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Crystal structure and mechanistic basis of a functional homolog of the antigen transporter TAP

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Contribution by Robert M. Stroud, December 7, 2016 (sent for review October 24, 2016; reviewed by Douglas C. Rees and Daniel M. Rosenbaum)

ABC transporters form one of the largest protein superfamilies in all domains of life, catalyzing the movement of diverse substrates across membranes. In this key position, ABC transporters can mediate multidrug resistance in cancer therapy and their dysfunction is linked to various diseases. Here, we describe the 2.7-Å X-ray structure of heterodimeric Thermus thermophilus multidrug resistance proteins A and B (TmrAB), which not only shares structural homology with the antigen translocation complex TAP, but is also able to restore antigen processing in human TAP-deficient cells. TmrAB exhibits a broad peptide specificity and can concentrate substrates several thousandfold, adopting an asymmetric inward-facing state, and we show that the C-terminal helices, arranged in a zipper-like fashion, play a crucial role in guiding the conformational changes associated with substrate transport. In conclusion, TmrAB can be regarded as a model system for asymmetric ABC exporters in general, and for TAP in particular.

ABC transporter | conformational dynamics | membrane proteins | peptide transport | transporter associated with antigen processing

ABC transporters use the energy of ATP binding and hydrolysis to translocate chemically diverse substrates across cell membranes, with substrates ranging from ions to lipids, peptides, and proteins (1–4). All ABC transporters are built from common structural modules comprising two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP, and two transmembrane domains (TMDs) that facilitate substrate translocation (5, 6). Despite these common structural units, members of the different ABC protein superfamilies exhibit significant structural and mechanistic diversity (2). Based on the different organization of their structure and the direction of substrate translocation, ABC proteins can be classified into import and export systems. Although several structures of type-I ABC exporters in different states of the transport cycle have been solved, they are restricted to transporters with two canonical ATP-binding sites, with the exception of TM288/287 (7), whose transport activity is undefined.

Due to scarce structural and functional data on heterodimeric exporters, in which one half-transporter contributes to a catalytically inactive, degenerate ATP-binding site, our knowledge of heterodimeric transport systems is still incomplete. However, a better understanding of these systems is particularly important because a large number of the eukaryotic ABC transporters have nonexclusive nucleotide-binding sites, including the heterodimeric transporter associated with antigen processing TAP (8). The prevalence of heterodimeric ABC half-transporters suggests an underlying functional principle of NBD asymmetry (9–11). Apart from general unresolved aspects that concern all ABC exporters (i.e., the structural basis of substrate recognition and specificity as well as the trajectory of conformational changes associated with substrate translocation) there are major open questions that pertain to heterodimeric systems (e.g., how the interplay of the two non-equivalent NBDs is coupled to substrate transport).

To address these questions, we identified in a structural genomics screen a bacterial TAP homolog called TmrAB (Thermus thermophilus multidrug resistance proteins A and B), an obligatory heterodimeric ABC exporter, with one half-transporter, TmrB, contributing to the degenerate ATP-binding site (12). TmrAB was initially shown to be able to transport the fluorescent dye Hoechst 33342 and to be inhibited by verapamil (12). Because TmrAB is the only type-I ABC export complex in Thermus thermophilus, its substrate specificity is expected to be extremely broad. In fact, we recently demonstrated that phosphoglycerolipids are bound to and released by TmrAB upon ATP hydrolysis, suggesting that this ABC transporter may also move lipids (13).

Here, we show that TmrAB is an exceptionally versatile ABC export system. In fact, TmrAB covers the substrate spectrum of the antigen translocation complex TAP and is able to fully restore antigen processing in TAP-deficient cells from immune-compromised patients; TmrAB can thus be regarded as a functional homolog of the antigenic peptide transporter TAP and present its fundamental questions concerning their mechanism remain open. Here, we identify the protein complex TmrAB as a functional homolog of the antigenic peptide transporter TAP and present its high-resolution structure. The structure adopts an asymmetric conformational state and is characterized by C-terminal zipper helices that are essential for efficient substrate translocation. The structure, together with functional studies, enables us to outline the general conformational dynamics of heterodimeric ABC transporters and to establish TmrAB as a model system for TAP.

