The Membrane Proteins SiaQ and SiaM Form an Essential Stoichiometric Complex in the Sialic Acid Tripartite ATP-independent Periplasmic (TRAP) Transporter SiaPQM (VC1777–1779) from Vibrio cholerae

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The SiaPQM system from Haemophilus influenzae referred to as HiSiaPQM is the most comprehensively studied system of a TRAP transporter operon (1). Hence, the SiaQM protein from H. influenzae, where the functional DctPQM transporter (3).

Substrate-binding protein (SBP)

Substrate-binding protein (SBP) is borrowed from TRAP transporters called the tripartite ATP-independent periplasmic (TRAP) transporters (2). The key difference between ATP-binding cassette transporters is that although ATP-binding cassette transporters are primary transporters, TRAP transporters are secondary transporters that use electrochemical gradients across the membrane to energize uptake (3, 4).

Much progress has been made recently in the understanding of the structure and function of SBPs from TRAP transporters (5–11), but our knowledge of the membrane components is much poorer. TRAP transporters contain two membrane components. The large (or “M”) subunit is predicted to have 12 transmembrane helices (TMHs) and is thought to form the translocation channel as it is a member of the ion transporter superfamily of secondary transporters (12). The small (or “Q”) subunit has four predicted TMHs, and the DctQ protein from Rhodobacter capsulatus has been experimentally determined to have an $N_{IN}C_{IN}$ topology (13). The function of the Q subunit is as yet unknown, but studies from the dctPQM $C_{IN}$-dicarboxylate TRAP transporter from R. capsulatus demonstrated genetically that the $dctQ$ gene, like $dctP$ and $dctM$, was essential for a functional DctPQM transporter (3).

In a typical TRAP transporter operon, the Q subunit is encoded upstream of the M subunit, although there are now a few rare examples of the order being reversed (14). Often the Q and M genes are fused genetically into a longer gene, QM, and this is the case in the sialic acid-specific TRAP transporter, SiaPQM, from Haemophilus influenzae, where the membrane domains are encoded by the siaQM gene (also called siaT by other authors) (15, 16). In these fused systems, which are not uncommon, there is a predicted extra “linker helix” that is needed to connect the $C_{IN}$ end of the Q subunit with the predicted $N_{OUT}$ location of the M subunit (Fig. 1 and supplemental Fig. 1).

Hence, the SiaQM protein from H. influenzae is predicted to have 17 TMHs (2).

The SiaPQM system from H. influenzae (from now on referred to as HiSiaPQM) is the most comprehensively studied TRAP transporter to date, as it has been fully reconstituted into...
proteoliposomes and determined to function as a unidirectional Na\(^+\)-dependent secondary transporter (2). This system was chosen for study partly due to the natural fusion of the membrane domains such that the reconstituted systems are effectively bipartite rather than tripartite. The structure of the SBP for this transporter, SiaP, is also known, which has been solved in ligand-free and ligand-bound forms (6, 11). The transporter is biologically important in *H. influenzae* as sialic acid is an important host-derived molecule that is needed for persistence in the host, and loss of the transporter causes defects in colonization (15, 17, 18).

More recently, an important role for sialic acid transport and catabolism has been identified in the human pathogen *Vibrio cholerae*, as the sialic acid catabolic gene *nanA*, which encodes a cytoplasmic enzyme that catalyzes the first step in sialic acid catabolism, has been shown to be important for colonization (15, 17, 18).

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Although we have demonstrated previously the VcSiaP (VC1779) has identical ligand-binding properties to HiSiaP (4), we demonstrate here using purified and reconstituted proteins that VC1777–1779 encodes a functional sialic acid transporter with bioenergetic properties identical to the HiSiaPQM system. Significantly, the VcSiaPQM system differs from the HiSiaPQM system as Q and M membrane subunits are encoded by separate genes, and as such this is the first *in vitro* characterization of a truly tripartite TRAP transporter. We use the VcSiaPQM system to demonstrate
that the Q and M subunits form a tight stoichiometric complex in the membrane and present the first biochemical data suggesting that all three subunits are essential for transporter function.

