI28 (erythromycin-resistant), yielding pOrI28disP. L. lactis IL108, carrying the repA gene in trans, was used as the host for the construction and propagation of the vector. Subsequently, L. lactis JIM7049 was transformed with pOrI28disP to inactivate the glnP gene. Transformants were selected for growth on GM17 agar supplemented with 2.5 μg/ml erythromycin. The integration of the vector was verified by PCR, and the disruption strain was designated JIM7049disglnP.

Plasmid Construction—For the overexpression of the GlnPQ complex from L. lactis IL1403 (GenBank™ accession numbers NP_267915 for glnP and NP_267916 for glnQ), the genes were placed under the control of the nisA promoter (7). The genes were amplified by PCR, using as the forward primer (glnPfor) 5′-GAGGACGCTTGAAGAATATTTTTCGCTCTGCTGTTGAGCCG-3′, and as the reverse primer (glnPRev) 5′-ATGCTGGTCTGACGATCGATTTGTAAATTTTTTGACTG-3′, starting at the ATG of glnP, and as the reverse primer (glnQRev) 5′-ATTGTTGCTTGAAGAATATTTTTCGCTCTGCTGTTGAGCCG-3′, and as the reverse primer (glnQRev) 5′-ATGCTGGTCTGACGATCGATTTGTAAATTTTTTGACTG-3′. The resulting protein is a fusion of glycine after the amino-terminal methionine of the glnP protein and the addition of a His6 tag at the carboxyl terminus of the GlnP protein.

For the construction of the deletion mutants ΔSBP1 and ΔSBP2, we took advantage of the homology between the two domains and the identity with GlnH from E. coli, for which crystal structures are available (8, 9); see also Fig. 1B. For ΔSBP1 (deletion of amino acids 33–265), two silent NruI sites were introduced into the glnP gene, one in the amino acid sequence 32–34 (IAs) using glnPQfor as the forward primer and 5′-GTATGATCGATCGATCTCGCTGATTICAGCTATGCCTG-3′ as the reverse primer, and one at residues 264–266 using 5′-CCGTAATGATCGATCGATCTCGCTGATTICAGCTATGCCTG-3′ as the forward primer and glnPQRev as the reverse primer. The two PCR products were subjected to restriction with NruI, followed by ligation. After purification, the ligation product was amplified with glnPQfor and glnPQRev and ligated into the NcoI and BamHI restriction sites of pNZglnPQhis, yielding pNZglnPQhisΔSBP1. The strategy for preparing ΔSBP2 was essentially the same, except that Scal sites were introduced 5′ and 3′ of the region specifying the second substrate-binding domain. The first Scal site was made by changing the sequence (residues 246–248) KYG into KYL, using glnPQfor as forward and 5′-GTTTCTTATGCGCGATCTCTGATTICAGCTATGCCTG-3′ as the forward primer and glnPQRev as the reverse primer. The resulting plasmid was named pNZglnPQhisΔSBP2. ΔSBP2 corresponds to a deletion of residues 248–480.

Whole Cell Uptake Experiments—L. lactis strain JIM7049 was grown on MRS medium plus 2% w/w glucose to an A600 of 0.6–1 and harvested by centrifugation (10 min, 4,000 × g). The cells were washed three times with ice-cold 50 mM potassium phosphate, pH 6.5, and suspended in this buffer to a density of 0.8–1.0. After transport experiments, the cells were diluted to an A600 of 5 into 50 mM potassium phosphate, pH 6.5, 5 mM MgCl2 plus 10 mM glucose and allowed to equilibrate at 30 °C. After 3 min of incubation, the uptake was started by adding substrate ([14C]glutamine, [14C]glutamate or substrate + inhibitor). The final protein concentration in the assays was 1 mg/ml. At given time intervals, 40-μl samples were taken and
diluted with 2 ml of ice-cold 50 mM potassium phosphate, pH 6.5. The samples were collected on 0.45-μm pore size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany) and washed twice with 2 ml of the same buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry.

