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Structural and mechanistic analysis of a tripartite ATP-independent periplasmic TRAP transporter

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Tripartite ATP-independent periplasmic (TRAP) transporters are found widely in bacteria and archaeb and consist of three structural domains, a soluble substrate-binding protein (P-domain), and two transmembrane domains (Q- and M-domains). HiSiaPQM and its homologs are TRAP transporters for sialic acid and are essential for host colonization by pathogenic bacteria. Here, we reconstitute HiSiaQM into lipid nanodiscs and use cryo-EM to reveal the structure of a TRAP transporter. It is composed of 16 transmembrane helices that are unexpectedly structurally related to multimeric elevator-type transporters. The idiosyncratic Q-domain of TRAP transporters enables the formation of a monomeric elevator architecture. A model of the tripartite PQM complex is experimentally validated and reveals the coupling of the substrate-binding protein to the transporter domains. We use single-molecule total internal reflection fluorescence (TIRF) microscopy in solid-supported lipid bilayers and surface plasmon resonance to study the formation of the tripartite complex and to investigate the impact of interface mutants. Furthermore, we characterize high-affinity single variable domains on heavy chain (VHH) antibodies that bind to the periplasmic side of HiSiaQM and inhibit sialic acid uptake, providing insight into how TRAP transporter function might be inhibited in vivo.
Tripartite ATP-independent periplasmic (TRAP) transporters represent a structural- and functional mix of the well-studied ATP-binding cassette (ABC) transporters and secondary active transporters, by functioning as substrate-binding protein (SBP) dependent secondary transporters. They are widespread in bacteria and archaea, especially in marine environments, but absent in eukaryotic organisms. TRAP transporters, together with ABC importers and tripartite tricarboxylate transporters (TTT), define the three classes of SBP-dependent transporters. In addition to a high-affinity SBP (also named the P-domain for TRAP transporters) that freely roam the periplasm in Gram-negative bacteria, TRAP transporters consist of a smaller- (Q) and a larger- (M) membrane domain. The latter two domains are either fused into a single polypeptide chain or smaller. The latter two domains represent a structural- and functional mix of the well-studied ABC transporters and TTTs, which operate via an elevator mechanism.

Of the three classes of SBP-dependent transporters, high-resolution structures for all domains are only available for ABC importers. TRAP transporters and TTTs have so far proved recalcitrant to experimental attempts at elucidating their structures. To date, only structures of the soluble P-domains, mainly from the family of DctP-like SBPs, have been determined. Features of the SBPs, such as the role of a conserved arginine residue for high-affinity substrate binding and specificity, and the conformational rearrangement of the protein upon substrate binding have been characterized in detail.

In this work, we determine the structure of the 70 kDa membrane domain of the HiSiaQM TRAP transporter in lipid nanodiscs with cryo-EM. An essential step for the successful 3D-reconstruction of the HiSiaQM structure is essential for virulence and host colonization, and could provide novel targets for the development of antimicrobials, for which the World Health Organization has identified ampicillin-resistant H. influenzae as a priority pathogen.

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TRAP transporters are monomeric elevators. The closest structurally described homolog of the QM-domains is the dimeric VcINDY membrane transporter (SU19), which operates via an elevator mechanism, now known to be employed by a diverse set of transporters. The superposition of a VcINDY dimer onto our HiSiaQM structure illustrates that the M-domain fits to one VcINDY subunit and that the Q-domain of HiSiaQM structurally mimics the oligomerization domain of the second polypeptide.
chain of the VcINDY dimer (Fig. 2a). The four helices of the Q-domain are, however, significantly longer than their structural counterparts in VcINDY (Fig. 2b). The superposition also suggests that the substrate and co-transported sodium ions are likely coordinated in the area surrounding the tips of HP1 and HP2 (Fig. 2c). Note that our in-lipid structure and its interpretation has since been confirmed by a cryo-EM structure of the sialic acid TRAP transporter PpSiaQM from *Photobacterium profundum* that was determined in an amphiphile environment.

The internal pseudo-symmetry of the elevator fold allows the construction of the outward open (Co) state of the transporter by “repeat-swap modeling”. This procedure has for instance been used to build an inward-facing model of the GltPh transporter and of the outward open state of the VcINDY transporter. Using this model as a basis, we constructed the outward open state of the HiSiaQM transporter by structural alignment of the elevator and stator domains (Fig. 2d). Notably, the procedure did not produce any clashes in our model and
agrees well with the predicted tripartite complex described below. The loops connecting elevator and stator domains were adjusted manually using the geometry regularization feature in Coot49 (Supplementary Data 1). A structural alignment of our in-lipid cryo-EM structure with the AlphaFold model results in an r.m.s.d. of 2.1 Å between 605 C-alpha atoms. Restricting the alignment to the Q-domain results in a smaller r.m.s.d. of only 1.2 Å between 136 C-alpha atoms. The differences between the two structures can be best described as a concerted movement of the elevator domain with respect to the stator domain with the elevator assuming a slightly more "downwards" conformation in our experimental structure vs. the computational prediction (Supplementary Fig. 8).

High-affinity VHHs inhibit sialic acid transport. An alpaca was immunized with DDM solubilized HiSiaQM, and nine distinct VHHs (VHHQM1–9) were isolated. Using surface plasmon resonance (SPR) single-cycle kinetic experiments, high affinities in the nano- to picomolar range were confirmed for seven VHHs (Fig. 3a, Supplementary Fig. 9, Supplementary Table 2). Additionally, an epitope binning experiment Supplementary Table 2. The affinities depended on the immobilization site of the TRAP transporter (E235C-biotin or K273C-biotin), which can be explained by our structure, since the biotinylated residues are located on different sides of the transporter (Fig. 3a).