**EXPERIMENTAL PROCEDURES**

**Expression Vector Construction**—All proteins used in this work were overexpressed using a P_BAD expression system that allows addition of decahistidine tags at either the N or C termini of the recombinant protein. The details of expression vector construction for the expression HiSiaQM have been described previously (4). N- and C-terminally tagged versions of VcSiaP (VC1779), VcSiaQ (VC1778), and VcSiaM (VC1777) were produced by PCR amplification of the genes using primer pairs (see supplemental Table 1) for primer sequences. The expression vector used to co-express the dicistronic VcsiaQM genes was produced by PCR amplification of the region containing both open reading frames with the native intergenic region (supplemental Table 1). All PCR products were cloned into both appropriate vectors using ligation-independent cloning to generate N- and C-terminally decahistidine-tagged constructs.

**Protein Expression**—The C-terminally tagged VcSiaQ, N-terminally tagged VcSiaM, and N-terminally tagged VcSiaQM were expressed essentially as described for N-terminally tagged HiSiaQM (4). Briefly, *Escherichia coli* MC1061 (pBADcVcSiaQ), MC1061 (pBADnVcSiaM), and MC1061 (pBADnVcSiaQM) were grown at 37 °C in Luria-Bertani broth (LB), supplemented with 0.5% (v/v) glycerol and 100 μg/ml ampicillin, until an A_600 of 1 was reached. Each culture was subsequently induced by addition of 50 mg/liter L-arabinose and grown for a further 2 h. Cells were harvested by centrifugation at 4500 g for 12 min at 4 °C and resuspended in 50 mM potassium phosphate, pH 7, and snap-frozen. The resultant supernatant was incubated for 1 h at 4 °C with Ni^{2+}-nitrilotriacetic acid superflow resin (Qiagen, 0.3 ml, 1 ml of membrane vesicles) pre-equilibrated with WB supplemented with 10 mM imidazole and 0.05% DDM (w/v). The resin was distributed between disposable polystyrene columns (Pierce) and washed with 10 column volumes of WB supplemented with 40 mM imidazole and 0.15% DDM (w/v). The resin was then washed with 10 column volumes of WB supplemented with 40 mM imidazole and 0.15% n-decyl-β-D-maltoside (w/v). Protein was eluted by addition of WB supplemented with 500 mM imidazole and 0.15% n-decyl-β-D-maltoside (w/v). 5 mM EDTA was added to the elution fractions. DDM concentrations were determined using the methods of Urbani and Warne (25).

**Membrane Reconstitution of VcSiaQM and NanT**—The liposomes used in the reconstitution were a 3:1 (w/w) ratio of purified *E. coli* lipids and egg 1-α-phosphatidylcholine (Avanti Polar Lipids) with a concentration of 20 mg of lipid/ml. Purification eluate containing 200 μg of VcSiaQM was diluted to 2 ml with 50 mM potassium phosphate, pH 7.8, 0.15% n-decyl-β-D-maltoside (w/v). The protein sample was mixed with 400 μl of liposomes and incubated on ice for 10 min. The protein/lipid mixture was rapidly diluted in 70 ml of 50 mM potassium phosphate, pH 7. Proteoliposomes were collected by centrifugation at 200,000 × g for 1.5 h at 4 °C. Proteoliposomes were resuspended in 50 mM potassium phosphate, pH 7, and snap-frozen. The proteoliposomes were then frozen and slowly thawed on ice five times.

**Preparation of Proteoliposomes and Transport Assays**—For use in the transport assays, proteoliposomes containing 400 μg of protein/ml were centrifuged for 20 min at 200,000 × g at 4 °C. The pellet of proteoliposomes was resuspended in lumenal buffer (100 mM potassium acetate, 20 mM potassium phosphate, pH 7, and 2 mM MgSO_4_2_) unless otherwise stated) and extruded through a 400-nm polycarbonate filter (Avestin Inc.)
The extruded proteoliposomes were centrifuged for 20 min at 200,000 × g at 15 °C, and the resultant proteoliposomes pellet was resuspended in luminal buffer to a concentration of 4 μg/μl protein.

