The substrate-binding protein imposes directionality on an electrochemical sodium gradient-driven TRAP transporter

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Edited by H. Ronald Kaback, University of California, Los Angeles, CA, and approved December 5, 2008 (received for review October 7, 2008)

Substrate-binding protein-dependent secondary transporters are widespread in prokaryotes and are represented most frequently by members of the tripartite ATP-independent periplasmic (TRAP) transporter family. Here, we report the membrane reconstitution of a TRAP transporter, the sialic acid-specific SiaPQM system from *Haemophilus influenzae*, and elucidate its mechanism of energy coupling. Uptake of sialic acid via membrane-reconstituted SiaQM depends on the presence of the sialic acid-binding protein, SiaP, and is driven by the electrochemical sodium gradient. The interaction between SiaP and SiaQM is specific as transport is not reconstituted using the orthologous sialic acid-binding protein VC1779. Importantly, the binding protein also confers directionality on the transporter, and reversal of sialic acid transport from import to export is only possible in the presence of an excess of unliganded SiaP.

sialic acid | SiaPQM | tripartite ATP-independent periplasmic transporter | *Haemophilus influenzae* | receptor-dependent translocation

Bacterial transporters involved in nutrient uptake often use a substrate-binding protein (SBP) component (often called an extracytoplasmic solute receptor or periplasmic binding protein) to confer specificity and high affinity to the system (1–3). SBPs are most well understood in the context of ATP-binding cassette (ABC) transporters, which are ubiquitous transporters that couple the use of a SBP and the hydrolysis of ATP to translocation of solutes across the membrane (1). Recent breakthroughs in the structural biology of ABC transporters have provided great insight into the mechanism by which the SBP component recognizes the membrane domains and releases its ligand into the translocation pore (4–7). In addition to ABC transporters, SBPs are used by a subclass of secondary transporters that couple transport to an electrochemical ion gradient (8–10). The tripartite ATP-independent periplasmic (TRAP) transporters are a large family of substrate-binding protein-dependent secondary transport systems present in many prokaryotes (11, 12). They are composed of three subunits, the SBP component and the large and small-transmembrane domains (TMDs), typically composed of 12 and four transmembrane α-helices, respectively (11). Early studies on the C_4-dicarboxylate transporter DctPQM, from *Rhodobacter capsulatus*, were the first to demonstrate in vivo that TRAP transporters are secondary carriers that depend on a membrane potential for energization (8), but little more is known about how TRAP transporters function.

We and others have characterized a TRAP transporter from the Gram-negative human respiratory pathogen *Haemophilus influenzae* that is essential for uptake of the sialic acid, *N*-acetylneuraminic acid (Neu5Ac) (13, 14). In this and other pathogens, sialic acid is required for the bacteria to persist in their mammalian host (15). In *H. influenzae* the sialic acid is used to sialylate the lipopolysaccharide (LPS), which then provides serum resistance for the bacterium (16). The SiaPQM transporter contains a classical SBP, SiaP, whereas the membrane subunits are fused into a single protein, SiaQM. Sialic acid binds with high affinity and specificity to SiaP via simple bimolecular association kinetics (14, 17, 18) and the liganded SBP is then assumed to interact with SiaQM, releasing the ligand into the membrane domains. The recently solved structure of SiaP revealed an overall architecture that is similar to SBPs of ABC transporters, suggesting a common mechanism for the transfer of the substrate from the SBP to the translocator domains (17, 18). In ABC transporters, the liganded SBP docks onto the TMDs and signals the nucleotide-binding domains (NBDs) to cooperatively bind ATP (1). Upon ATP binding, a binding site in the TMDs is made available to the outside, concomitant with the opening of the SBP and substrate transfer to the TMDs. Subsequently, ATP hydrolysis dissociates the NBD dimer and directs the reorientation of the binding site from an outward- to an inward-facing conformation. The substrate arrives in the cytoplasm and the SBP is released from the TMDs. How the transmembrane signaling between the SBP and TMDs is occurring in secondary transporters is unknown. Clearly, the driving force for transport is different in a TRAP system as are the structural properties of the TMDs.

To further understand the mechanism of SBP-dependent secondary transport, we describe the purification and functional reconstitution of the membrane components of a TRAP transporter and define the bioenergetic and biophysical properties of a member of this widespread family of transporters.

Results

Overexpression of SiaPQM. To enable the in vitro analysis of the mechanism of transport of SiaPQM, the soluble SBP, SiaP, and the membrane component SiaQM, were produced in *Escherichia coli* and *Lactococcus lactis*. SiaP could be expressed in *E. coli* to

Author contributions: C.M., E.R.G., E.S., D.J.K., B.P., and G.H.T. designed research; C.M. and E.S. performed research; C.M., E.R.G., E.S., B.P., and G.H.T. analyzed data; and C.M., E.R.G., B.P., and G.H.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0809979106/DCSupplemental.