Depending on their binding epitope, VHHs can influence the function of their target proteins, and examples of transport-inhibiting VHHs are known, also for elevator-type transporters50–53. To test this possibility for our HiSiaQM VHHs, we used a modified in vivo transport assay for TRAP transporters based on E. coli strain SEVY38,54. In this strain, the native sialic acid transporter NanT was functionally replaced by HiSiaPQM, enabling it to grow in M9 minimal medium with sialic acid as the sole carbon source. We transformed different clones of the strain with our nine VHHs, either with or without a periplasmic export signal. In addition to the HiSiaQM VHHs, we included a camelid VHH for HiSiaP (VHHP1) with an affinity of 0.89 µM to the SBP (Supplementary Fig. 10), since inhibition of SBPs by VHHs was described before52. Growth curves of the different strains were recorded to investigate the effect of the VHHs on cell growth (Fig. 3b). No significant inhibition of cell growth was observed when the HiSiaQM-specific VHHs were expressed without the signal sequence and thus remained in the cytosol. In contrast, when HiSiaQM-specific VHHs were exported to the periplasm, bacterial growth was strongly inhibited by VHHQM3, 4, 6, 7, and 9. Cultures with VHHQM1, 8, and VHHP1 did show normal cell growth, irrespective of their cellular localization. As a control, we
is no clear correlation between the individual expression level and inhibitory effects, we cannot exclude that the level of inhibition of the individual VHHs is to an extent biased by their expression level (Supplementary Fig. 9d–g).

Since Mb3 was constructed from VHHQ3M3, this information experimentally identifies the TM orientation of our cryo-EM structure. The molecular interaction between VHHQ3M3 and the QM-domains is resolved in our structure (in the form of Mb3) and explains the inhibitory effect of this particular VHH (Fig. 3c), because it binds to both the elevator and stator domains of the transporter by forming multiple hydrogen bonds and a prominent hydrophobic interaction between W512 of Mb3 (residue 106 in VHHQ3M3) and P112Q, F111Q on the periplasmic loop Q3–Q4 of HiSiaQM (Supplementary Fig. S5). These observations are further supported by the constructed tripartite model and the single-molecule data presented below.

**Constructing a model of the tripartite transport complex.** Encouraged by the close resemblance of the HiSiaQM AlphaFold model to our experimental structure (Supplementary Fig. 8), we employed the algorithm to predict the tripartite complex between HiSiaP and HiSiaQM by fusing the two proteins into a single chain. The biological rationale behind this approach was the observation that in rare cases, natural M-P fusions do occur in nature, for example, a TRAP transporter from *Acidaminococcus intestini* (UniProt ID: G4Q5D7) (Supplementary Fig. 11). As fusion peptides for HiSiaPQWM, we used both the native variant from the *A. intestini* TRAP transporter and spacer residues from AlphaFold (U) (Supplementary Fig. 11). Both modelled HiSiaPQWM variants, as well as the AlphaFold model of the natural M-P fusion transporter from *A. intestini*, were very similar to each other and to the previously mentioned model by Ovchinnikov et al. 38. In all tripartite models, the SBP with its closed substrate-binding site (for HiSiaP most similar to 3B5022) is positioned in the same orientation on top of the periplasmic side of the membrane domains, which are in the inward-open conformation as found in our experimental structure (Fig. 4a and Supplementary Fig. 11, Supplementary Data 2). An analysis with the PISA server48 revealed that a combined surface area of ~1980 Å^2^ is buried by complex formation. Figure 4b shows the conservation of this P–QM interface across a large number of different TRAP transporters. In the tripartite HiSiaPQWM complex, the N-terminal lobe of HiSiaP is bound to the stator domain of

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**Fig. 3 Characterization of TRAP transporter-specific VHHs and inhibition of transport in vivo.** a A hierarchical clustering tree of nine HiSiaQM-specific VHHs, based on PBLAST e-values as the distance matrix for tree building. The binding affinities of the VHHs, determined from SPR experiments are given (x: no binding detected; nd: not determined, since no clear binding detected in size-exclusion chromatography). VHHs that bind to HiSiaQM mutually exclusively are grouped by yellow and violet boxes. The underlying data are described in detail in Supplementary Fig. 9. HiSiaQM was immobilized on the SPR chip in two different orientations as indicated. b Growth defect of cultures expressing different VHHs specific for either the QM-domains (VHHQM) or the P-domain (VHHP) in a sialic acid uptake assay. The VHHs were either localized in the cytosol or exported to the periplasm via a signal sequence, as indicated in the figure. An empty plasmid was used as a negative control. A VHH specific for a completely unrelated human protein (VHHX) was used as an additional control. The growth of each culture was measured after 17 h. Data are presented as mean values ± SD of *n* ≥ 3 independent experiments. c Interface of HiSiaQM and Mb3, which derives from VHHQ3M3. The color scheme is the same as in Fig. 1. Selected polar interactions and residues are highlighted. Source data are provided as a Source Data file.

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HiSiaQM, and the C-terminal lobe is bound to the elevator domain. This arrangement puts the substrate-binding cleft of HiSiaP directly on top of the presumed substrate-binding site of the transporter, as identified by superpositions with substrate-bound VcINDY (Supplementary Fig. 12). This suggests that the N-terminal lobe of the SBP stays fixed on the stator, while its C-terminal lobe can move in concert with the up-and-down movement of the elevator during a transport cycle. Indeed, superposing the N-terminal lobe of the open-state crystal structure of HiSiaP (2CEY) onto the stator domain in precisely the same manner puts its C-terminal lobe into a position that closely matches the elevator in its above-described outward open conformation (Fig. 4a). The relative orientation of the P-domain to the QM-domains in our models is compatible with TRAP SBPs that are known to form stable homodimers, for example, TakP from Rhodobacter sphaeroides (Supplementary Fig. 13)\textsuperscript{61,62}. The dimeric P-domains superpose such that the second monomers point upwards, not interfering with the interface of the tripartite P-QM complex.