For all transport assays performed, the proteoliposome suspension was diluted 50-fold into the following buffers to form the required electrochemical gradients. An inwardly directed Na⁺ (ΔμNa⁺) gradient was formed by dilution into buffer containing 100 mM sodium acetate, 20 mM sodium PIPES, pH 7, and 2 mM MgSO₄. A Na⁺ gradient in combination with a membrane potential (ΔμNa⁺ + ΔΨ) was formed by dilution into buffer containing 100 mM sodium acetate, 20 mM sodium PIPES, pH 7, 2 mM MgSO₄, and 2 μM valinomycin. A Na⁺ gradient in combination with a pH gradient (ΔμNa⁺ + ΔpH) was formed by dilution into buffer containing 120 mM sodium acetate, 2 mM MgSO₄, and 2 mM valinomycin.

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SiaM into proteoliposomes, using a dilution method (28), as used previously for HiSiaQM (4). To determine the energetic requirements of transport by VcSiaPQM, different electrochemical gradients across the membrane were systematically varied, and the resulting effects on $[^{14}C]$Neu5Ac uptake in the presence of VcSiaP were monitored. As was observed for HiSiaPQM, the application of an inwardly directed sodium gradient ($\Delta \mu \text{Na}^+$) to VcSiaQM-containing proteoliposomes resulted in rapid uptake of $[^{14}C]$Neu5Ac into the lumen of the proteoliposomes where it accumulated ~11-fold over 6 min (Fig. 3A). The presence of either a pH gradient alone ($\Delta pH$, increasing internal pH from 7 to 8.7) or a membrane potential alone ($\Delta \Psi$, –40 mV) was insufficient to support transport by VcSiaPQM (Fig. 3A). In the presence of $\Delta \mu \text{Na}^+$, the addition of $\Delta pH$ did not alter the rate of uptake, indicating no direct role for protons in the transport activity of VcSiaPQM. The highest transport activity was observed in the presence of $\Delta \mu \text{Na}^+$ in combination with a $\Delta \Psi$ (Fig. 3A). This stimulation of transport activity by inclusion of a $\Delta \Psi$ suggests that transport of VcSiaPQM is an electrogenic process. As Neu5Ac has a single negative charge at physiological pH ($pK_a = 2.6$), this implies that Neu5Ac is co-transported with at least two Na$^+$ ions. These data demonstrate the VcSiaPQM system is a sialic acid transporter, which has similar properties to the known bipartite counterpart from *H. influenzae* (2).

**VcSiaPQM Functions as a Unidirectional Secondary Transporter**—Characterization of HiSiaPQM revealed that this TRAP transporter has unusual properties that distinguish it from other secondary carriers, in that it appears to function unidirectionally (4). To assess whether this interesting property was also a feature of a second TRAP transporter, we repeated these experiments with VcSiaPQM and also included purified and reconstituted NanT, the *E. coli* K12 sialic acid transporter from the major facilitator superfamily as a control (29–31). Using membrane-reconstituted protein, we demonstrated that NanT is a $\Delta pH$-driven transporter and that $\Delta \mu \text{Na}^+$ plays no part in Neu5Ac transport (supplemental Fig. 2).

The directionality of VcSiaPQM transport was investigated using substrate exchange and substrate counterflow experiments *in vitro*. In the substrate exchange experiments, $[^{14}C]$Neu5Ac was accumulated in VcSiaQM or NanT-containing proteoliposomes energized by a $\Delta \mu \text{Na}^+ + \Delta \Psi$ or $\Delta pH$, respectively. At 100 s, 1 mM unlabeled Neu5Ac (200-fold excess) was added, which resulted in rapid efflux of the preaccumulated $[^{14}C]$Neu5Ac from NanT-containing proteoliposomes, as expected for a conventional secondary transporter (Fig. 3B). Addition of excess unlabeled substrate to VcSiaPQM-containing proteoliposomes resulted in a cessation of transport activity but no efflux of preaccumulated $[^{14}C]$Neu5Ac (Fig. 3B), indicating that VcSiaPQM cannot perform this substrate exchange phenotype characteristic of secondary carriers. In both cases, the addition of distilled H$_2$O resulted in continued transport of $[^{14}C]$Neu5Ac into the proteoliposomes (Fig. 3B).