Significance

ABC transporters shuttle chemically diverse substances across membranes in an energy-dependent manner. They mediate multidrug resistance in microorganisms and cancer cells and can cause human pathologies when dysfunctional. Although important insights into ABC transporters have been gained in recent years, fundamental questions concerning their mechanism remain open. Here, we identify the protein complex TmrAB as a functional homolog of the antigenic peptide transporter TAP and present its high-resolution structure. The structure adopts an asymmetric conformational state and is characterized by C-terminal zipper helices that are essential for efficient substrate translocation. The structure, together with functional studies, enables us to outline the general conformational dynamics of heterodimeric ABC transporters and to establish TmrAB as a model system for TAP.
Overall Structure of TmrAB. To explore the mechanism of substrate transport we determined the crystal structure of nucleotide-free TmrAB at a resolution of 2.7 Å by selenomethionine (SeMet) single-wavelength anomalous dispersion (SAD) phasing (Table 1). Because of the extreme unit-cell e dimension of 1.044 Å (space-group: P6522; SI Appendix, Fig. S1), dedicated setups of the X-ray beam and detector had to be implemented for data collection (Materials and Methods). TmrAB is observed in an asymmetric inward-facing conformation that differs from other ABC transporter structures (SI Appendix, Table S2). The overall architecture of TmrAB exhibits two half-transporters interacting in an arrangement with pseudo-twofold symmetry (Fig. 2). The periplasmic gate is tightly sealed, preventing proton leakage. The substrate cavity is open to the cytoplasmic side, and the NBDs are separated; however, the NBDs are still in contact and positioned via their C-terminal helices (zipper helices), burying a total surface area of 1,498 Å². Apart from the cytoplasmic entry route, a lateral gate is formed by TmrB, which grants potential hydrophobic substrates access to the substrate cavity (Fig. 2A; see also the discussion of lipidic substrates below). This lateral gate is found only in TmrB, but not in TmrA, signifying the asymmetric architecture of TmrAB. Further notable structural differences between the two half-transporters are found in the A-loop of the NBDs and in periplasmic loop 1, which are significantly extended in TmrA (SI Appendix, Fig. S2). The cavity facilitating substrate translocation across the membrane is formed by the two TMDs, each comprising six transmembrane helices (TM1–6). Intra- and intersubunit TMD–NBD communication is organized by coupling helix 1 and 2 of each subunit (Fig. 2A and B). The N-terminal elbow helices are located at the membrane–water interface. In our X-ray structure, we observe an arginine in each elbow helix (R17A;R 12B) forming a salt bridge with an aspartate in TM6 (D332A;D 317B; SI Appendix, Fig. S3). Notably, the residues involved in this interaction are conserved among ABC exporters, suggesting that they stabilize the heterodimeric complex as seen for TAP (14, 15). Comparing our X-ray structure of TmrAB with the cryo-EM model (16) shows

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a consistent conformation, with the X-ray model superimposable with the 8.2-Å EM density map. The average deviation of Cα positions is 2.4 Å, with a maximum deviation of 11.0 Å at the very C terminus of TmrB (SI Appendix, Fig. S4). The larger structural differences are found in several loop regions in both NBDs, in the C-terminal helix of TmrB, increasing linearly toward the terminus, and in the periplasmic loop; these are all regions that would be expected to be less defined in the lower-resolution model and could not be modeled accurately in the cryo-EM map. Furthermore, parts of TM6 of TmrA, as well as TM2, TM4, and TM5 of TmrB, also showed some variability with regard to the crystal structure; however, TM4 or its equivalent has been observed to be highly variable in several X-ray structures of ABC transporters (17–19). It is unclear whether the described differences between the cryo-EM model and the X-ray structure are due to a minor conformational change, caused by different experimental environments (cryo-EM vs. crystallography), or due to the limited resolution of the cryo-EM data.

TmrAB Restores Antigen Processing in Human TAP-Deficient Cells. TmrAB shares 27–30% overall sequence identity with human TAP1 and TAP2, of which the TMDs account for 22% and the NBDs for 34% and 37%, respectively. We therefore wondered whether the bacterial TmrAB could restore TAP function in TAP1-negative skin fibroblast cells (BRE169) derived from an immune-compromised bare lymphocyte syndrome patient (20). We transfected the TAP1-deficient cells with TmrA and TmrB containing the extra domain TMD0 of TAP2 for the interaction with components of the MHC-I peptide-loading complex (Fig. 3 A) (15, 21, 22). As expected, coreTAP1 (lacking its TMD0) can restore antigen processing by pairing with the endogenous TAP2 in TAP1-deficient cells, as evidenced by a significant increase in MHC-I surface expression (Fig. 3 B and C). Remarkably enough, TmrAB is also able to fully assume the function of TAP and restore MHC-I surface expression to the level of mock- or untransfected cells. Noteworthily, TmrA or TmrB alone cannot rescue TAP function (Fig. 3 C). These data demonstrate that TmrAB, in combination with the TMD0, can restore antigen presentation. TmrAB is therefore a full-fledged functional homolog of the TAP complex.