**Validation of the tripartite model with an in vivo sialic acid uptake assay.** To validate the described models, we selected 31 residues in regions that we thought were important for the integrity and function of the TRAP transporter, such as the substrate-binding site of the P-domain (region I), the extended periplasmic loops of the Q-domain (region II), the P-QM interface (regions III and IV), or the assumed sialic acid- and Na\(^+\) binding sites at HP1 and HP2 (V) of the QM-domains (Fig. 5a) (detailed views of the mutation sites are shown in Supplementary Fig. 14 and the sequence conservation of TRAP transporters is shown in Supplementary Figs. 15 and 16). Highly conserved sites
in the periplasmic loops of the Q-domain (region II) were selected to test for a potential “scoop-loop” mechanism, as found in SBP-dependent ABC transporters. The effects of all mutants were analyzed in our SEVY3-based complementation assay.

We identified 10 mutants that severely or completely abolished growth and are structurally spread over all three transporter domains (Fig. 5a, b). Mutations of highly conserved residues R127Ap and R147Ap in the P-domain (region I, Fig. 5), which are known to be important for substrate binding and were previously described as a substrate-filter, showed comparably small effects in the growth assay. This observation is in accordance with previous data and crystal structures of substrate-bound R147P variants, where the arginine–carboxylate interaction is mimicked by water molecules. It seems that at high substrate concentrations (3.2 mM in our assay) this can be tolerated by the system. Minor effects were also observed for mutants of the periplasmic loops or the adjacent region of the P-domain (region II, Fig. 5).

Twelve mutants are located in region III (Fig. 5), which connects the N-terminal lobe of HiSiaP and the stator of HiSiaQM. Here, the most substantial effect on growth was observed with mutants D58Rp and S60Rp. Due to their larger size and reversed charge, the introduced side chains would likely interfere with a positively charged surface patch of QM, explaining their effect. This is corroborated by our finding that the conservative D58NP mutant did not show any effect on growth. Note that all P-domain mutants that failed to form a functional transporter in vivo, showed wildtype-like binding behavior towards sialic acid in ITC experiments, strongly indicating that the mutated residues are indeed essential for the tripartite complex formation and do not otherwise impair the function of the P-domain (Supplementary Fig. 17).

Ten of the tested mutants are located in region IV, formed by the C-terminal lobe of HiSiaP and the elevator domain of HiSiaQM. Here, the most substantial effect on growth was observed with mutants D58R of region III. While the glutamate sidechain can be accommodated structurally, the charge reversal appears to impact complex formation, albeit the effect on growth was not as strong as for the former two mutants that come from the "P-side" of the interface. Position W227R was selected to modify the interface indirectly by pushing TM helices 3 and 4a into a different position by introducing a large charged residue into the hydrophobic pocket occupied by W227M, F101Q, V230M, and F177M. Indeed, this also led to a substantial reduction in growth.

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Fig. 5 Validation of the tripartite P-QM complex. a Structural context of the mutations in the AlphaFold model of the tripartite complex. The P-domain is colored red and the QM-domains are color-coded as in Fig. 1. Red spheres indicate mutants that showed a significant effect in the growth assay and are labelled. White spheres represent the remaining mutants. b Growth defect after 17 h (compared to a wildtype culture and normalized to the negative control) of TRAP transporter mutants in a sialic acid uptake assay. The positions of the mutants with respect to the tripartite complex are indicated by labels and the color code. Data are presented as mean values ± SD of n ≥ 3 independent experiments. Source data are provided as a Source Data file.
HiSiaQM. In this region, the strongest effects were seen with mutants E172RP, R30EQ, S356YM, and E429RM. The variants are spread over a slightly larger area than the interface III mutants. E172RP and S356YM would again lead to severe steric clashes. The effect of the R30EQ mutant of the strongly conserved R30Q cannot be explained by steric hindrance in the C-state of the transporter. However, we noticed that this residue is in the salt-bridge distance with the other knock-out mutant E172RP in the C-state transporter model (Fig. 4). In addition, the highly conserved E429RM is located directly next to R30Q, and the strong growth defect of this mutant supports the high importance of the region for the interaction of the domains. A weaker but significant growth reduction was observed for N354YM. In our model, the asparagine forms hydrogen bonds with E172RP in the C-state of the transporter.

For the potential Na\(^+\)-binding site mutants (region V) two of three mutants (D304AM and D521AM) independently led to complete loss of transporter function, providing a structural explanation for the earlier finding that a sodium ion gradient is strictly needed for transport by HiSiaPQM. Since the sodium substrate-binding sites are spatially linked, the effect might also stem from an altered affinity of the transporter to sialic acid.

All mutants that completely inhibited growth in our uptake assay were expressed and purified. As mentioned above, the P-domain mutants had wild-type-like affinity for sialic acid and all QM-mutants behaved overall similar to the wildtype protein in size-exclusion chromatography experiments (Supplementary Fig. 18).

**Tracking the formation of the tripartite transport complex in vitro**

We employed single-molecule TIRF microscopy to visualize the tripartite transport complex in solid-supported DO/PC bilayers (SSBs\(^{44}\)), allowing us to directly study the impact of sialic acid and the above-described P–QM interface mutants on the complex formation (Fig. 6a).