**Purification of GlnPQ**—Membrane vesicles were resuspended in 50 mM potassium phosphate, pH 8.0, 200 mM KCl, 20% glycerol (buffer A) to a concentration of 5 mg of protein/ml and solubilized with 0.5% DDM for 30 min on ice. After centrifugation, the supernatant was incubated with nickel-nitrilotriacetic acid resin (0.5 ml of resin/10 mg of membrane protein) for 1 h, rotating at 4 °C in the presence of 10 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented with 0.05% DDM and 10 mM imidazole. The His-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.5% DDM and 200 mM imidazole.

**Membrane Reconstitution of GlnPQ**—Liposomes composed of egg yolk Lα-phosphatidylcholine and purified E. coli lipids in a 1:3 (w/w) ratio were prepared, and membrane reconstitution of the GlnPQ complex was performed essentially as described by Knol et al. (10), except that 20% (v/v) glycerol was added to the buffers. Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Detergent-saturated liposomes were mixed with purified GlnPQ complex in a 1:1 ratio (w/w) and incubated for 30 min at room temperature under gentle agitation. To keep the molar lipid-to-protein ratio the same as for the wild-type protein complex, purified GlnPQASBP1 and GlnPQASBP2 were reconstituted at 128:1 (w/w) lipid:protein. To remove the detergent, Bio beads SM-2 were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Bio beads SM-2 (40 mg/ml) were added to the sample four times, and the incubations were continued at 4 °C for 15 min, 30 min, overnight, and 1 h, respectively. After 5× dilution into 50 mM potassium phosphate, pH 7.0, the proteoliposomes were collected by centrifugation (150,000 g for 90 min) and stored in liquid nitrogen. For membrane reconstitution in defined lipid mixtures, synthetic lipids (DOPC, DOPG, and DOPE) dissolved in chloroform were mixed in the appropriate ratio, dried in vacuo, and resuspended in 50 mM potassium phosphate, pH 7.0. After sonication with a probe sonicator (15 s on/45 s off, 6 cycles at an intensity of 4 μm), the liposomes were frozen/thawed three times and stored in liquid nitrogen. The protocol used for incorporation of GlnPQ into these liposomes was essentially the same as described for the E. coli lipid/egg phosphatidylcholine mixture.

**ATPase Activity**—An ATPase assay on proteoliposomes was used to define the functional integrity of the GlnPQ complex. The ATPase activity of the proteoliposomes was determined using calculated molar absorption coefficients of 83,200 mol⁻¹·cm⁻¹ for GlnP and 1,615 mol⁻¹·cm⁻¹ for GlnQ, respectively.

**RESULTS**

**Gene Disruption**—Integration of pOri28disP into the chromosome of L. lactis JIM7049 led to disruption of the glnP gene and abolished the uptake of glutamate and glutamine (Fig. 2). Although glutamine (or glutamate) is essential for growth of L. lactis, the disruption mutant was able to grow in complex MRS broth, probably because of the presence of glutamine-and/or glutamate-containing peptides. Using a chemically defined medium (18) supplemented with glutamine or glutamate as the sole source for glutamine or glutamate, growth was not observed for the disruption mutant, but growth in chemically defined medium could be rescued by the addition of the dipeptide alanine-glutamine, which is taken up by a peptide transport system (11). These experiments strongly indicate that GlnPQ is the sole transport system for the uptake of glutamine and glutamate, which is consistent with previous observations that these amino acids compete for transport in L. lactis. In these experiments, it was also shown that glutamine and glutamic acid (and not the glutamate anion) are the actual substrates of, at that time, unknown transporter(s) (6).

**Amplification and Purification of GlnPQ**—The genes encoding the translocator subunit (GlnP) and the ATPase (GlnQ) were placed in tandem in the pNZOpuA plasmid, using the NcoI and BamHI restriction sites. Hereby, an extra glycine residue is introduced into GlnP as the second amino acid, and an extra glycine residue is introduced into GlnQ as the second amino acid. After nickel-nitrilotriacetic acid purification, the GlnPQ complex was collected by centrifugation (300,000 × g for 15 min) and was washed with ethanol and dissolved in 50 mM potassium phosphate, pH 7.0, to a concentration of 20 mg of lipid/ml. Uptake was measured in 90 mM potassium phosphate, pH 7.0, with 2 ml of the same buffer. The radioactivity on the filters was calculated molar absorption coefficients of 83,200 mol⁻¹·cm⁻¹ for GlnP and 1,615 mol⁻¹·cm⁻¹ for GlnQ, respectively.