Substrate Selectivity by a Chemically Heterogeneous Binding Cavity. We next addressed the substrate specificity and potential binding site. The inner surfaces of TMDs of TmrA and TmrB, forming the substrate-binding cavity, display considerable differences in their charge distribution (Fig. 4 A), which might explain the ability of TmrAB to bind a range of chemically diverse substrates. The polyspecificity in substrate recognition is also reflected by the diverse physicochemical properties of the cavity residues contributed by TmrA and TmrB. Although the majority of conserved and invariant residues in the TMDs are located on the outside of the protein complex (SI Appendix, Fig. S5), TmrAB shares with TAP

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**Fig. 2.** Structure of the heterodimeric, asymmetric ABC transport complex TmrAB. (A and B) Ribbon representation of TmrA (wheat) and TmrB (cyan) in two different side-view orientations. The surface is shown as a transparent overlay. A faint gray bar depicts the membrane interface. (C) Electrostatic surface charge of TmrAB, revealing the hydrophobic transmembrane region of TmrAB. (D) View onto the TMDs from the periplasm, displaying the tightly closed periplasmic gate of the transporter. (E) Cytoplasmic view, showing the relative orientation of the two NBDs and their zipper helices.

**Fig. 3.** TmrAB restores antigen processing in TAP-deficient cells. (A) The antigen translocation complex (coreTAP) and the bacterial TmrAB complex share structural and functional homology. TAP1/2 and TmrAB share one canonic, ATPase active and one degenerate nucleotide-binding site. TmrB was N-terminally fused to the TMD0 of TAP2 to promote the assembly of the MHC-I peptide loading complex. (B) TmrAB restores antigen processing in TAP1-deficient cells from immune-compromised bare lymphocyte syndrome type-I patients. CoreTAP1 and TmrAB were expressed in BRE169 cells. Mutation of the catalytically active glutamate residue of TmrA to glutamine (E523Q) blocked antigen processing and MHC-I surface expression. MHC-I surface expression was monitored by the MHC-I conformation-specific antibody W6/32. (C) Restoration of MHC-I surface expression in TAP1-negative BRE169 cells by TmrAB. Mean fluorescence intensities (MFI ± SD) from at least three independent experiments are displayed.
certain residues that have been shown to interact with peptide substrates in TAP. These include D297 in TAP1 (E131 in TmrB), identified by docking as part of a negatively charged pocket that contacts the peptide N terminus (23). Further peptide-interacting residues in TAP1 (24) shared with TmrB are G282, R287 (invariant G116TmrB and R121TmrB, respectively), I284 (L118TmrB), and V288 (L122TmrB). Moreover, particular residues that affect substrate specificity in TAP2 and may thus participate in substrate binding (25, 26) are either invariant (A374TAP2 and A258TmrA) or physicochemically more or less conserved (N262TAP2 and Q146TmrA; M218TAP2 and W100TmrA). These common residues might at least partially explain the ability of TmrAB to take over the function of TAP in TAP-deficient cells.

For a more detailed analysis of the substrate-binding cavity, we defined the cavity as the inner region of TmrAB starting at the membrane-water interface and extending up to the periplasmic gate (Fig. 4B). Additional density in this region corresponding to putative substrate has been identified in the low-resolution cryo-EM map (Fig. 4B) (16). The cavity surface of TmA is mainly charged, whereas it is predominantly hydrophobic in TmB. Using random-sequence libraries of 125I-labeled peptides, we determined the dependence of transport activity on substrate size. Although TmrAB was able to translocate polypeptides of 35 aa, 7-mer peptides were most efficiently transported (Fig. 4C). The upper size limit is consistent with the volume of the cavity (5,760 Å3). The optimal peptide length for TmrAB is similar to the one determined for TAP, which prefers peptides of 8–16 aa (27, 28). Considering the vast combinatorial diversity of the random-sequence peptide libraries, TmrAB exhibits remarkably versatile substrate specificity. The antigen translocation complex TAP is the only other ABC exporter for which such a broad peptide specificity with a similar length selectivity has been observed. In summary, TmrAB can be regarded as a bacterial prototype of the antigen translocation complex TAP, which plays a prominent role in adaptive immunity.

The high resolution of our X-ray structure in combination with site-directed mutagenesis allowed us to pinpoint specific residues involved in substrate binding and transport. We used the extra density identified in the cryo-EM study attributed to bound substrate as a starting point for mutational studies. To block the putative substrate-binding pocket, we coupled the spectrally unrelated fluorophore ATTO647N to the...
cysteine of H246CB. Even though the H246CB mutant was not affected in its transport activity, coupling the bulky moiety to the cysteine resulted in a markedly lower activity.

To determine whether the reduced transport activities are due to impaired substrate recognition we directly measured substrate binding by fluorescence polarization (Fig. 4E). The E242CB mutant exhibited reduced substrate binding, suggesting that E242CB is directly involved in binding of the PEP-peptide. An inhibition of substrate binding was observed if H246CB was labeled, indicating that the attached fluorophore obstructs access to the peptide-binding site. Furthermore, the cavity single-cysteine mutants H246CB, E242CB, and Y187CB are specifically disulfide cross-linked to a fluorescent peptide (SI Appendix, Fig. S6a). In contrast, the mutant R206CA (Fig. 4B) did not show any cross-linking (SI Appendix, Fig. S6c). Thus, part of the peptide-binding site seems to be located in the region of the extra EM density ascribed to a bound small-molecule compound, the same position where glutathione is bound to bacterial and mitochondrial Atm1-type ABC exporters (29, 30). Residues influencing transport activity but not directly involved in substrate binding, for example R206CA (SI Appendix, Fig. S6b), most likely participate in the conformational changes that couple substrate binding and periplasmic gate opening.