The native QM-domains were reconstituted into SSBs and an AF-555-labelled VHHQM3 (Fig. 6a) was used to identify and localize the reconstituted transporter in the lipid bilayer (Fig. 6b, Supplementary Movie 1). The VHH was also used to optimize the experimental conditions, such as the number of QM-domains in the field of view of the microscope. No unspecific binding of VHHQM3 to the bilayer was detected in control experiments with empty SSBs (Fig. 6c).

Next, we incubated QM-SSBs with AF-647-labelled P-domains (Fig. 6a, final concentration of \(~100\) pM). Strikingly, we could clearly observe single P-domains appearing out of the bulk solution and localizing to defined points on the membrane (Fig. 6d, Supplementary Movie 2). This direct experimental observation of the formation of the full tripartite complex confirms a key step in the transport cycle.

To investigate the role of sialic acid in the P–QM interaction, we omitted the compound from the SSB TIRF experiment. To our surprise, there was only a \(~50\%\) reduction of P–QM binding events in the absence of sialic acid (Fig. 6e).

Both VHHQM3 and the P-domain bind to the same position on the periplasmic surface of the transporter (Fig. 6a). Hence, the VHH should block the P–QM interaction in the SSB experiments, as indicated by our in vivo experiments (Fig. 3). To test this in vitro, we preincubated the QM-SSB with unlabelled VHHQM3 and then added the AF-647-labelled P-domain. In this experiment, only a very small number of binding events could be tracked (Fig. 6f). The number of observed interactions was in fact comparable to a control experiment with empty SSBs, which also proved that the P-domain does not bind to the bilayer in an unspecific manner (Fig. 6g).

Finally, we investigated 9 different mutants which had a clear loss of function phenotype in the sialic acid uptake assay (Fig. 5). For the three P-domain mutants D58RP, S60RP, and E172RP, the number of binding events was drastically decreased to levels comparable to the experimental background for D58RP and E172RP (Fig. 6h, i) and a slightly higher number of events for S60RP (Fig. 6i). As mentioned above, all three mutants had a wild-type-like affinity towards sialic acid (\(~50\) nM) (Supplementary Fig. 17), which strengthen the hypothesis that the introduction of large, charged arginines disrupt the tripartite complex formation. Similar results were observed for the four QM-domain mutants R30EM, S356YM, E429RM, and R484EM, which are all located on the periplasmic site in the P-domain interacting region, and clearly showed no significant binding events compared to the experimental background (Fig. 6k–n, Supplementary Movies 2 and 3). Interestingly, the two mutants from the sodium binding site, which had no transport activity in the sialic acid uptake assay, showed a wild-type-like interaction with the P-domain (Fig. 6o, p).

The TIRF data above is supported by surface plasmon resonance (SPR) experiments where HiSiaQM (in DDM micelles) was immobilized on an SPR chip as described above in the VHH-binding studies. Figure 6r shows that the closed P-domain interacted with the immobilized transporter (5 mM sialic acid was added to the running buffer). The interaction was clearly weaker in the absence of sialic acid and could be blocked by saturating the immobilized QM-domain with VHHQM3 (Fig. 6r, s). Interestingly, the resulting sensograms and especially the fast dissociation of the analyte could not be satisfactorily fit with a 1:1 binding model (Fig. 6r). Hence, the true \(K_D\) is very likely higher (i.e. weaker) than the average \(K_D\) of \(~1\) \(\mu\)M that was determined from a set of four independent SPR experiments. The deviation from the 1:1 model indicates a complex binding behavior, which is maybe not surprising considering that at least two separate binding interfaces (stator/N-lobe and elevator/C-lobe) are involved, combined with the conformational flexibility of the participating molecules. Similar observations of complex binding events in transport mechanisms have been made with the BtuCD-BtuF ABC transporter\(^{65}\) or the GlpH elevator-type transporter\(^{66}\). Clearly, more detailed experiments are needed to fully understand the binding kinetics of the tripartite complex. However, taken together, the TIRF and SPR data support our observation from the complementation assay and our tripartite HiSiaPQM complex model.

**Discussion**

Our study reveals the unique structural architecture of TRAP transporters and provides important insights into their function. Based on the above-described structures, and in accordance with our biochemical-, microscopy- and in vivo data, as well as with previously published results, we suggest that TRAP transporters employ an elevator mechanism for substrate translocation. Strikingly, TRAP transporters are monomeric elevator-type transporters, expanding the landscape of transporter architecture known in biological systems. Our structural data indicate that the characteristic Q-domain of TRAP transporters, whose essential function has been a mystery for many years, is central to this architecture, by representing a structural mimic of the combined stator domains of multimeric elevators (Fig. 2). We hypothesize that a minimal size of the stator is needed to anchor the domain in the membrane and to support the up-and-down movement of the elevator domain. The extended length of the Q-domain helices in HiSiaQM compared to their structural counterparts in VcINDY might further stabilize this asymmetric design. While the bile acid transporter ABST is also monomeric,
it uses a mixture of the moving barrier and elevator mechanisms and a structurally different elevator mechanism has also been postulated for the CcdA transporter. The SiaQM structure uniquely represents a monomeric version of a classical elevator.

Interestingly, the likely substrate-binding site formed by the QM-domains does not contain any conserved positively charged residues, such as in the SBP, where Arg147 determines the specificity for its negatively charged monocarboxylate substrate. In contrast, the only other structurally characterized sialic acid transporter SiaT from *Proteus mirabilis* (5NVA), a structurally unrelated sodium-solute symporter (SSS) transporter, does have a conserved arginine in its translocation channel, and this residue interacts with the substrate during transport. Since SiaT does not use an SBP, this supports a model where the substrate selectivity of TRAP transporters is "outsourced" from the membrane domains to the SBP, explaining why the latter is strictly needed for TRAP transporter-mediated transport.