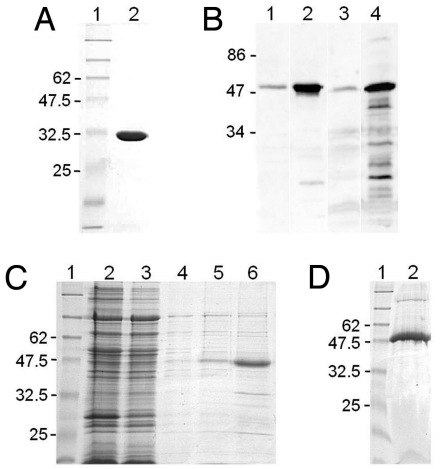

Fig. 1. Expression and purification of SiaPQM. (*A*) Coomassie-stained SDS PAGE gel of ligand-free, N-terminally decahistidine tagged SiaP purified by using Ni-affinity chromatography, followed by anion-exchange chromatography. Lane 1, molecular mass ladder with sizes of relevant proteins indicated in kDa; lane 2, purified SiaP. (*B*) Western blot using anti-His antibodies of membranes expressing SiaQM with an C- or N-terminal decahistidine tag from *L. lactis* (Lanes 1 and 2, respectively) or *E. coli* (Lanes 3 and 4, respectively). (*C*) Coomassie-stained SDS PAGE of fractions from the Ni-affinity chromatographic purification of SiaQM from *E. coli* membrane vesicles. Lane 1, molecular mass ladder; lane 2, membrane vesicles; lane 3, column flowthrough; lane 4, 40 mM imidazole wash; lanes 5 and 6, 500 mM imidazole elution fractions. (*D*) Coomassie-stained SDS PAGE gel of SiaQM containing proteoliposomes. Lane 1, molecular mass ladder; lane 2, SiaQM-containing proteoliposomes.

high levels using the P_{BAD} promoter (19) and was purified to homogeneity by using nickel-affinity and anion-exchange chromatography (Fig. 1*A*). High level expression of SiaQM was achieved by using the P_{BAD} promoter in *E. coli* and the nisin-inducible P_{nisA} expression system in *L. lactis* (20) (Fig. 1*B*). In both expression systems, N-terminally decahistidine tagged versions of SiaQM were produced to higher levels than C-terminally tagged proteins. On SDS-PAGE, SiaQM migrated to a position indicative of a mass of approximately 48 kDa, which is smaller than its predicted mass of 69.8 kDa; this aberrant migration is observed frequently for membrane proteins (21). SiaQM could be purified in a single step by using nickel-affinity chromatography (Fig. 1*C*) and was stable in both DDM and DM as judged by size-exclusion chromatography (see supporting information (SI) Fig. S1 and data not shown). We used protein expressed in *E. coli* throughout this study.

Functional Membrane Reconstitution of SiaPQM. To guide the in vitro analysis of SiaPQM, we initially characterized SiaPQM function in whole cells of *E. coli* BW25113 Δ*nanT*, which lack the ability to transport sialic acid. In the presence of a proton-motive force, BW25113 Δ*nanT* cells expressing SiaPQM from a low copy-number vector showed no uptake of [^{14}C]-Neu5Ac (Fig. S2). Significant transport of [^{14}C]-Neu5Ac was observed in cells exposed to a sodium gradient but not a lithium gradient.

Purified SiaQM was membrane-reconstituted into DM-destabilized liposomes by rapidly diluting the reconstituted mixture below the CMC of DM (22). Analysis of the SiaQM proteoliposomes by SDS-PAGE indicated one major band (Fig. 1*D*), which was confirmed to be SiaQM by MALDI-tandem mass spectrometry (data not shown). To determine whether SiaQM was functionally reconstituted and not merely membrane-associated, we assessed sialic acid transport in the presence of an inwardly directed sodium gradient ($\Delta\mu_{Na}$). A rapid uptake of [^{14}C]-Neu5Ac was observed in the presence of exogenously added SiaP (Fig. 2*A*). After three min of uptake, the substrate