Transport Pathway for Lipidic Substrates. In light of the fact that the activity of TmrAB is modulated by membrane lipids (Fig. 1C) and that TmrAB binds phospholipids and releases them upon ATP hydrolysis (13), we analyzed our structure with regard to potential lipid binding sites and translocation pathways: in addition to the cytosolic entry route to the substrate cavity, TM4 and TM6 of TmrB form a laterally open V-shaped gate that grants membrane–cytosolic entry route to the substrate cavity, TM4 and TM6 of TmrA (Fig. 5A). The contacting zipper helices of the NBDs in the TmrAB structure are in stark contrast to the inward-facing conformations of P-gp (17, 34, 35) and MsbA (36), which display extremely wide separations of their NBDs, or TM288/287 with almost closed NBDs (7). We analyzed the conformational dynamics and interaction between the zipper helices in the case of reconstituted TmrAB by disulfide cross-linking of an introduced cysteine pair (C416A,L595C/E568C) (Fig. 5B and SI Appendix, Fig. S8A). The resolution of our crystal structure allows us to design tailored cysteine substitutions at two opposite sites for cross-linking, with a Ca-Ca distance of 5.6 Å. Even though substrate binding was not affected (SI Appendix, Fig. S6b), the 100% cross-linked zipper variant did not show any transport activity (Fig. 5B), demonstrating that TmrAB has to undergo structural rearrangements during its catalytic cycle, involving unzipping of the C-terminal helices, that cannot be performed by the conformationally locked mutant. The fact that activity can be fully restored by reduction indicates that the loss of transport activity upon oxidation is not due to misfolded or damaged protein. Disulfide cross-linking of the C-terminal helices is significantly reduced when the mutant is vanadate-trapped in the

Unzipping of C-Terminal Helices As an Essential Step of the Transport Cycle. In TmrAB, the NBD–NBD contacts mediated by the C-terminal helices are predominantly formed by van der Waals interactions between L572, L595, L563, and L602, packing in an arrangement similar to the “knobs-into-holes” pattern (Fig. S4). We therefore refer to the C-terminal helices as “zipper helices.” Polar contacts between the two helices are formed between Q596B and E568B, between Y588B (main chain) and E575B, and between R569A, E575B (main chain), and the C-terminal carboxyl group of A578B (Fig. 5A). However, judging from the angles between hydrogen bond donors and acceptors, the interactions are rather weak. The lack of strong interactions might facilitate the movement of the two zipper helices relative to each other during the structural changes necessary to adopt the outward-facing conformation.

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Fig. 5. The C-terminal zipper helices control the translocation cycle. (A) Details of the interaction between the C-terminal zipper helices of TmrA and Tmrb in the NBDs (view from cytoplasm). Salt bridges and hydrogen bonds are shown as dotted lines. (B) Transport activity (1 μM RRR4CKSTEL, 15-min values) of oxidized (ox) and reduced (red) C416A,L595C/E568C. Complete cross-linking upon oxidation is demonstrated by SDS/PAGE. (C) Transport activity of Δzipper TmrAB mutant, which lacks the C-terminal zipper helix of TmrA, in comparison with the wild-type protein (WT). (D) Transport kinetics of the wild-
hydrolytic transition state (SI Appendix, Fig. S8B), establishing that the zipper helices reorient during NBD closure.

The importance of the zipper helices was further analyzed by the deletion of the zipper helix in TmrA (Δzipper). Although the Δzipper complex presented the same expression level and purification profile as wild-type TmrAB, the transport activity of the Δzipper complex is significantly reduced (Fig. 5C). Nevertheless, the residual transport activity of the Δzipper complex allowed us to measure its transport kinetics (Fig. 5D). Despite the largely reduced turnover rate, the $K_m$ value of the Δzipper complex for the transport substrate is similar to the wild-type protein (wild-type: 7.7 ± 1.0 μM; Δzipper: 12.5 ± 4.2 μM). Taken together, these results provide evidence for a role of the zipper helices as a functional motif in the TmrAB translocation cycle.

Opening and Closing of the Periplasmic Gate. In our structure, the periplasmic gate of TmrAB is closed, with several primarily hydrophobic side chains forming a tight seal that maintains the proton-motive force across the inner membrane. The opening of the periplasmic gate must be tightly coordinated if peptides, fluorophores, or potentially even lipids are released via this gate. We therefore examined the conformational change at the periplasmic gate of TmrAB during the transport cycle using pulsed electron-electron double resonance spectroscopy (PELDOR/DEER).