**Substrate Specificity of GlnPQ and the Role of the Individual Substrate-binding Domains**—Glutamine uptake followed Michaelis-Menten kinetics (Fig. 4A), from which an apparent \( K_m \) for uptake of 6.3 ± 0.8 μM was estimated. Using 5 μM \(^{[14]C}\)glutamine as the reporter ligand, we tested all natural L-amino acids (except Tyr), the dipeptide Ala-Gln and methyl-
ammonium as possible substrates at 1 and 10 mM concentrations. Significant inhibition of glutamine transport was only observed in the presence of glutamate, arginine, and γ-glutamylhydrazide (data not shown). The inhibition by arginine and γ-glutamylhydrazide was competitive, with $K_i$ values of 2.2 ± 1.1 mM ($n = 4$; Fig. 4A) and 80 ± 50 μM ($n = 5$), respectively, where $n$ is the number of independent measurements. Competitive inhibition of glutamine transport by glutamate and uptake of $[^{14}C]$glutamic acid was only observed at low pH (pH < 6), which most likely reflects the low fraction of glutamic acid at the ambient pH values; the pK_a of the γ-carboxyl group is 4.25. Uptake of arginine could not be measured, possibly due to the low affinity of the transporter for arginine, but it could not be ruled out that arginine is only inhibiting and not transported. In summary, these measurements, together with the pH dependence of transport (see below), reveal the unusual substrate specificity of the transporter, which is that GlnPQ transports glutamine and glutamic acid and is competitively inhibited by arginine and γ-glutamylhydrazide.

By aligning the sequences of the two substrate-binding domains (SBDs) of GlnPQ with the corresponding substrate-binding protein GlnH from *E. coli* and inspection of the crystal structures of GlnH (8, 9), we deduced a linker of ten residues that connects SBD1 with SBD2 (Fig. 1B, katppkdvyt). To construct the transporter complex lacking the first substrate-binding domain (GlnPQΔSBD1), the sequence ettvk following the signal sequence was fused to the first conserved residue (Ile) in SBD2, resulting in a mature GlnPQΔSBD1 protein that started with the sequence ETTVKIASDNSF (which compares to ETTVKIASDNSF) in wild-type GlnPQ. For the construction of GlnPQΔSBD2, the sequence YDKILKKY (just before the end of SBD1) was fused to the sequence starting with LESDAKT, leaving the last seven amino acids of SBD2 in place. The expression, purification, and membrane reconstitution of GlnPQΔSBD1 and GlnPQΔSBD2 was comparable with wildtype GlnPQ (Fig. 3). However, with GlnPQΔSBD2, uptake of glutamine or glutamate was no longer observed (data not shown). On the contrary, the kinetics of glutamine uptake ($K_m = 7.2 ± 1.2$ μM) and arginine inhibition ($K_i = 1.8$ mM) of GlnPQΔSBD1 was similar to that of the wild-type complex (Fig. 4B), indicating that the SBD, in sequence nearest to the translocator domain, is used to deliver the substrate to the translocator. The turnover number of GlnPQΔSBD1 was 20–30% lower than that of wild-type GlnPQ, as deduced from activity measurements in several independent preparations of proteoliposomes (Fig. 4 and data not shown).