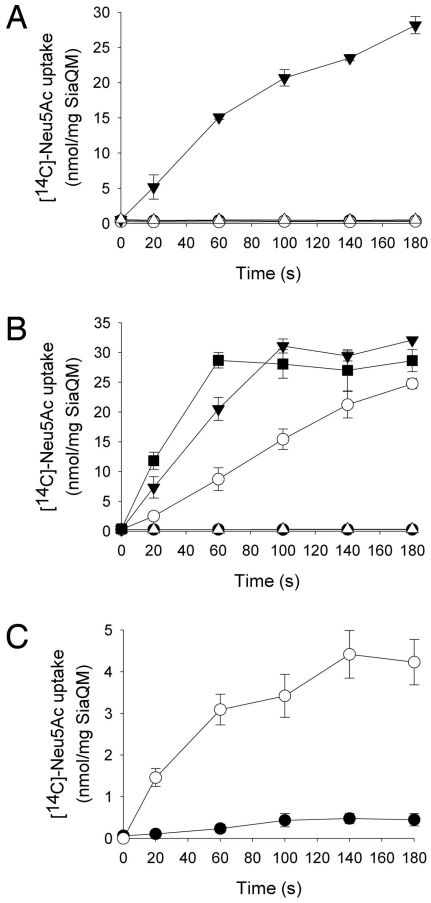

Fig. 2. Analysis of the energetic requirements for Neu5Ac uptake. (*A*) Uptake of 5 μM [^{14}C]-Neu5Ac into SiaQM-containing proteoliposomes in the presence of a sodium gradient ($\Delta\mu_{Na}$) and either 5 μM SiaP (closed triangles), 5 μM VC1779 (closed circles), no SBP (open circles), or in the presence of 5 μM SiaP but no $\Delta\mu_{Na}$ (open triangles); (*B*) Uptake of 5 μM [^{14}C]-Neu5Ac into SiaQM-containing proteoliposomes in the presence of 5 μM SiaP and the following different energetic conditions: $\Delta\mu_{Na} + \Delta\Psi$ (closed squares), $\Delta\mu_{Na}$ alone (closed triangles), $\Delta\mu_{Na}$ + ΔpH (open circles), $\Delta\Psi$ (open triangles), and ΔpH (closed circles). (*C*) Comparison of the [^{14}C]-Neu5Ac uptake properties of SiaQM-containing proteoliposomes in the presence of a $\Delta\Psi$ plus either equimolar Na^+ across the membrane (closed circles) or an inwardly directed sodium gradient (open circles).

was accumulated 150-fold inside the proteoliposomes. In the absence of the $\Delta\mu_{Na}$ or SiaP, no uptake was detectable, in accordance with the in vivo measurements (Fig. 2*A*).

To demonstrate the specific requirement for SiaP, we reconstituted transport with the SiaP orthologue from *Vibrio cholerae*, VC1779, which is 49% identical to SiaP. This SBP has a dissociation constant (K_d) for Neu5Ac of 0.11 ± 0.03 μM (Fig. S3), very similar to that reported for SiaP (0.12–0.14 μM) (14, 17). However, VC1779 did not allow sialic acid transport, demonstrating that a specific interaction between SiaP and SiaQM is required (Fig. 2*A*).

Maximal Activity of SiaPQM Requires both $\Delta\mu_{Na}$ and $\Delta\Psi$. To dissect the energetic requirements of SiaPQM for Neu5Ac uptake, we systematically varied the ion gradients across the membrane by imposing ion diffusion potentials. In agreement with the observations in whole cells, a pH gradient (ΔpH; inside alkaline) alone was insufficient to drive transport (Fig. 2*B*). Moreover, the increase in internal pH from 7 to 8.7 (calculated from the imposed acetate diffusion potential) had an inhibitory effect when combined with a

$\Delta\mu_{Na}$ (the uptake decreased from 20.5 to 8.7 nmol of min^{-1} mg of $protein^{-1}$). Additionally, in the absence of sodium ions a membrane potential ($\Delta\Psi$; inside negative) could not drive sialic acid uptake (Fig. 2*B*). However, a $\Delta\Psi$ was able to drive accumulation of Neu5Ac in the presence of equal concentrations of sodium on the inside and outside of the proteoliposomes ($[Na^+]_{in} = [Na^+]_{out}$), although at a far lower rate and accumulation level than when a $\Delta\mu_{Na}$ ($[Na^+]_{in} \ll [Na^+]_{out}$) was imposed (Fig. 2*C*). The highest rate of uptake and levels of accumulation were observed in the presence of a $\Delta\mu_{Na}$ in combination with a $\Delta\Psi$ (Fig. 2*B*). Because Neu5Ac is an anionic species with a single negative charge at physiological pH ($pK_A = 2.6$), the stimulation of transport by $\Delta\Psi$ suggests that Neu5Ac uptake by SiaPQM is an electrogenic process, requiring the cotransport of at least two positive charges. Thus, our data indicate that a minimum of two sodium ions are cotransported with one Neu5Ac molecule.