The abilities of VcSiaPQM and NanT to facilitate substrate counterflow activity were also assessed. This assay measures the ability of a transporter to exchange luminal unlabeled substrate present at a high concentration with external radiolabeled substrate present at a relatively low concentration (32, 33). This exchange of substrate is facilitated by the accessibility of the substrate-binding site alternating from one side of the membrane to the other. This results in rapid but transient accumulation of the radiolabeled substrate followed by apparent efflux as the concentration on both sides of the membrane equilibrates. VcSiaPQM- and NanT-containing proteoliposomes were loaded with 1 mM unlabeled Neu5Ac by extrusion and diluted into a solution containing 5 $\mu M$ $[^{14}C]$Neu5Ac. NanT-containing proteoliposomes had distinct counterflow activity under these conditions, whereas VcSiaPQM-containing proteoliposomes did not (Fig. 3C). Under these experimental conditions, these findings suggest that like HiSiaQM, VcSiaPQM is a unidirectional secondary transporter. This is unlike the control MFS protein NanT, which has the ability to transport Neu5Ac in a reversible manner.
At 100 s (Fig. 4A), we could not detect uptake for the separately expressed and mixed SiaQM (Fig. 4A). Also, no uptake was observed when VcSiaQ or VcSiaM was reconstituted alone either in the presence of absence of VcSiaP, which suggests that they also are not functional individually (Fig. 4A) (data not shown). We confirmed the proteins present in the proteoliposomes on silver-stained SDS-polyacrylamide gels (Fig. 4B) and also by Western blot (data not shown), but we cannot rule out that the His tags used for purification of these proteins might interfere with their function. As mentioned previously, the expression of VcSiaQ and VcSiaM alone reduced their stability and also resulted in diminished reconstitution efficacy (Fig. 4B), although there is clearly a protein present in each of the different conditions. We note that the SiaM from the co-expressed protein migrates slightly further than the singly expressed sample, likely as the single expressed protein has an additional N-terminal decahistidine tag. These data suggest that none of the three subunits expressed alone can catalyze transport, consistent with all the subunits being essential for TRAP transporter function and that co-expression of VcSiaQ and VcSiaM is essential for function.

**Failure of Separately Expressed Membrane Domains to Form a Functional TRAP Transporter**—Although the essential role of the SBP for TRAP transporter function is well established both in vivo and in vitro (2, 3, 15), the essential role of both membrane subunits has only been demonstrated genetically (3, 15). Using the tripartite VcSiaPQM system, we sought to determine the SBP for TRAP transporter function is well established both in vivo and in vitro (2, 3, 15), the essential role of both membrane subunits has only been demonstrated genetically (3, 15). Using the tripartite VcSiaPQM system, we sought to determine whether each individual subunit of the transporter was necessary and/or sufficient to catalyze transport.

Transport of [14C]Neu5Ac into proteoliposomes prepared to produce a $\Delta \mu_{\text{Na}^+} + \Delta \Psi$ was assessed using a range of differently prepared and reconstituted SiaPQM subunits. First, we compared SiaQM co-expressed and reconstituted to SiaQ and SiaM expressed and purified separately, mixed, and reconstituted together. Assays were performed in the presence of VcSiaP. Although we could measure uptake of [14C]Neu5Ac into proteoliposome containing co-expressed SiaQM (Fig. 4A), we could not detect uptake for the separately expressed and mixed SiaQM (Fig. 4A). Also, no uptake was observed when VcSiaQ or VcSiaM was reconstituted alone either in the presence of absence of VcSiaP, which suggests that they also are not functional individually (Fig. 4A) (data not shown). We confirmed the proteins present in the proteoliposomes on silver-stained SDS-polyacrylamide gels (Fig. 4B) and also by Western blot (data not shown), but we cannot rule out that the His tags used for purification of these proteins might interfere with their function. As mentioned previously, the expression of VcSiaQ and VcSiaM alone reduced their stability and also resulted in diminished reconstitution efficacy (Fig. 4B), although there is clearly a protein present in each of the different conditions. We note that the SiaM from the co-expressed protein migrates slightly further than the singly expressed sample, likely as the single expressed protein has an additional N-terminal decahistidine tag. These data suggest that none of the three subunits expressed alone can catalyze transport, consistent with all the subunits being essential for TRAP transporter function and that co-expression of VcSiaQ and VcSiaM is essential for function.