Salt and Lipid Dependence—After reconstitution in liposomes composed of *E. coli* phospholipid/egg phosphatidylcholine (3:1), the activity of the GlnPQ transporter was highest when the external medium was hyperosmotic relative to the inside (Fig. 5A). Both salts (NaCl and KCl) and membrane-impermeant nonionic osmolytes (sucrose) stimulated the uptake to the same extent when added in equiosmolar concentrations (the membrane-permeant nonionic osmolyte glycerol had no effect), which is reminiscent of the activation of the osmoregulatory glycine betaine transporter OpuA from *L. lactis*. In the case of OpuA, the osmotic activation originates from the increase in lumenal electrolyte concentration upon osmotic upshift, converting the osmotic signal into an ionic signal, which is sensed by the transporter through altered lipid-protein interactions (4, 12). Comparable increases in electrolyte concentration upon osmotic upshift take place in the proteoliposomes with GlnPQ. To determine whether possibly a similar osmoregulatory mechanism is operative in GlnPQ, we reconstituted the transporter complex in synthetic lipid mixtures (DOPC/DOPG/DOPG) with varying fractions of the anionic lipid DOPG and determined the osmotic activation profiles. Fig. 5B shows that the fraction of DOPG influenced the maximal activity of GlnPQ.
The glutamine-glutamic acid transporter from *L. lactis* IL1403 is a member of the PAO family of the ABC superfamily with a unique domain structure. The GlnP protein has two different SBDs fused in tandem at the amino-terminal side of the translocator domain. The first substrate-binding domain is preceded by a signal sequence, which thus far has only been shown for one other prokaryotic membrane protein, the ammonia transporterAmtB from *E. coli* (13). Whereas the function of the signal sequence in AmtB is unknown, in the case of GlnPQ, it serves a role in directly translocating across the membrane of the two SBDs.

The two SBDs of GlnPQ are 50% identical, and SBD1 and SBD2 are 32 and 34% identical to the periplasmic substrate-binding protein GlnH from *E. coli*. The structures of GlnH in the open and liganded form have been determined (8, 9), and most of the residues in the substrate-binding pocket of GlnH are conserved in SBD1 and SBD2 of GlnPQ. Our experiments indicate that, surprisingly, deletion of the first SBD has little or no effect on the kinetics of glutamine uptake, whereas the deletion of the second SBD is detrimental for transport. Because SBD1 has been preserved through evolution, it may have a regulatory role, although not directly participating in the transport cycle. However, functional analysis of wild-type GlnPQ and GlnPQ\(\Delta\)SBD1 did not reveal noticeable differences in the specificity of transport; only the turnover number was somewhat reduced. In contrast to the glycine betaine transporter OpuA with two identical SBDs in the functional complex (14), we did not observe any cooperativity in the substrate dependence of uptake mediated by GlnPQ or GlnPQ\(\Delta\)SBD1. The fact that wild-type GlnPQ and both deletion mutants were produced in similar amounts, behaved protein chemically very similarly (each complex was expressed in the membrane and had no tendency to aggregate after solubilization and/or purification), and were highly similar in sequence makes us believe that not only SBD2 in GlnPQ\(\Delta\)SBD1 but also SBD1 in GlnPQ\(\Delta\)SBD2 are correctly folded. In respect to the failure to detect any transport activity of the GlnPQ\(\Delta\)SBD2 mutant, we note that it may be more tedious to engineer an internal (\(\Delta\)SBD2) than amino-terminal deletion (\(\Delta\)SBD1) and allow the remaining domain to functionally interact with the translocator. However, as detailed under “Results” and Fig. 1B, both deletion mutants were carefully designed, and it is unlikely that the linker region in GlnPQ\(\Delta\)SBD1 is too short to allow SBD1 to donate the substrate to the translocator. Future studies are aimed at the further elucidation of the roles of the different SBDs in the transport process and/or physiology of the cell by structure-guided site-directed mutagenesis of SBD1 and SBD2.

Recently, it has been observed that the *glnPQ* genes of group B streptococci are playing a role in virulence (15). This GlnPQ transporter is homologous to the *L. lactis* system studied here. For GlnPQ from group B streptococci, it has been proposed that the transporter plays a role in the regulation of expression of fibronectin adhesins and virulence of this pathogen. Whether GlnPQ from *L. lactis* has analogous regulatory roles remains to be determined.

Disruption of the *glnPQ* gene resulted in the loss of glutamine and glutamic acid uptake, and the cells were no longer able to grow on chemically defined medium with glutamine or glutamate as the sole source of glutamine or glutamate. The disruption mutant grew normally when glutamate was added.