A major role of Na^+ in the process of Neu5Ac binding to SiaP can be excluded from previous work that demonstrated a 2-fold decrease in the K_d of SiaP for Neu5Ac in the presence of 120 mM NaCl (17). A similar small increase in affinity for Neu5Ac by NaCl was observed for VC1779. Taken together, our data establish the role of Na^+ as the coupling ion in Neu5Ac transport, rather than enabling Neu5Ac binding to the SBP.

Is SiaPQM-Mediated Transport Unidirectional? By definition, the driving force for secondary transport is provided by the electrochemical gradients of all species translocated. In the case of SiaPQM this is the anionic Neu5Ac together with most likely two sodium ions. Consequently, the direction of transport (import or export) depends on the direction of the sum of these gradients. In conventional secondary transporters not employing a SBP, the only substrate binding site(s) are located in the TMDs and are alternately exposed to the outside and inside of the cell. However, because of the presence of the SBP, the translocation path for a SBP-dependent secondary transporter is more complicated. The heretofore described experiments indicated the ability of SiaPQM to import Neu5Ac. Being a secondary transporter, SiaPQM is expected to catalyze substrate export as well. To determine the conditions needed for substrate export by SiaQM and the role of SiaP in this reaction, we initially performed a series of substrate exchange experiments.

First, a 200-fold excess of unlabeled Neu5Ac was added to proteoliposomes that had preaccumulated [^{14}C]-Neu5Ac. No significant difference in removal of radiolabeled substrate from the proteoliposomes with the control sample was observed (Fig. 3*A*), although for a conventional secondary transporter rapid substrate efflux would be expected. To compare under the same conditions the response of SiaPQM with that of a conventional secondary transporter, we analyzed [^{14}C]-Neu5Ac transport in *E. coli* BW25113 Δ*nanAT*, complemented *in trans* with *siaPQM* or *nanT*. NanT is a SBP-independent secondary Neu5Ac transporter from *E. coli*. Both sialic acid transporters were able to restore [^{14}C]-Neu5Ac uptake in the Δ*nanAT* strain and, importantly, preaccumulated substrate was expelled rapidly on the addition of an excess unlabeled Neu5Ac when NanT was present but not when SiaPQM was present (Fig. 3*B*).

Next, we tested whether SiaPQM would be able to facilitate a substrate counterflow reaction (23, 24). Substrate counterflow allows the exchange of external, labeled substrate for internal, unlabeled substrate and one probes the redistribution of different isotopically labeled substrates without net transport. Substrate counterflow results in a transient accumulation of labeled substrate as the binding sites alternate between the inside and outside orientation. Proteoliposomes were loaded with a high concentration of unlabeled Neu5Ac (1 mM) and then diluted into medium with 5 μM [^{14}C]-Neu5Ac. No significant counterflow activity was observed in the absence or presence of Na^+ ions ($[Na^+]_{in} = [Na^+]_{out}$) and with or without SiaP present in the assay medium (Fig. 3*C* and data not shown). A control experiment confirmed that these proteoliposomes were fully functional for Neu5Ac transport when an electrochemical Na^+ gradient was imposed (Fig. 3*C*).