**VcSiaQ and VcSiaM Form a Complex in Solution**—During purification of co-expressed SiaQM, it was clear that the two proteins were associated during IMAC (Fig. 2A). We analyzed this interaction further using size-exclusion chromatography, and here we included HiSiaQM as a control. The co-expressed IMAC-purified VcSiaQM eluted as a single large peak in the chromatogram indicating the presence of a single protein or protein complex (Fig. 5A). SDS-PAGE analysis of the samples across the peak revealed that, despite their large difference in molecular weight, VcSiaQ and VcSiaM shared an identical elution profile (Fig. 5B), indicating that the proteins are forming a complex. As a control, we also performed an identical procedure with HiSiaQM, the fused QM protein, which elutes slightly earlier than VcSiaQM due to the extra mass due to the presence of the linker helix (Fig. 5A) and on SDS-PAGE yields a single band that corresponds to the HiSiaQM protein (Fig. 5C).

To investigate further the nature of the interaction between the SiaQ and SiaM, we studied the naturally fused HiSiaQM and introduced a Factor Xa protease cleavage site into the solvent-exposed loop predicted to follow helix 5 (cutting at residue 200), producing the mutant HiSiaQMfax200. This mutant protein was produced with a similar yield to wild-type HiSiaQM and was stable in DDM as determined by SEC analysis (data not shown). After incubation at room temperature for 3 h in the presence or absence of Factor Xa (supplemental Methods), SEC was used to determine whether the two domains of HiSiaQMfax200, when no longer physically attached, would remain in complex. Although protein aggregation was apparent in both the presence and absence of Factor Xa, a peak in the SEC trace was observed for both samples at a retention volume of $\approx 11.9$ ml (supplemental Fig. 3A). SDS-PAGE analysis of the fractions across these peaks revealed that in the absence of Factor Xa, only full-length HiSiaQMfax200.
eluted (supplemental Fig. 2B), although in the presence of Factor Xa, HiSiaQMFxa200 was partially digested resulting in both cleaved and noncleaved protein in the sample (supplemental Fig. 3B). Significantly, the elution pattern of the cleaved protein was identical to the uncleaved, suggesting that the two polypeptides remained packed into a tight complex despite no longer being covalently linked.

Stoichiometry of SiaQ, SiaM, and SiaQM Fusions—Using the separately encoded SiaQ and SiaM subunits of the V. cholerae transporter, we wished to determine experimentally the stoichiometry of the two components in the purified transporter. For this, we first used SEC combined with SEC-MALLS. Individually, VcSiaQM and HiSiaQM were extracted from the membrane using DDM and purified using IMAC, as described previously. The molecular weight of each protein-DDM complex was assessed using SEC-MALLS, revealing masses of 186 ± 4 and 207 ± 7 kDa for VcSiaQM and HiSiaQM, respectively (Fig. 6A).

As a separate measure of the size of the protein-detergent complex, we used sedimentation equilibrium AUC experiments to corroborate the mass ranges obtained from SEC-MALLS. Unfortunately, VcSiaQM was too unstable over the 24-h period of the experiment to obtain a reliable molecular weight from AUC; however, the HiSiaQM-DDM complex was found to be 194.4 kDa using AUC, which is in good agreement with the SEC-MALLS data (Fig. 6, B and C).