**FIG. 6.** *Internal pH dependence of glutamine uptake.* The internal pH was varied by diluting proteoliposomes in 50 mM potassium phosphate, pH 6.5, 100 mM potassium acetate into media consisting of 50 mM potassium phosphate plus varying ratios of potassium acetate and K-MES (pH 6.5); the final external potassium acetate concentrations were 100, 30, 10, 5, 1.5, and 1 mM. The internal pH was determined from the fluorescence of pyranine. Uptake of \(^3\)H-glutamine (final concentration 20 \(\mu\)M) was assayed after diluting 5 \(\mu\)l of proteoliposomes (40 mg/ml lipids) from ice into 195 \(\mu\)l of buffer with varying ratios of potassium acetate/K-MES at 30 °C.

**DISCUSSION**

To determine the internal pH dependence of GlnPQ, proteoliposomes resuspended in a buffer containing 100 mM potassium acetate were diluted into equiosmolar media with different amounts of potassium acetate to vary the acetate diffusion gradients. Because acetic acid (but not the acetate anion) is highly membrane-permeable, the outward diffusion of acetic acid increased the internal pH, according to \(\text{pH}_{\text{in}} = \text{pH}_{\text{out}} + \log \left( [\text{potassium acetate}]_{\text{in}} \right) / [\text{potassium acetate}]_{\text{out}} \right) \). The rate of uptake of \(^3\)H-glutamine at an external pH of 6.5 was determined under conditions where initially (time zero, at proteoliposome dilution) the internal pH was 0–1.0 pH unit higher than the outside pH, as determined from pyranine fluorescence measurements. The rate of uptake increased with increasing internal pH and displayed an optimum of ~7.3 (Fig. 6). Control experiments showed that the ATP concentration inside the proteoliposomes was constant and ~9 mM during the course of the assay. The relatively large error in the experiments, as shown in Fig. 6, reflect the initial drop in internal pH upon dilution of potassium acetate-loaded proteoliposomes, which affected the transport rate (because of the pH dependence of the system) and the determination of the internal pH. However, three independent sets of experiments, performed at external pH values between pH 6 and 7, clearly revealed the internal pH dependence of GlnPQ.

**pH Dependence of Glutamine and Glutamate Uptake—**The uptake of glutamine and glutamate has been studied in detail for whole cells of *L. lactis*, previously *Streptococcus lactis* ML-3 and *Streptococcus cremoris* Wg2 (5, 6). Transport was found to be dependent on the internal pH, with little or no activity below pH 6 and maximal activity at pH 7.5–8. Moreover, glutamate was found to be dependent on the internal pH, with little or no activity below pH 6 and maximal activity at pH 7.3 (Fig. 6). Control experiments showed that the ATP concentration inside the proteoliposome port. Indeed, in proteoliposomes, significant uptake was only observed when pHout was 5.0 and the internal pH was ~5.5. That might be due to the lack of an internal pH sensor, which is present in whole cells but not in proteoliposomes. Another possible explanation is that the internal pH in proteoliposomes is not as well controlled as in whole cells. However, this does not rule out the possibility that the internal pH plays a role in the regulation of uptake. Therefore, it is important to control the internal pH when studying the transport of glutamine and glutamate in proteoliposomes.

**Substrate Specificity and Ionic Regulation of ABC Transporter**

Recently, it has been observed that the *glnPQ* genes of group B streptococci are playing a role in virulence (15). This GlnPQ transporter is homologous to the *L. lactis* system studied here. For GlnPQ from group B streptococci, it has been proposed that the transporter plays a role in the regulation of expression of fibronectin adhesins and virulence of this pathogen. Whether GlnPQ from *L. lactis* has analogous regulatory roles remains to be determined.