We used AlphaFold to generate a structural model of the tripartite PQM transporter complex. The resulting model elegantly rationalizes the results of our in vivo mutagenesis study and is in very good agreement with our single-molecule tracking microscopy data (Figs. 5 and 6). It is further supported by our finding that VHHs, which bind to the periplasmic side of the QM-domains efficiently block transport activity (Fig. 3). The observation that the crystal structures of the closed- and open states of HiSiaP comfortably fit our inward open structure of HiSiaQM and a model of the outward open transporter, respectively, indicates that not only the interfaces of these two proteins but also their conformational flexibility have been matched by evolution. Clearly, this has to be proved by experimental structures of the P-QM complex in the future.

The information presented above allows us to amend the working hypothesis for TRAP transporter-mediated transport that was last updated by Mulligan et al. in 2011. As known from several previous studies, the SBP binds tightly (KD = 20–300 nM depending on the SBP) to its substrate and switches from an opened- to a closed conformation (Fig. 7, step 1). As the P-domain only closes when a substrate is bound, empty transport cycles are prevented. Both, the TIRF and SPR data show that the presence or absence of sialic acid and, accordingly the conformational state of the P-domain, strongly impacts the formation of the tripartite complex (Fig. 6, Supplementary Fig. 19). The TIRF experiments in lipid bilayers demonstrate that the opened

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**Fig. 6 Single-molecule interaction studies of HiSiaQM and HiSiaP on solid-supported bilayers (SSBs).**

- **a** HiSiaQM variants were integrated into DOPC (1,2-Dioleoyl-sn-glycerol-3-phosphocholine) SSBs and their interaction with single AF-647-labelled P-domain variants or AF-555-labelled VHHQM3 was observed by TIRF microscopy.
- **b** SSB containing HiSiaQM visualised with AF-555 labelled VHHQM3. As a control, VHHQM3-AF-555 was added to an SSB in the absence of HiSiaQM. No unspecific binding was observed.
- **d-p** Top row: first frame of an image sequence of a typical set of data. Bottom row: maximum intensity projections of the respective image sequence. The conditions are indicated below each panel.
- **q** Normalised interactions per second of P-domain variants with a SSB containing HiSiaQM variants. Unless otherwise stated, the P-domains were pre-incubated with 10 mM sialic acid (Neu5Ac). Statistical significance of the P-mutants and controls was assessed by applying a two-sided unpaired Student’s t-test with a 95% confidence interval (*p < 0.01, **p < 0.001*).

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The scale bars equal 3 μm. Source data are provided as a Source Data file.
P-domain (i.e., in the absence of sialic acid) does interact with the transporter, albeit to a significantly reduced extent compared to the substrate-bound P-domain. Considering the architecture of the tripartite AlphaFold model (Fig. 4), this result may be explained by weak interactions of either the N- or C-lobe of the open P-domain with either the stator or elevator domain of the transporter. Since the C-lobe has much higher sequence conservation (Fig. 4b), one might speculate that this part of the open P-domain interacts with the transporter. Our observation explains a finding by Mulligan et al. that the transporter can be forced to “run backwards” with a steep but “inverted” and thus non-physiological siac acid gradient. Clearly, this requires a functional interaction of the apo P-domain with the transporter, consistent with our data.

In the next step, the substrate-bound SBP binds to the transporter in its inward open resting state, the structure of which has been determined in this work (Fig. 7, step 2). Our microscopy and SPR data are in agreement that the sodium gradient, which is consistent with our data.

Functional interaction of the apo P-domain with the transporter, the QM-domains8 (Fig. 7, steps 3, 4). Dissociation of the open movement is presumably accompanied by the binding of two ions, this upwards movement is presumably accompanied by the binding of two such ions and the relaying of the substrate from the P-domain to the QM-domains8 (Fig. 7, steps 3, 4). Dissociation of the open SBP presumably allows the elevator domain to “fall” back into the inward open state (Fig. 7, step 5). The substrate and the Na+ ions then diffuse into the cytosol, resetting the transport cycle (Fig. 7, step 6).

Since TRAP transporters are absent in humans and play an essential role in the virulence of important pathogens, such as H. influenzae or V. cholerae, they are attractive targets for the development of new antibiotics. Our finding that TRAP transporters can be inhibited with several different VHHS opens a road in this direction and can be used in following studies, for example in the development of small compounds that mimic the CDrs of the VHHS and thus lead to a similar effect on transport, or by using them as tools in high throughput displacement assays.

Methods
Cloning, expression, and purification of HiSiaP. The sbp was previously cloned into a pBADHiSuc/TEV vector which fuses the HiSiaP protein to an N-terminal His6-tag and TEV-cleavage site. HiSiaP mutants were produced with site-directed mutagenesis71 (Supplementary Table 4). The protein was expressed and purified as described in Peter et al.21. E. coli BL21 cells were used for expression and a 50 mL LB-medium culture was incubated overnight at 37 °C. On the next day, the culture was stored at 4 °C overnight and used to inoculate 100 mL M9 minimal medium to avoid contamination of HiSiaP with sialic acid during expression. For this, the culture was centrifuged and resuspended in 100 mL M9 minimal medium. After repeating the wash step once more, 20 ml of the resuspension was used to inoculate 11 M9 minimal medium. The cells were cultured overnight for 14–16 h until OD600 of 0.6–1.0, expression was induced with 0.3 mg/ml arabinose and the cells were harvested after 3 h at 27 °C. The pellet was stored at −80 °C or directly used for purification. For purification, the pellet was resuspended in 5 times excess of 50 mM Tris (pH 80 °C) and incubated with a sonicator on ice (40% amplitude, 5 min, pulses 10 s on-5 s off). After 20 min, centrifugation with 75,000 × g for 20 min and 10 °C. The cell pellets were stored at −80 °C until further use.