To determine the mass of the detergent component of the complex, we used the colorimetric assay that measures the maltoside components of the DDM (Fig. 6C) (25). For VcSiaQM, the DDM assay revealed that there were 219/1100663 DDM molecules per 1:1 complex of VcSiaQM, giving a detergent mass component of 112/1100632 kDa. Subtracting this from the mass of the complex from SEC-MALLS leaves a mass of 74 kDa, which is only consistent with a single molecule of SiaQ and a single molecule of SiaM in the complex, which would have a mass of 67 kDa. Similarly, for HiSiaQM there were 235/1100693 DDM molecules in the micelle giving a mass contribution of 120/1100647 kDa. Subtracting these values from the masses of the whole complex gives a mass for HiSiaQM of around 87 kDa, which is only consistent with the protein being a monomer in the detergent micelle as its predicted mass of 69.9 kDa. Although there is considerable variation in the DDM assay, the masses suggest a likely 1:1 stoichiometry of the SiaQ and SiaM subunits in VcSiaQM as seen in the naturally fused HiSiaQM.

**DISCUSSION**

In this work, we have described the *in vitro* characterization of the sialic acid-specific TRAP transporter, VcSiaPQM, from *V. cholerae*. This TRAP transporter is of particular interest as it is a tripartite orthologue of the bipartite *H. influenzae* SiaPQM. The *in vitro* characterization of a tripartite TRAP transporter...
FIGURE 6. Molecular masses of HiSiaQM and VcSiaQM determined by AUC and SEC-MALLS. A, SEC-MALLS trace of VcSiaQM (black lines) and HiSiaQM (gray lines) showing the relative absorbance at $A_{280}$, trace normalized to 1 (thin lines) and the molar masses of the species present in the samples (thick lines). B, representative dataset from the sedimentation equilibrium experiment performed on HiSiaQM using a Beckman Optima XL/1 analytical ultracentrifuge at 20 °C. C, table summarizing the data obtained for the masses of VcSiaQM and HiSiaQM using AUC, SEC-MALLS, and the colorimetric maltoside assay.
SiaQM Forms a Stable Heterodimer


to offer a number of advantages over bipartite systems, most prominently, the ability to express each protein separately to determine their individual characteristics and contributions. This is particularly important as the functions of the two integral membrane proteins have not been explicitly demonstrated.

The catabolism of sialic acid transport in *V. cholerae* is known to be important, and a strain lacking the first catabolic gene *nanA* has a defect in early colonization of the mouse intestine (19). The *siaPQM* genes (called *dcptPQW* in Ref. 19) are encoded within the *Vibrio* pathogenicity island 2 (VPI-2), which is present in strains of *V. cholerae* that can cause epidemic cholera outbreaks. The *siaP* gene is specifically induced by sialic acid as are related genes in the VPI-2 that also include the catabolic genes for sialic acid (19), and there is evidence that these genes are expressed in vivo (34). There is a strong correlation between the ability to grow on sialic acid and the presence of VPI-2 (19), which suggests that SiaPQM is the sole sialic acid transport system in this bacterium. Although we have previously demonstrated that VcSiaP can bind sialic acid (4), this is the first characterization of VcSiaPQM as a functional sialic acid transporter.

Of note, the orthologous transporter genes in the related marine pathogen *Vibrio vulnificus* ATCC 29307 are also part of a pathogenicity island, and the adjacent catabolic gene, *nanA*, is important for virulence in a mouse model of infection (35), and it is probable that these genes encode a functional sialic acid transporter in this bacterium.