Based on our X-ray structure, we strategically placed a spin-label pair at the periplasmic gate (Fig. 6A). We used vanadate to mimic the transition state in ATP hydrolysis and trap TmrAB in an outward-facing conformation. An analysis of this vanadate-trapped state and the apo state gives evidence that the periplasmic gate of TmrAB opens by ~20 Å, from 20 to 40 Å, upon vanadate trapping (Fig. 6B). ADP and nonhydrolyzable AMP-PNP did not induce periplasmic gate opening (Fig. 6C), suggesting that formation of an ATP hydrolysis transition state is necessary to drive the change to the outward-facing conformation. Notably, due to the heterodimeric nature of TmrAB with a degenerate site, the hydrolytic transition state must be formed asymmetrically by the catalytically active site. The fact that the transition state is required to achieve the outward-facing state in the case of TmrAB is in contrast to the homodimeric transporters MsbA and Sav1866, where ATP and AMP-PNP, respectively, are sufficient to induce a similar conformational change (37, 38).

To assess the similarity between a detergent/lipid micellar environment and a lipid bilayer we determined the distances in TmrAB reconstituted in a lipid bilayer and obtained an opening of ~35 Å, from 20 to 55 Å (Fig. 6D). In the case of TmrAB, the surrounding lipid environment thus seems to significantly increase the extent of periplasmic gate opening in the transition state. The transition to the outward-facing state in TmrAB was strictly dependent on vanadate trapping, similar to the case with BmrCD (9), suggesting that the formation of the hydrolytic transition state may be the balance point in the ATP hydrolysis-driven power stroke in heterodimeric ABC transport complexes.

Discussion

TmrAB is a highly efficient transporter that can concentrate substrate several thousandfold. Moreover, it exhibits a remarkably...
broad substrate specificity that fully covers the substrate spectrum of the human antigenic peptide transporter TAP, as exemplified by its ability to rescue MHC I surface presentation in TAP-deficient cells. TmrAB can be distinguished from TAP by its capacity to recognize fluorophores (FL), to be inhibited by verapamil (12), and its possible function as a lipid translocator (13). Despite the high activity, we noted that the transport rate and ATP turnover (12) are not identical. However, a defined 1:1 or 1:2 ratio of transported substrate per ATP has not yet been observed for any ABC export system. This could be explained by (i) a cotransport of lipidic compounds and (ii) the possibility that not all of our TmrAB complexes are transport-active after reconstitution, as has been demonstrated for the TAP-like complex by single-liposome-based analysis (39).

Although a comprehensive understanding of the activity of ABC transport complexes will only result from high-resolution structures of several different on-pathway intermediates of the same protein and of substrate-bound complexes, the current structure provides insights into the inner workings of this important protein family at the molecular and atomic level and hence suggests models of substrate transport by TmrAB. As outlined above, our crystal structure of TmrAB differs significantly from a previously determined X-ray structure of Thermotoga maritima TM288/287 [Protein Data Bank (PDB) ID code 4Q4A] and Staphylococcus aureus Sav1866 (PDB ID code 2HYD) (11, 40). This allowed us to derive a trajectory of conformational changes associated with the transport cycle of ABC exporters. Whereas TM288/287 represents an inward-facing intermediate with a maximum of one occluded nucleotide, Sav1866 is in an outward-facing state with two fully occluded nucleotide-binding sites. The movement of the two NBDs relative to each other during the translocation cycle is accompanied by a successive reorientation of the Walker A motif (P-loop) relative to the signature motif of the opposite NBD (Fig. 7A and Movie S1). The distance between Walker A (P-loop; conserved lysine) and signature motif (C-loop; conserved serine) decreases from TmrAB (Cα distance K399–S476) 24.9 Å; K377–S498 24.3 Å) over TM288/287 (Cα distance for both K394–S471 18.1 Å; K372–S493 17.2 Å) to Sav1866 (Cα distance for K380–S479 between the two protomers: 9.5 Å). In Sav1866, the two NBDs have adopted a closed dimeric state; the relative movement of Walker A and signature motif has been brought to completion, positioning the signature motif in the immediate vicinity of the Walker A motif and tightly packing the two nucleotides between the NBDs.

Another striking structural change along the trajectory from the inward- to the outward-facing conformation is the movement of the catalytic glutamate (E523V) (Fig. 7A and Movie S1). In apo TmrA, this glutamate, which acts as a catalytic base polarizing the water molecule that attacks the ATP (canonic site), is oriented away from the catalytically active nucleotide-binding site (Fig. 7A). In the nucleotide-occluded Sav1866 structure, however, the glutamate has moved into a hydrolysis-competent position. With regard to the position of its rearranged zipper helices, the TM288/287 structure resembles the Sav1866 structure, whereas the periplasmic gate is closed, that is, the arrangement of the TM helices is similar to TmrAB (Fig. 7B). The structural changes in the NBDs of TM288/287 seem to be not sufficiently advanced on the conformational trajectory to induce periplasmic gate opening.