Expression and purification of MSP1D1-H5. The msps were previously cloned into a pET28a vector which fused an N-terminal His6-tag and TEV-cleavage site to the MSP1 protein. To decrease the size of the nanodiscs, helix v (residues 68–87) of the MSP1 domain was deleted (Supplementary Table 4), yielding construct MSP1D1-H5. The expression and purification were based on ref. 22. In brief, the plasmid was transformed into E. coli BL21 cells and a preculture of 120 mL LB-medium (50 µg/ml kanamycin) was grown for 4–6 h at 37 °C and 140 rpm until an OD600 of 0.6–0.8. The culture was stored at 4 °C overnight and used to inoculate 61 L LB-medium (50 µg/ml kanamycin) the next day. The culture was incubated at 37 °C and 140 rpm until an OD600 of 2.5–3.0 and expression was induced with a final concentration of 1 mM IPTG in the culture. After expression of 1 h at 37 °C.

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Fig. 7 Proposed mechanism of TRAP transporters. The schematic shows the components of the transport reaction in the different steps (numbers in circles) of the proposed transport mechanism, which are explained in the main text. The conformational state of the QM domains is annotated: Ci—inward open; Co—inward open; Ci—inward open, substrate bound; Ci—inward open, substrate bound. Neu5Ac—sialic acid (N-acetylneuraminic acid).
followed by 3.5 h at 28 °C under shaking with 140 rpm, the cells were harvested by centrifugation at 4000 × g for 15 min. The cell pellet was stored at −80 °C until further use.

For purification of MSP1D1-H5, the cell pellet was resuspended in 2.5 times excess of lysis buffer (20 mM NaPi (pH 7.4), 1% Triton X and 0.001% nuclease). Cells were lysed by sonication for 5 min at 40% amplitude with pulses of 10 s on, 5 s off, under constant cooling on ice. After centrifugation of the lysate for 30 min at 30,000 × g and 10 °C, the supernatant was mixed with Ni-NTA agarose beads, previously equilibrated with basic buffer (40 mM Tris (pH 8.0), 300 mM NaCl) and incubated for 1 h at room temperature under gentle shaking. The suspension was transferred onto a bench-top column at room temperature and the flowthrough was discarded. The beads were washed in four steps of 12 ml with basic buffer, supplemented in the first step with 1% Triton X, in the second step with 50 mM sodium-cholate and no supplementation in the third step. Afterward, the protein was eluted from the column with 15 ml basic buffer with 300 mM imidazole and concentrated to a total volume of 5 ml. The eluate was dialyzed overnight at 4 °C against 100 times excess of buffer with 40 mM Tris (pH 8.0). For further purification, the protein solution was loaded onto a HiLoad Superdex 200 16/600 column on an ÄKTA chromatography system, previously equilibrated with 10 mM Tris (pH 7.4), 100 mM NaCl and 1 mM EDTA. The protein was eluted in fractions and checked via an SDS-PAGE for protein-containing fractions. Selected fractions were combined and incubated overnight at 4 °C with TEV protease in a 1:50 ratio. Afterward, the cleaved tag and TEV protease were removed via Ni-NTA affinity chromatography and cleaved MSP1D1-H5 was collected in the flowthrough. TEV-cleavage was checked via SDS–PAGE and the protein was concentrated and stored at −80 °C.

Reconstitution of HiSiaQM in MSP1D1-H5 nanodiscs. The nanodisc reconstitution protocol for the TRAP transporter was based on ref. 72. The purified MSP1D1-H5 nanodiscs and Mb3 were mixed in buffer C (50 mM KH2PO4, pH 7.8) for 2 h with a ratio of 1:1000. The mixture was then loaded onto a HiLoad Superdex 200 16/600 column, supplemented with 50 mM DMPC and 100 mM sodium cholate, to a ratio of 160:1 (MSP1D1-H5:DMPC:HiSiaQM). The protein solution was loaded onto a HiLoad Superdex 200 16/600 column, supplemented with 50 mM DMPC and 100 mM sodium cholate, to a ratio of 160:1 (MSP1D1-H5:DMPC:HiSiaQM). The protein was eluted in fractions and checked via an SDS-PAGE for protein-containing fractions. Selected fractions were combined and incubated overnight at 4 °C with TEV protease in a 1:50 ratio. Afterward, the cleaved tag and TEV protease were removed via Ni-NTA affinity chromatography and cleaved MSP1D1-H5 was collected in the flowthrough. TEV-cleavage was checked via SDS–PAGE and the protein was concentrated and stored at −80 °C.

Site-specific biotinylation of HiSiaQM. For protein expression, the genes were cloned into a pHEN6 vector which fused the protein to an N-terminal pelB signal peptide and C-terminal LPETG sortase motif. The HiSiaQM expression vector was digested with SapI and ligated into the megabody sequence via two SapI restriction sites, yielding the HiSiaQM megabody (Mb3). The expression, lysis, and purification of Mb3 were described in Supplementary Table 4. The final purification step, a HiLoad Superdex 200 16/600 column was used.