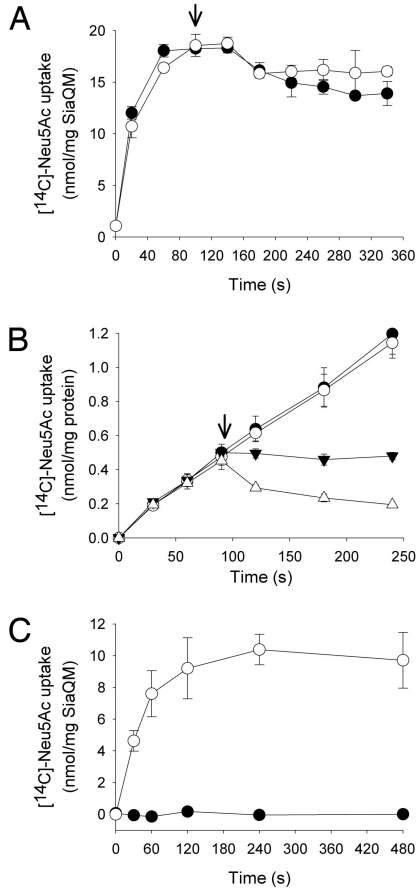

Fig. 3. Chase with unlabeled Neu5Ac and Neu5Ac counterflow. (*A*) Uptake of 5 μM [^{14}C]-Neu5Ac into SiaQM-containing proteoliposomes in the presence of 5 μM SiaP, $\Delta\mu_{Na}$, and $\Delta\Psi$. At t = 100 s (indicated with an arrow) 1 mM unlabeled Neu5Ac (open circles) or the same volume of dH_2O (closed circles) was added. (*B*) Uptake of 1 μM [^{14}C]-Neu5Ac into *E. coli* BW25113Δ*nanAT* expressing NanT (open symbols) or SiaPQM (filled symbols). At t = 100 s (indicated with an arrow), either 1 mM unlabeled Neu5Ac (triangular symbols) or an equal volume of buffer (circular symbols) was added to the reactions. (*C*) Substrate counterflow activity of SiaQM-containing proteoliposomes (closed circles). Control curve of [^{14}C]-Neu5Ac uptake into SiaQM-containing proteoliposomes in the presence of 5 μM SiaP and $\Delta\mu_{Na} + \Delta\Psi$ (open circles).

Taken together, these data suggest a key mechanistic difference between SBP-dependent and SBP-independent secondary transporters. Unlike conventional secondary transporters, SiaPQM facilitates only import under conditions where typical secondary transporters would allow a reversed direction of transport.

Excess Unliganded SiaP Can Induce Substrate Export. From the heretofore described experiments, it seems that transport through SiaPQM is unidirectional, presumably because of the involvement of the SBP component in its transport mechanism. In the in vitro experiments there was an equimolar ratio of SiaP and [^{14}C]-Neu5Ac, both being present at 5 μM. Given the high-affinity of SiaP for Neu5Ac, the vast majority of SiaP will be bound with ligand throughout the course of the experiment. To investigate the role of unliganded SiaP, we performed uptake experiments of [^{14}C]-Neu5Ac at 2.5, 5, 10, and 20 μM external

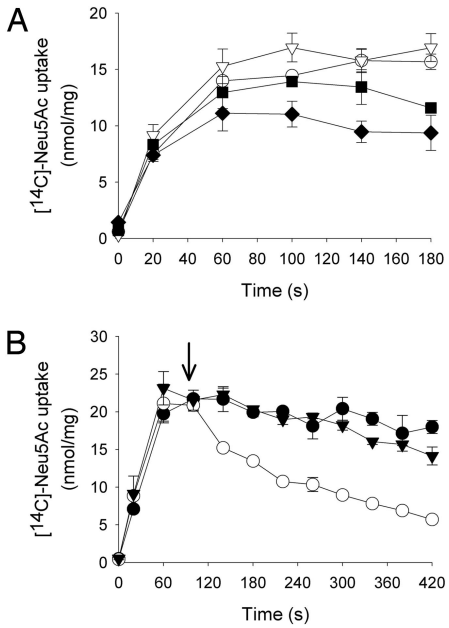

Fig. 4. Dependence of Neu5Ac transport on the concentration of SiaP. (*A*) Uptake of 5 μM [^{14}C]-Neu5Ac into SiaQM-containing proteoliposomes in the presence of $\Delta\mu_{Na} + \Delta\Psi$ and 2.5 μM (open circles), 5 μM (open triangles), 10 μM (closed squares), or 20 μM (closed diamonds) SiaP. (*B*) Effect on uptake of [^{14}C]-Neu5Ac into SiaQM-containing proteoliposomes upon the addition of 17.5 μM unliganded SiaP (open circles), 17.5 μM unliganded VC1779 (filled triangles), or buffer (50 mM KPi, pH7, filled circles) at 100 s. The SiaQM-containing proteoliposomes were used in the presence of 2.5 μM SiaP and $\Delta\mu_{Na}$. The point of addition is indicated by an arrow.

SiaP, whereas keeping the [^{14}C]-Neu5Ac concentration at 5 μM (Fig. 4*A*). Increasing concentrations of SiaP above that of the substrate gradually reduced the uptake rate and accumulation level, indicating that the presence of unliganded SBP in the assay impacts on the overall transport kinetics.

We then tested the effect of addition of excess unliganded SBP on the fate of preaccumulated [^{14}C]-Neu5Ac. Given the high affinity of SiaP for Neu5Ac, an excess of unliganded SBP over substrate will create an outwardly directed substrate concentration gradient that could exceed the sum of the inwardly directed $\Delta\mu_{Na}$ and $\Delta\Psi$. Addition of a large excess of SiaP (17.5 μM at t = 100 s in addition to the 2.5 μM already present at $t = 0$) resulted in a significant efflux of the preaccumulated substrate (Fig. 4*B*). To determine whether this observed efflux was mediated specifically through SiaPQM, we used the SiaP orthologue VC1779 from *Vibrio cholerae* instead of SiaP, because this binds Neu5Ac with similar affinity and will produce the same outwardly directed concentration gradient, but is unable to form a functional transporter with SiaQM (Fig. 2*A*). Significantly, efflux was not observed when VC1779 was added at t = 100 s instead of SiaP (Fig. 4*B*), demonstrating that a net outwardly directed Neu5Ac concentration is not sufficient for substrate export and that this phenomenon requires an unliganded SBP that is capable of binding to SiaQM to accept substrate for the export reaction. These data demonstrate that like other secondary transporters, the TRAP transporters function bidirectionally.