During the transition from the inward- to the outward-facing conformation of TmrAB the overall rearrangement of the NBDs consists of translational motions (Movie S1). These motions require the C-terminal zipper helices to unzip and to switch their positions (Fig. 7C). Our experiments demonstrate that the zipper helices are essential for an efficient catalytic transport cycle. The C-terminal zipper helices of yeast Atm1 show an arrangement similar to that in TmrAB. However, in Atm1-type transporters, these helices are significantly longer and interact in a slight cross-over as opposed to antiparallel fashion (29, 30). C-terminal deletion mutants of Atm1 were interpreted as suggesting that they affected primarily stability rather than function because lowered activity was accompanied by substantially lower expression. We propose that the zipper helices position the NBDs and act as checkpoints that allow the transporter to execute its conformational transitions in a coherent and productive manner. They might also help the transporter to avoid the extremely large NBD separations that have been observed for some ABC exporters (e.g., P-gp, MsbA, and PglK) (17, 33–36). It remains to be determined how common this role of the C-terminal helices is among ABC transporters.

Conformational changes in the NBDs are allosterically coupled to a reorganization of the TMDs. This reorganization is most pronounced in regions of TM4 and TM6 that form the lateral entrance gate. At the base of the gate, the two TM helices are

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**Fig. 7.** Conformational dynamics of TmrAB and the catalytic transport cycle. (A) Relative movement of the NBDs during the transport cycle, analyzed by superimposing the NBDs of TmrB (pale cyan), TM287, and of one Sav1866 protomer; P-loop, Walker B, and D-loop of the superimposed NBDs are shown in transparent colors. Structural elements of the NBD moving relative to the superimposed domains are depicted in opaque colors. (B) Structural side-by-side comparison of TmrAB with alternating conformations displayed by TM288/287 and Sav1866. (Top) Schematic outline of the NBDs with the positions of the C-terminal helices highlighted by white rectangles. The relative positions of the two C-terminal helices are marked by a wheat and cyan dot. The red arrows indicate the movement of the two C-terminal helices. The red C stands for the C terminus. (Bottom) View onto the TMDs from the periplasmic side showing the closed state of the periplasmic gate in TmrAB and TM288/287, and the open state in Sav1866. The Cα atoms of two glycines are shown as red spheres (G291 in TmrA and G276 in TmrB, and corresponding positions in TM288/287 and Sav1866). (C) Model of substrate transport by TmrAB and the role of the zipper helices. TmrAB (subunits shown as schematic outlines) is able to transport a diverse range of substrates (depicted as encircled letter “S”), both soluble and membrane-associated compounds. An arginine cluster in TmrB at the rim of the lateral gate mediates association with headgroups of lipidic substrates. The inward-facing state with juxtaposed NBDs is stabilized by interactions between the C-terminal zipper helices. ATP and substrate binding induce a conformational transition to an occluded state, in which the NBDs interact intimately, but the periplasmic gate is still closed. For this transition, the zipper helices must dissociate their interaction and switch their position. The central cavity (shown in dark blue) functions as the cargo compartment that mediates substrate transport from the cytoplasmic to the periplasmic side of the membrane. ATP hydrolysis leads to periplasmic gate opening, allowing the substrate to leave the cavity. Release of phosphate restores the inward-facing state of TmrAB.
separated by a distance of 12.5 Å (Δ184B–300B Cα distance) in TmRB. In the outward-facing conformation of Sav1866, the lateral gate is blocked by TM4 and TM6, and the substrate cavity is exposed to the extracellular space and the outer leaflet of the membrane. Between the inward- and outward-facing states there is an intermediary putative occluded state (Fig. 7C), similar to the ones that have been observed in the substrate-free structures of the homodimeric ABC exporters McjD and PCAT1 (41, 42).

A pivotal role in transmitting conformational changes between NBDs and TMDs during substrate transport has been assigned to coupling helices in the NBD–TMD interfaces (40, 43, 44). They are a common feature of ABC transporters. In TmRB, the interactions in the NBD–TMD interfaces are mainly TmRB-specific (SI Appendix, Fig. S9); one notable exception is a triad of interacting Arg, Asp, and Tyr residues that can also be found in Caenorhabditis elegans and Cyanidioschyzon merolae ABCB1, TM288/287, and ABCB10 (7, 17, 18, 45). The high degree of conservation suggests that this motif may fulfill an important function in the communication between NBDs and TMDs. Certain regions of the transmembrane helices seem to contribute to the structural flexibility necessary to carry out the conformational changes accompanying substrate transport; these regions might be thought of as hinges and may even act as a trigger, coupling substrate binding to periplasmic gate opening. The region with the highest mobility in TmRB is indeed located in TM6 of TmRB right above the elbow helix and spans roughly 16 aa (residues 304–319). The weak electron density in this region, which includes a proline and glycine, reflects its high mobility. A similar feature is observed in the corresponding helix (TM12) of C. elegans Pgp (17), and it has been suggested that the flexibility is functionally important in mediating substrate interactions and/or transducing conformational changes induced by substrate binding and substrate occlusion (17, 41). Helical irregularities in TM6 close to the substrate-binding site have also been observed in Atm1-type transporters (29, 30). In summary, these findings substantiate the idea that discontinuous transmembrane helices are a common attribute of many transport proteins.