SRP binding characterization of VHH/megabody and HiSiaP to HiSiaQM. The SPR experiments were performed on a BiacoreTM 8K instrument (GE Healthcare Life Sciences), using a streptavidin-functionalized sensor chip (Serie S Sensor SA, GE Healthcare Life Sciences) and HiSiaQM buffer B at 25 °C /phenylboronic acid. The two biotinylated HiSiaQM constructs, E235C-biotin and K273C-biotin, were injected and immobilized on the chip (100 nM, 5 µl/min, 70–100 s). The analytes, the VHHs or the megabody, were added in six injections (30 µl/min, 120 s) with a doubling of the concentration at each step, resulting in single-cycle kinetic titration curves. The binding data were double referenced by blank cycle and reference flow cell subtraction. For epitope binning, the VHHS were tested for competitive binding using an ABA-injection protocol.

For characterizing the binding of HiSiaP, biotinylated HiSiaQM (K273C) was immobilized as described above. Single-cycle kinetic titration curves were recorded for (0.078, 0.156, 0.312, 0.625, 1.25, 2.50, 5.00, 10.00 µM) injections of HiSiaP. The buffer was supplemented with 5 mM sialic acid where indicated. Competitive binding of VHHs and HiSiaQM was assessed by competitive injections of either buffer (with 5 mM sialic acid, 25 µM VHH, 0.8 s) followed by HiSiaP (2.5 µM, 30 µl/min, 120 s) using a dual injection protocol.

Cryo-EM—sample preparation, data collection, processing, structure modeling. The cryo-EM structural studies were performed with HiSiaQM in MSP1D1-H5 nanodiscs with DMPC lipids. For the selection of HiSiaQM-containing nanodiscs, the purified Mb3 was loaded onto NiNTA beads, equilibrated with HiSiaQM buffer C, for 1 h at 4 °C. The flowthrough was discarded and the column was washed with 10 µl buffer C. The Mb3-bound Ni-NTA beads were resuspended in 2 ml buffer C, transferred into a flask, and supplemented with a HiSiaQM-rich solution, resulting in a concentration of 250 µg/ml HiSiaQM. The mixture was dialyzed overnight at 4 °C and transferred back to the bench-top column. The flowthrough was discarded, the column was washed with 10 µl buffer C and the protein was eluted with 1.5 µl buffer C with 500 mM imidazole. After concentration to around 50 µl in a Vivaspin (MWCO 100 kDa), the protein solution was loaded onto a Superose increase 6 3.2/300 column on an Agilent HPLC 1260 infinity II, previously equilibrated with buffer D (20 mM Tris (pH 7.5) and 100 mM NaCl). The eluted protein was monitored and fractionated manually and dropwise. The fractions that corresponded to the main protein peak were combined and used for cryo-EM experiments.

The grid preparation was performed on a Vitrobot (Thermo Fisher Scientific) at 4 °C and 100% humidity by using Quantifoil R1/2.13 grids. The blot time was set to 6 s and the blot force to 0. The grids were stored in liquid nitrogen until data...
collection at a Titan Krios microscope (Table S1). A dataset of 5004 movies was collected using a Cs-corrected Titan Krios electron microscope (Thermo Fisher Scientific) operating at 300 kV, and equipped with a Gatan K3 camera and a BioQuantum imaging filter (Gatan). Images were recorded over 2.264 s with the camera operating in counting mode, with a dose rate of 22.17 e−/Å²/s for a total dose of 50.213 e−Å²/Å over 50 frames. After patch motion correction and CTF estimation with cryoSPARC, a total of 233 million particles were automatically picked using a blob-picker job and subjected to multiple rounds of 2D classification. Representative 2D classes are shown in Fig. S3 and clearly show the megabody–HisSiQM complex in the top views. The 2D classes and the movies were used as inputs for a template picker job resulting in 2.55 million particles that were used for 2D classification. The remaining 2.29 million particles were exported from cryoSPARC using the csparc2star.py script by Daniel Asarnow (https://doi.org/10.5281/zenodo.3576630) and imported into RELION76 to construct an ab initio model and to perform two rounds of 3D classifications. The first round (regularization parameter T = 4) revealed one good 3D class with a clearly visible transporter consisting of 790 K particles. A mask around the transporter was built in ChimeraX and was used as input for the second round of 3D classification without alignment (T = 4). Further 3D subclassing did not lead to classes with improved resolution and thus, the 215 K particles constituting the best class from the 2nd 3D classification were again imported into cryoSPARC for non-uniform sampling of particles. The resulting 2D classification revealed 100 000 K particles, which were finally used for the 3D classification. The final map had a GSFC resolution of 4.7 Å with a local resolution of the core area up to 3.7 Å. The map was subjected to local refinement. A size-exclusion chromatography (Superdex 75 10/300 on an ÄKTA system) was performed to fully remove the unbound label and to check the successful labelling with the detection of the fluorophore-corresponding wavelength of the eluted protein solution. The protein-containing fractions were concentrated, flash-frozen, and stored at −80 °C.

Solid supported biayer preparation. For the preparation of planar bilayers on glass supports, very small unilamellar vesicles (USVs) were prepared from detergent solution by the addition of cyclodextrin according to Grein et al. and Roder et al.80,81. For each bilayer, a lipid mixture with 31.8 mM DOPC (Avanti Polar Lipids, Birmingham, AL, USA) and 0.01 mol% TopFluo-PC (Avanti Polar Lipids) was prepared in chloroform, and the chloroform was slowly removed in a nitrogen stream. The resulting lipid film was solubilized in 200 μl HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) supplemented with 40 mM Triton X-100. The lipid-detergent solution was split into 10 aliquots each 20 μl and stored at −4°C until use. A second stock solution contained 4 mM heptakis(2,6-di-O-methyl)-β-cyclodextrin (cyclodextrin) in ddH2O and was also stored at −4°C. A suspension of VSUs was prepared by first diluting the lipid-detergent stock solution in 200 μl HEPES buffer, followed by the addition of 1 μl HisSiQM solution (HisSiQM in 10 mM HEPES buffer) and was first diluted and then 100 μl of cyclodextrin stock solution and immediate, thorough mixing by vortexing for 2 min. Vesicles were generally used within 1 h of preparation.