Discussion

This study provides the first *in vitro* characterization of a substrate-binding protein-dependent secondary transporter, using the sialic acid TRAP transporter SiaPQM from *H. influenzae*. Analysis of the energetics of the uptake process has revealed the absolute requirement for sodium ions, and the role of a $\Delta\mu_{Na}$ and a $\Delta\Psi$ as the driving forces for transport. A role for sodium ions is consistent with *in silico* analyses of the distribution of TRAP transporters in nature: A distinct enrichment of TRAP transporters was noticed in organisms living in marine environments (11, 12). Because the transport was electrogenic and given that Neu5Ac carries a single negative charge at physiological pH (pK_A = 2.6), we propose that the uptake of sialic acid most likely occurs with two sodium ions.

Our energetic data are in agreement with those of Forward *et al.* (8), who studied the DctPQM system in *R. capsulatus*, and demonstrated that the membrane potential was required for transport of C_4-dicarboxylates, although the role of Na^+ or $\Delta\mu_{Na}$ was not investigated. In membrane vesicles from *R. sphaeroides*, Na^+- and SBP-dependent transport of glutamate has been described but the nature of the transporter is unknown (10).

The recent structure of TakP, a SBP from a TRAP transporter from *Rhodobacter* that is specific for monocarboxylate keto acids (25, 26), revealed that the protein binds pyruvate as a Na^+-pyruvate complex (1:1 stoichiometry), and the cation is directly involved in the coordination of the pyruvate. This raised the attractive hypothesis that Na^+ may also be involved in the initial ligand-binding process by the SBP. Whereas this may be the case for TakP, which is an unusual dimeric SBP (26), we did not observe any bound Na^+ in our structure of SiaP (17) or in that of a more recently published and higher resolution structure of its orthologue from a nontypeable strain of *H. influenzae* (18). Given that we were also unable to find a pronounced effect of Na^+ on the binding of Neu5Ac to SiaP and that each transport cycle is likely to require more than one Na^+ ion, the Na^+ ions are most likely not recruited by the SBP.

It is clear that the SBP is an essential component of the SiaPQM transporter mechanism, as seen in ABC transporters. However, in contrast to ABC transporters, we could observe transport in the reverse direction, provided excess external unliganded SBP was available. This property indicates that in the export reaction, the release of Neu5Ac from the TMDs is coupled to the binding of SiaP to SiaQM. Apparently, the transition from the inward to the outward facing binding site requires the binding of unliganded SiaP. Conversely, in the import reaction, liganded SiaP in the closed state interacts with SiaQM, presumably in a conformation wherein the binding site of SiaQM is not yet accessible to accept the substrate. We propose that the binding of external sodium facilitates a conformational change that opens the SiaP protein and concomitantly shifts the TMDs of SiaQM in the outward conformation. The role of the electrochemical sodium gradient in SiaPQM would be equivalent to the role of ATP binding in ABC transporters. In Fig. 5, we present a schematic illustrating our current knowledge about the mechanism of TRAP transporters. Whereas the experiments presented in Fig. 4 are important to delineate the transport mechanism of SiaPQM, the export reaction is not of immediate physiological relevance as the metabolism of transported Neu5Ac will prevent build up of large outwardly directed substrate-concentration gradients.

We assume that the SiaQM protein is reconstituted in two orientations: inside-out and right-side-out. However, the presence of these two orientations does not complicate the analysis of the transport kinetics as transport of Neu5Ac via reconstituted SiaQM has an absolute requirement for SiaP (see Fig. 2*A*). Consequently, inside-out oriented SiaQM transporters, which cannot interact with the sialic acid binding protein, will not participate in the overall transport reaction, that is, neither in uptake nor efflux. The presence of two orientations will only result in an underestimation of the transport activities observed in the proteoliposomes as we do not know the fraction of inside-out oriented SiaQM.