**DISCUSSION**

The glutamine-glutamic acid transporter from *L. lactis* IL1403 is a member of the PAO family of the ABC superfamily with a unique domain structure. The GlnP protein has two different SBDs fused in tandem at the amino-terminal side of the translocator domain. The first substrate-binding domain is preceded by a signal sequence, which thus far has only been shown for one other prokaryotic membrane protein, the ammonia transporter AmtB from *E. coli* (13). Whereas the function of the signal sequence in AmtB is unknown, in the case of GlnPQ, it serves a role in directly translocating across the membrane of the two SBDs.

The two SBDs of GlnPQ are 50% identical, and SBD1 and SBD2 are 32 and 34% identical to the periplasmic substrate-binding protein GlnH from *E. coli*. The structures of GlnH in the open and liganded form have been determined (8, 9), and most of the residues in the substrate-binding pocket of GlnH are conserved in SBD1 and SBD2 of GlnPQ. Our experiments indicate that, surprisingly, deletion of the first SBD has little or no effect on the kinetics of glutamine uptake, whereas the deletion of the second SBD is detrimental for transport. Because SBD1 has been preserved through evolution, it may have a regulatory role, although not directly participating in the transport cycle. However, functional analysis of wild-type GlnPQ and GlnPQ\(\Delta\)SBD1 did not reveal noticeable differences in the specificity of transport; only the turnover number was somewhat reduced. In contrast to the glycine betaine transporter OpuA with two identical SBDs in the functional complex (14), we did not observe any cooperativity in the substrate dependence of uptake mediated by GlnPQ or GlnPQ\(\Delta\)SBD1. The fact that wild-type GlnPQ and both deletion mutants were produced in similar amounts, behaved protein chemically very similarly (each complex was expressed in the membrane and had no tendency to aggregate after solubilization and/or purification), and were highly similar in sequence makes us believe that not only SBD2 in GlnPQ\(\Delta\)SBD1 but also SBD1 in GlnPQ\(\Delta\)SBD2 are correctly folded. In respect to the failure to detect any transport activity of the GlnPQ\(\Delta\)SBD2 mutant, we note that it may be more tedious to engineer an internal (\(\Delta\)SBD2) than amino-terminal deletion (\(\Delta\)SBD1) and allow the remaining domain to functionally interact with the translocator. However, as detailed under “Results” and Fig. 1B, both deletion mutants were carefully designed, and it is unlikely that the linker region in GlnPQ\(\Delta\)SBD1 is too short to allow SBD1 to donate the substrate to the translocator. Future studies are aimed at the further elucidation of the roles of the different SBDs in the transport process and/or physiology of the cell by structure-guided site-directed mutagenesis of SBD1 and SBD2.

Recently, it has been observed that the *glnPQ* genes of group B streptococci are playing a role in virulence (15). This GlnPQ transporter is homologous to the *L. lactis* system studied here. For GlnPQ from group B streptococci, it has been proposed that the transporter plays a role in the regulation of expression of fibronectin adhesins and virulence of this pathogen. Whether GlnPQ from *L. lactis* has analogous regulatory roles remains to be determined.
to the medium in the form of a dipeptide (Ala-Gln). Here, one of the dipeptide transport systems of *L. lactis* is used to compensate for the loss of Gln/Glu transport function. The GlnPQ transporter is therefore the only transporter for glutamine (and glutamic acid) uptake in *L. lactis*.

The GlnPQ complex is responsible for glutamine and glutamate transport, but the latter is only transported in the protonated form, which is glutamic acid. Previously (6), the surprising observation was made that arginine inhibited the uptake of glutamine in whole cells with an apparent *K* for a molar range. Using purified and membrane-reconstituted GlnPQ, we now confirm that arginine competitively inhibits Gln uptake with a *K* of ~2 mM. Except for glutamic acid, arginine, and γ-glutamylhydrazide, there was no clear inhibition of glutamine uptake by any of the other amino acids tested. The substrate specificity of GlnPQ and GlnPQΔSBD1 were very similar and seems to be determined by SBD2. The amino acid-binding site of SBD2 seems to tolerate fairly large neutral and basic side chains. Asparagine and aspartic acid, on the other hand, residues that are only one methylene shorter than glutamic acid and glutamic acid, did not inhibit the transport. Thus, the positioning of the amide and carboxylic acid moiety is crucial for high affinity transport. In contrast to lysine, arginine is competing with glutamine, although with a low affinity. It is possible that the guanidinyl moiety in arginine makes critical contacts with the protein, which are not possible with lysine. In respect to the region in the binding pocket of SBD2 that accommodates the side-chain amine moiety of glutamine or the hydroxyl of the glutamic acid, larger functionalities are also tolerated, which are the hydrazide of γ-glutamylhydrazide and even (part of) the bulky guanidinyl of arginine. However, for binding (and transport) of substrate, it is prerequisite that the overall charge is neutral (high affinity transport) or positive (low affinity competition by arginine).