Biochemically, we found the VcSiaPQM is indistinguishable from HiSiaPQM, which confirmed that TRAP transporters can be Na⁺−dependent and that they have unique properties relating to their directionality that are conferred by the presence of the SBP. The only small difference we observed was that in HiSiaPQM transport decreased about 2-fold when a ΔpH was added in addition to ΔμNa⁺ (4) This was not seen with VcSiaPQM (Fig. 3A), which supports our previous interpretation that this effect was due to the added ΔpH affecting the protein conformation of HiSiaQM rather than being required for the transport cycle per se. Presumably differences in the cytoplasmic face of VcSiaQM versus HiSiaQM make the latter more susceptible to pH changes. The Na⁺−dependence we observed fits well with our previous observation of a propensity for TRAP transporters to be used preferentially by marine-dwelling bacteria (14), and this is also not surprising for *V. cholerae*, which can live in sea water where the salt concentration is ~600 mM. The question of whether all TRAP transporters are Na⁺−dependent will require characterization of further, more diverse, members of the TRAP transporter family. This is also true for our repeated observation of the unidirectional movement of substrate, as judged from the cold chase assay and substrate counterflow assay. Importantly, in this study we included the NanT protein from *E. coli* as a control, which we show here for the first time in vitro is a sialic acid transporter that has typical properties of a major facilitator superfamily protein.

The formation of a complex between the small and large subunits was a primary reason to study the VcSiaPQM transporter, and indeed tagging with the small subunit resulted in co-purification of VcSiaM. This complex was stable and could also be observed on subsequent size-exclusion chromatography. The formation of the complex between the two membrane domains was already suggested by the genetic fusion of the two genes seen in the *H. influenzae* system, but these data demonstrated biochemically that they form a tight complex. By using the HiSiaQM protein, we were able to split the protein after expression and purification and found that the split SiaQ and SiaM still formed a stable complex. The stabilization of the individual proteins was also observed during our initial expression trials where the individually expressed proteins did not accumulate to high levels, whereas the co-expressed proteins did. The failure of the separately expressed and mixed VcSiaQ and VcSiaM to form a function transporter in the presence of VcSiaP suggests that the proteins need to fold at the same time in the membrane and have an intimate interaction that cannot be formed if they have folded separately; however, we cannot rule out here that the His tag on the separately expressed subunits might interfere with complex formation.

It is worth noting that the levels of separately expressed and purified VcSiaM were greater than those for VcSiaQ, which perhaps relates to the 12-transmembrane subunits having ancestral function as a stand-alone transporter and hence being more stable. We have also observed similar increases in stability and yield during work on the *E. coli* TRAP transporter YiaMNO, when the yield of YiaN (the large subunit) was greatly increased when co-expressed with YiaM (the small subunit). These biochemical data suggest that the small subunit is an important final component of the transporter and that complex formation directly after translation is likely important for the function of the SiaQM complex as separately purified and mixed proteins are not active.

The determination of the molecular weight of the purified protein complex, accounting for the amount of bound detergent in the micelle, yields a mass that was consistent with a 1:1 stoichiometry for the two subunits, which is what is likely occurring in the TRAP transporters where the membrane domains are naturally fused. The relative stoichiometry of the SBP with the membrane domains is assumed to be 1:1:1, but this has not been proven formally. Interestingly, we have recently observed novel genetic fusions between the large membrane subunit and the SBP, with the SBP being C-terminal to the large membrane subunit. This genetic organization is seen in a limited number of species of *Acidaminococcus* and *Ruminococcus* but are clearly suggestive of a 1:1 stoichiometry of the large membrane domain to the SBP. Hence, our current working models of TRAP transporters assume a 1:1:1 stoichiometry of the three subunits in the active transporter, although the SBP is present in the periplasm at a large excess over the membrane domains in Gram-negative bacteria.

It is also interesting, given this recent observation of fusion between the equivalents of SiaM and SiaP, that this might indicate that SiaM is the site of interaction with SiaP, and these two subunits might be all that are needed for function. However, in our experiments we have never been able to observe transporter function unless all three subunits are present, and in fact only if SiaQMs have been co-expressed, presumably forming a

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3 C. Mulligan and G. H. Thomas, unpublished data.
4 R. Dixon and G. Thomas, unpublished data.
Acknowledgment—We thank Rosie Dixon for computational analysis and that all three components of the transporter are required biochemically that the membrane subunits form a 1:1 complex with ectoine-A comparison with other TRAP-T-binding proteins. J. Mol. Biol. 389, 58–73

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