In its ability to transport a diverse set of peptides, drugs, and lipophilic compounds, TmRB seems to be a member of the TAP/MDR subfamily of transporters, which translocate a wide range of compounds and for which loss-of-function mutations have been linked to various diseases. In this respect, the structure of TmRB may open new avenues to therapeutic intervention, especially in the case of mammalian ABC transporters, many of which are characterized by an asymmetric organization of the central cavity as well as a functional nonequivalence of the NB in TmRB. Moreover, the particular ability of TmRB to fully subdivide the TAP complex functionally suggests that TmRB can serve as a model system for TAP, a key component of antigen processing in adaptive immunity.

Materials and Methods

Cloning and Expression. TmRA (TTC0976) and TmRB (TTC0977) were PCR-amplified from T. thermophilus HB8 genomic DNA. Wild-type TmRB as well as the ATPase inactive complex TmRAΔCbbCB were cloned as described previously (12). Cysteine mutants were prepared by site-directed mutagenesis. Both wild-type and mutant proteins were expressed in Escherichia coli BL21(DE3) as described (12). Selenomethionine-labeled protein was prepared using Selenomethionine Expression Media (Molecular Dimensions).

Protein Purification. Protein purification was performed at 4 °C unless stated differently. Harvested E. coli cells were resuspended in lysis buffer (20 mM Hespe and 300 mM NaCl, pH 7.5) containing lysozyme (Sigma) and DNase I (AppliChem). Cells were disrupted by using an automated French press (1.0 kbar, Basic 2; Constant Systems) and cell debris was removed by centrifugation at 10,000 × g for 15 min. The supernatant was centrifuged at 120,000 × g for 1 h to pellet membranes. Membranes were solubilized with solubilization buffer (20 mM Hespe, 300 mM NaCl, pH 7.5, and 20 mM N-dodecyl-β-maltopyranoside (β-DDM; Anatrace)) to a final concentration of 40 mg/ml total protein and incubated for 1 h at 20 °C. Detergent-solubilized protein was separated by centrifugation at 100,000 × g for 30 min. His-tagged TmRB was bound to Ni-NTA agarose (Qiagen) equilibrated with purification buffer (20 mM Hepes, 300 mM NaCl, pH 7.5, 800 μM NiSO4, 200 mM β-DDM) washed with purification buffer containing 50 mM imidazole and 1 mM β-mercaptoethanol. Protein was eluted with purification buffer supplemented with 300 mM imidazole. Fractions containing TmRB were desalted by Econo-Pac10DG (Bio-Rad) columns equilibrated in SEC buffer (20 mM Hepes, 150 mM NaCl, pH 7.2, and 1 mM β-DDM). The His tag was cleaved by tobacco etch virus (TEV) protease treatement. Cleaved protein was separated from TEV protease by reverse immobilized-metal affinity chromatography using Ni-NTA agarose in size-exclusion chromatography (SEC) buffer. TmRB was finally purified via SEC using a TSK G3000SWV column (Tosoh Bioscience LLC) in SEC buffer. The main fractions of the monodisperse peak were pooled for crystallization. The mutants were purified using the same protocol, except for the double-cysteine mutant TmRBΔCbbCB (SI Appendix, Fig. S39) in this case, 1 mM of β-mercaptoethanol was added at all buffers to prevent oxidation. Oxidative cysteine cross-linking was induced by buffer exchange to SEC buffer without β-mercaptoethanol via an Econo-Pac10DG column at 20 °C.

Crystallization. Before crystallization, TmRB was concentrated to 23 mg/ml by ultrafiltration using a 50-kDa molecular weight cutoff Amicon Ultra-0.5 (Millipore). TmRB crystals were obtained by the vapor diffusion method in hanging drops at 18 °C against a reservoir containing 26–30% PEG 400, 0.1 M N-acetylecitrulic acid, pH 3.5, or 0.5 M Tris–HCl, pH 7.5, or 0.5 M Na3citrate/HCl, pH 3.5, 0.1 M Li2SO4, and 0.5–2.5 mM CYMAL-5. Concentrated TmRB containing 5 mM AMP-PNP and 5 mM MgCl2 was mixed with reservoir solution in a 1:1 volume ratio. Crystals appeared after 1–3 d and were grown for another 5–12 d until they reached their full size of 0.2–0.5 mm. Crystals were directly flash-frozen in liquid nitrogen without additional cryoprotectant. SeMet-labeled TmRB was crystallized under the same conditions, but at 12 °C.