The uptake of glutamine in proteoliposomes was highly stimulated when the concentrations of ionic osmolytes on the luminal side of the membrane were increased, *i.e.* by osmotic upshift. These observations mimic the activation of the osmoregulatory OpuA from *L. lactis*, ProP from *E. coli*, and BetP from *Corynebacterium glutamicum* (4, 12, 16, 17) and contrast with that of other, non-osmoregulatory (ABC) transporters, such as Opp.2 Because osmosensing by the OpuA system is mediated by anionic lipid-protein interactions (4), it was important to investigate the osmotic activation of the GlnPQ complex and to determine the (anionic) lipid dependence of the system. Although there was an optimal DOPG concentration for maximal activity of GlnPQ, the activation profiles were very similar at every DOPG concentration, which contrasts the situation for OpuA, where the activation profiles shift to higher osmolalities with increasing fractions of anionic lipids in the membrane. Thus, the osmotic activation of GlnPQ seems to be a direct consequence of the concentrated (ionic) osmolytes at the luminal ("cytoplasmic") side of the membrane on the protein, rather than a modulation of (anionic) lipid-protein interactions. Even though this mode of activation is different from that described for OpuA (3, 4), it would allow the cell to accumulate faster (and more) glutamine and glutamate when it is confronted with hyperosmotic stress, wherein the cytoplasmic volume decreases and the ion concentration (ionic strength) increases as a consequence of water efflux.

Recently, we proposed a common physicochemical mechanism of osmosensing (12), which for OpuA has been modeled as an on/off electrostatic lever that assumes "electrostatically locked" or "thermally relaxed" conformations. The on/off activation was found to correlate with the theoretical prediction of transitions in ionic clouds according to the Maxwellian version of the Poisson-Boltzmann equation. A similar electrostatic mechanism may be operative in GlnPQ, except that screened electrostatic forces playing a role in the different conformational states of the transporter are not mediated by lipid-protein interactions but only involve protein domains and low molecular weight electrolytes. Because the pH inside the proteoliposomes influences the ionization state of GlnPQ and low molecular weight electrolytes (phosphate, creatine-phosphate, ATP, and ADP in our experimental setup), it is well possible that the internal pH and salt dependence of GlnPQ activity have a common electrostatic basis.

How relevant is the observed regulation of GlnPQ for *L. lactis*? Molenaar et al. (18) measured an increase in the pool of glutamate after growing *L. lactis ML3* under hyperosmotic conditions, implying that glutamate could serve as an osmoprotectant in this organism. Similar to OpuA, a decrease in cell volume and the accompanying increase in ion concentration upon osmotic upshift could activate GlnPQ in *vivo*, and the further accumulation of glutamate (up to ~50 mM) would offset the deleterious effects of the stress. However, we emphasize that the role of OpuA in osmoprotection is far greater than that of GlnPQ (glycine betaine is accumulated to >0.5 M, whereas glutamate plus glutamine reach levels of at most (50 mM), which may also be reflected in the more advanced electrostatic switching mechanism of OpuA. The activity of OpuA is modulated over 2–3 orders of magnitude, whereas regulation of GlnPQ is <10-fold.

Acknowledgments—We thank Esther Biemans-Oldehinkel, Sytse Henstra, and Mark Doeven for stimulating discussions.

REFERENCES

18. Henstra, and Mark Doeven for stimulating discussions.

2 M. K. Doeven, unpublished observations.