Coverslips (18 × 18 mm) were cleaned overnight in fresh Piranha solution (one part H2SO4 30% and two parts concentrated H2SO4), rinsed thoroughly with milliQ water, and dried in a nitrogen stream. Clean coverslips were placed into custom-built sample chambers with an O-ring as a seal and two metal clips to form a metal insert on top of the coverslip. Bilayers were prepared immediately by adding 400 μl of freshly made vesicle suspension filling the well of the sample chamber. Due to electrostatic interaction between the negatively charged lipid headgroups and highly hydrophilic bilayer membranes, the bilayers are readily attached to the coverslip. The high surface tension led to the fusion of VSUs and the formation of homogeneous bilayers on the complete cover slip within 5 min. Residual, non-fused vesicles were removed by carefully adding 2 ml of HEPES buffer to the sample chamber and then removing only 1 ml of liquid. In total, 12 times 1 ml HEPES buffer and removing 1 ml. The final volume of HEPES buffer in the chamber was 1.4 ml. During the washing steps, care was taken to not dry out the lipid bilayer.

Biaryl binding assay and single-molecule imaging. For the inhibition test and nanobody staining of HisSiQM 10 μl Nb3 or 2 μl Nb3-AF-555 were added to the surface and incubated for at least 30 min. For Nb3-AF-555 the bilayer was washed 5 times afterward.

The P-domains variants were diluted 1:20 in HEPES buffer with 10 mM sialic acid, incubated for at least 20 min, and centrifuged at 14,000 × g for 10 min. Samples without sialic acid were treated the same way. To each bilayer 1 μl of this solution was added. The buffer solution in the sample chamber was mixed carefully by pipetting up and down and incubated for 5 min before measurements were started.

Images were acquired at a custom-built, single-molecule sensitive, inverted microscope capable of total internal reflection fluorescence (TIRF) microscopy, which was equipped with an sCMOS camera (Prime BSI, Teledyne Photometrics, Tucson, AZ, USA)82. For the combination with total internal reflection reduced fluorescence excitation to a thin region at the coverslip surface with the benefit of background suppression from fluorescence outside the illuminated region. The illumination beam angle was adjusted by tilting a collimated laser beam in the object plane of the microscope. The illumination beam angle was adjusted by tilting a collimated laser beam in the object plane of the microscope. Due to electrostatic interaction between the negatively charged lipid headgroups and highly hydrophilic bilayer membranes, the bilayers are readily attached to the coverslip. The high surface tension led to the fusion of VSUs and the formation of homogeneous bilayers on the complete cover slip within 5 min. Residual, non-fused vesicles were removed by carefully adding 2 ml of HEPES buffer to the sample chamber and then removing only 1 ml of liquid. In total, 12 times 1 ml HEPES buffer and removing 1 ml. The final volume of HEPES buffer in the chamber was 1.4 ml. During the washing steps, care was taken to not dry out the lipid bilayer.
two frames. The maximum linking distance was set to 1 μm. Each track is considered as a single interaction of the P-domain with the bilayer. Three independently prepared samples were measured 20 times each, resulting in a total of 90 individual movies for each condition. Normalization of interactions per second was achieved by first dividing the total number of interactions by the summed total duration of all measured movies in the respective condition. Then, the positive control value was set to 1 and all other values were adjusted accordingly.

Structural predictions with AlphaFold. The source code of the AlphaFold algorithm was downloaded from https://github.com/deepmind/alphafold and installed as described https://github.com/deepmind/alphafold. The pLDTS's scores were mapped onto the structures with PyMOL (www.pymol.org). The models of the triplicate complexes are available as Supplementary Data 1 (outward facing) and Supplementary Data 2 (inward facing).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The coordinate and map data generated in this study have been deposited in the PDB and EMDB databases under accession codes 7Q5E and EMD-13930. The movie data generated in this study are provided as Supplementary Movies 1–4. The models of the outward and inward-facing triplicate complex are provided in Supplementary Data 1 and 2. Data underlying all plots are provided as Source data. The coordinate data used in this study are available in the PDB database under accession codes 5U19, 2CEY, 3B5B, SNVA, 2HZL, 2ZZV). Source data are provided with this paper.

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experiment. P.D. and M.F.P. developed the reconstitution of the nanodiscs. P.-A.K. supervised alpaca immunization, selection, and initial characterization of VHHs. Proteins for the SPR experiments were prepared by M.F.P. K.G. and J.M. performed and analysed the SPR experiments, supervised by M.G. The VHHs1 protein purification and ITC experiment was performed by N.S. Cryo-EM experiments were performed by M.F.P., G.H., and A.D. The cryo-EM dataset was processed by M.F.P. and G.H. The results were discussed with C.Z., V.H., and A.D. The TRAP transporter complementation assay was established and performed by M.F.P. with the help of E.S. who developed the original assay and k/o strain. S.T. provided the HiSiQM multiple sequence alignments. M.F.P., J.P.S., and J.A.R. planned the single-molecule experiments, J.A.R. performed and analysed the single-molecule measurements; the results were discussed and evaluated with J.P.S. and U.K. J.A.R., J.P.S., and U.K. contributed to Fig. 6 and the manuscript. M.F.P. and G.H. wrote the manuscript together with G.H.T. All authors discussed the data and commented on the final manuscript version.

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**Additional information**

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