Why would a cell use a SBP-dependent secondary transporter rather than a conventional SBP-independent system or a SBP-

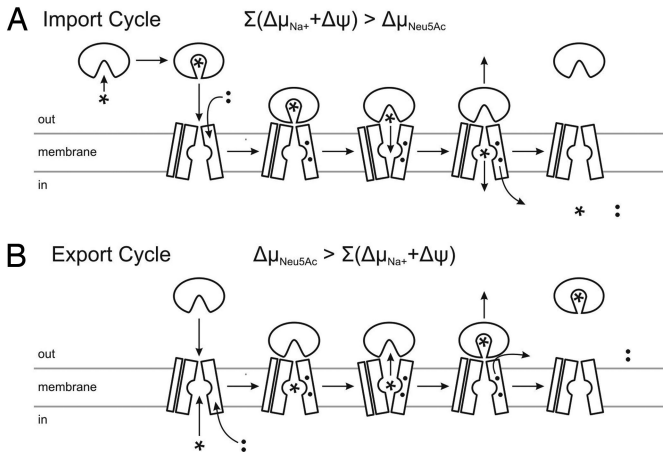

Fig. 5. Model of Na^+-dependent transport of sialic acid by SiaPQM. The *Upper* image (Import Cycle) shows the uptake of sialic acid (denoted as asterisk), driven by a (electro)chemical Na^+ gradient ($\Delta\mu_{Na} + F\Delta\Psi$; F, Faraday constant). After binding of sialic acid (asterisk) to SiaP, the liganded complex docks onto SiaQM. A minimum of two sodium ions (black dots) bind to the complex and drive the translocation of sialic acid across the membrane; the sodium ions are cotransported with sialic acid, after which the system relaxes back to the initial conformation. The *Lower* image (Export Cycle) shows the efflux of sialic acid under conditions that an excess of unliganded SiaP is available. The critical point is that efflux of sialic acid only occurs when unliganded SiaP docks onto SiaQM with bound substrate. Assuming tight coupling in the transport reaction, two or more Na^+ ions will be exported together with sialic acid.

dependent ABC transporter? In many environments, such as marine waters, the nutrient concentrations are generally low and a high-affinity SBP to scavenge substrates is likely to be advantageous. If, as we suggest, SiaPQM uses two sodium ions to translocate one Neu5Ac molecule, the energetic costs are lower than that of an ABC transporter (27), so TRAP transporters potentially offer high-affinity but with lower energetic costs than ABC transporters.

In conclusion, this study provides the first characterization of the energetics and the basic mechanism of transport of a substrate-binding protein-dependent secondary transporter. We have shown that sialic acid uptake is coupled to the cotransport of two or more Na^+ ions. We also show that the SBP is not only required in the import cycle to donate the substrate to the transmembrane domains but must also be present for the export cycle to accept the substrate from the membrane domains. These data indicate that the substrate cannot be released into the medium directly but rather requires a conformational coupling of the transmembrane domains to the binding of the SBP for substrate movement through and release from the transporter.

Materials and Methods

Expression Vector Construction. For overexpression of SiaQM (HI0147), we used both a P_{BAD} expression system in *E. coli* and the nisin-inducible P_{nisA} expression system in *L. lactis*. After comparison of the SiaQM sequence to other closely related proteins, it was clear that the original annotation of *H. influenzae* Rd KW-20, used an incorrect start codon. We thus used a later start codon that resulted in a SiaQM protein of 616 aa. The gene was amplified by PCR using primers SiaQM_Nfor/rev and SiaQM_Cfor/rev (see Table S1 for primer sequences) and cloned by using ligation-independent cloning to create N- and C-terminally decahistidine tagged versions of SiaQM. For expression in *L. lactis siaQM* was initially cloned into the vectors pREnLIC and pREcLIC, which were converted into lactococcal expression vectors by using the VBEx method (28). Details of the expression constructs for SiaP and VC1779 are described in the *SI Methods*.

Protein Expression. For expression of N-terminally tagged SiaQM, *E. coli* MC1061/pBADnQM was grown at 37°C in Luria–Bertani broth (LB), supplemented with 100 μg/ml ampicillin and 0.5% glycerol, to an A_{650} of 1, at which point they were induced with 5×10^{-3} % (wt/vol) L-arabinose. For SiaQM expression in *Lactococcus lactis*, we used strain NZ9000/pNZnQM (N-terminal decahistidine tag) that was grown in M17 medium (Oxoid), supplemented with 1% glucose (wt/vol) and 5 μg/ml chloramphenicol, to an A_{650} of 0.5. Expression was induced by the addition of 1×10^3 dilution of culture supernatant from the nisin A producing strain of *Lactococcus lactis* NZ9700 (20). Cells were harvested after 2 h growth by centrifugation at 4430 $\times$ *g* for 15 min at 4°C and were then resuspended in 50 mM potassium phosphate, pH 7.8, supplemented with 20% (wt/vol) glycerol.

Protein Purification and Membrane Reconstitution. The membrane vesicles were prepared and SiaQM was purified, essentially as described in ref. 29. For the reconstitution of purified SiaQM, we used a detergent dilution method (22). Details of these procedures are presented in the *SI Text*.

Preparation of Proteoliposomes for Transport Assays. Before the transport assay, the proteoliposomes (at 400 μg of SiaQM/ml concentration) were thawed on ice and collected by centrifugation at 200,000 $\times$ *g* for 20 min at 4°C. The proteoliposomes were resuspended in inside buffer (100 mM potassium acetate, 20 mM potassium phosphate, pH 7.0, and 2 mM $MgSO_4$, unless stated otherwise) and extruded 11 times through a 400-nm polycarbonate filter (Avestin Inc). The extruded proteoliposomes were collected by centrifugation at 200,000 $\times$ *g* for 20 min at 15°C and then resuspended in inside buffer to a final concentration of 4 μg/μl SiaQM.

Transport Assays. To generate the ion gradients, concentrated proteoliposome suspensions were diluted 50-fold, unless specified otherwise. The total volume in the lumen of the proteoliposomes accounts for only a small fraction of the total volume of this suspension. Consequently, upon dilution of the proteoliposomes, the ions not present in the lumen of the vesicles will contribute to the composition of the external solution [and thereby set the maximal value of the ion gradient(s)]. The external ion concentration is thus always well defined and corresponds for 50-fold dilutions to 1/50 of the lumen concentration plus 49/50 of the external medium concentration.

A Na^+ gradient alone ($\Delta\mu_{Na}$) was formed by dilution of proteoliposomes containing inside buffer (see above) into 100 mM sodium acetate, 20 mM sodium Pipes, pH 7.0, and 2 mM $MgSO_4$. A Na^+ gradient plus membrane potential ($\Delta\mu_{Na} + \Delta\Psi$) was formed by dilution of the proteoliposomes into 100 mM sodium acetate, 20 mM sodium Pipes, pH 7.0, and 2 mM $MgSO_4$, plus 2 μM valinomycin. A Na^+ gradient plus pH gradient ($\Delta\mu_{Na} + \Delta pH$) was formed by dilution of the proteoliposomes into 120 mM sodium Pipes, pH 7.0, and 2 mM $MgSO_4$. A membrane potential ($\Delta\Psi$) was formed by dilution of the proteoliposomes into 100 mM *N*-methyl glucamine acetate, pH 7.0, 20 mM *N*-methylglucamine phosphate, pH 7.0 and 2 mM $MgSO_4$, plus 2 μM valinomycin. A pH gradient was formed by dilution of the proteoliposomes into 120 mM potassium phosphate, pH 7.0, and mM $MgSO_4$. Control experiments (no gradients) were performed by dilution of the proteoliposomes into inside buffer. Unless indicated otherwise, the assay buffer in which the proteolipsomes were diluted contained 5 μM SiaP and 5 μM [^{14}C]-Neu5Ac. For the solute counterflow experiments proteoliposomes containing 100 mM potassium acetate, 20 mM potassium phosphate, pH 7.0, 2 mM $MgSO_4$, 10 mM sodium Pipes and 1 mM Neu5Ac were added to the same buffer without 1 mM Neu5Ac, but containing 5 μM SiaP and 5 μM [^{14}C]-Neu5Ac. For each uptake assay, 600 μl of the appropriate buffer was incubated for one min in a water bath at 30°C. During the assay, samples were homogenized by stirring. For the initiation of the transport reaction, 12 μl of 4 μg of SiaQM/μl of proteoliposomes were diluted into 600 μl of the appropriate buffer and 50 or 100 μl of samples were taken at regular intervals. The samples were incubated for 10 s with 50 or 100 μl of cold wash buffer (these buffers have the same composition as the outside buffers of the assays, except that SiaP was not present and 1 mM Neu5Ac was included to chase [^{14}C]-Neu5Ac from the SBP) and then rapidly filtered onto cellulose nitrate membranes with a pore diameter of 0.45 μm (Millipore). Filters were washed with 2 ml of ice-cold 50 mM potassium phosphate, pH 7. Radioactivity associated with the filters was determined using liquid scintillation counting. Initial rates of uptake were estimated from the linear parts of the progress curves of [^{14}C]-Neu5Ac uptake. All transport assays were performed in triplicate.

ACKNOWLEDGMENTS. We thank Peter Henderson (University of Leeds, U.K.) for help with the initial expression trials, Rosie Young and Nicola Carter Barnes for technical assistance. We thank the Society for General Microbiology for supporting the visit of C.M. to the lab of B.P. We would also like to thank the BBSRC, the European Membrane Protein Consortium Grant 504601 and the Netherlands Proteomics Centre for funding.

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