Data Collection. Diffraction data of native and SeMet-labeled TmRB crystals were collected at beamline X06SA of the Swiss Light Source. Because of an extremely long unit cell axis (unit-cell dimensions: a = b = 93.4 Å, c = 1,044 Å; space group P6_22 (SI Appendix, Fig. S1 and Table 1), a special beamline setup was necessary. X-ray optics were adjusted to focus the beam on the detector surface to minimize diffraction spot size and so eliminate overlaps. The X-ray beam size was defined with a 100 × 100 μm2 spot at the sample position to match the average size of TmRB crystals. Diffraction patterns were recorded to 2.7 Å with a PIILATUS 6M, while maintaining good separation of the densely spaced diffraction spots. The resulting sharp diffraction spots also helped to increase the signal-to-noise ratio for weak reflections at high resolution. In addition, crystal orientation was manipulated to have the long unit cell axis close to the rotation spindle axis to avoid spot overlaps during rotation data collection. Native data were collected to 2.7 Å resolution at a wavelength of 1.0000 Å, whereas SeMet data were collected to 3.6 Å resolution at a wavelength of 0.9793 Å. Diffraction data were processed with the program XDS (46). Minimum distances allowed between spots and maximum radius of difference vector clusters were adjusted manually (SEPMMIN = 2 and CLUSTER_RADIUS = 1) to allow indexing of the closely spaced reflections arising from the 1,044-Å axis. The crystals belong to space group P6_22, containing one TmRB heterodimer per asymmetric unit.

Structure Determination. Initial phases were obtained by SAD using SeMet derivative crystals (SI Appendix, Fig. S10). The heavy atom substructure of 19 selenium atoms was determined by SHELXD and initial phases were obtained using SHELXE (47). This yielded an interpretable electron density map. Automatic chain tracing using Buccaneer v1.5 (48) was used as an initial guide for building an intermediate polyalanine model, which was refined against the native dataset using PHENIX v1.9 (49). Based on the asymmetry in methionine distribution, the two protomers could be distinguished from the SeMet substructure. The chains of TmRA and TmRB were traced, and the model was built using Coot v0.8 (50) and refined using PHENIX. The high-resolution limit was chosen based on paired refinement calculations (51) at different cutoffs and found to give the best model and electron density. The complete polypeptide chains of TmRB were built with the exception of 10 N-terminal residues (1–10) of TmRB. Phe314A was substituted by alanine because of ill-defined density for this residue. Cysteine pairs were carried out using the program SSM (53). To analyze the extra density, the homodimeric ABC exporters McjD and PCAT1 (41, 42) were used as a model system for TmRB, with the long axis of the McjD crystal structure oriented perpendicular to the substrates. The long axis of the PCAT1 crystal structure was oriented parallel to the substrates. This resulted in interpretable electron density in both cases. The resulting model of TmRB was inspected with a Ramachandran plot, and residues were assigned stereochemistry and allowed to relax using COOT (50). The stereochemistry of all residues was judged to be acceptable.

Data Deposition. The atomic coordinates and structure factors of TmRB have been deposited in the Protein Data Bank (accession code 6HUu).

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were determined in PyMOL using the align command with the parameter cycles = 0. The align command carries out a sequence alignment before structural superposition.

**Restoration of Antigen Processing by TmrAB in TAP-Negative Cells.** TmrA and TmrB were transferred to a mammalian expression system. TmrA was cloned into pCDx3YC8 and TmrB into pCDX (54). The TMD0 of human TAP2 (residues 1–127) was added seamlessly at the N terminus of TmrB. All cloning steps were based on PCR amplification by Phi DNA polymerase and FX-cloning (55). TAP1-deficient BRE169 cells (20) were transfected with TmrA, TmrB, coreTAPA1 (15), or empty pCDx3YC8 as mock according to the X-treme-GENE HP manual.

Forty-eight hours after transfection, cells were harvested and stained with PE anti-human HLA-A,B,C (clone W6/32; BioLegend). Cells were fixed with 0.25% formaldehyde. Data were recorded by a FACSaria III 3 sorter (BD Bioscience) flow cytometer and processed using FlowJo 10 software (TreeStar, Inc.).

**Reconstitution into Liposomes.** Liposomes comprised of E. coli polar lipids/DOPC (7:3) or mixture of phospholipids plus 5 mol % E. coli cardiolipin (Avanti Polar Lipids) with a total lipid concentration of 10 mg/mL prepared in reconstitution buffer (20 mM Hepes, pH 7.5, 140 mM NaCl, and 5% (vol/vol) glycerol) were extruded 11 times through a 400-nm polycarbonate filter. After destabilizing the liposomes for 30 min at 4 °C with Triton X-100 (Roht), SEC-purified TmrAB was added to a protein-to-lipid ratio of 1:20 (wt/wt) unless stated otherwise. Excess detergent was removed in a stepwise fashion over a period of 20 h using Bio-Beads (Bio-Rad). Proteoliposomes were pelleted by ultracentrifugation at 270,000 × g for 30 min at 4 °C. Pelleted proteoliposomes were resuspended in reconstitution buffer to a final lipid concentration of 5 mg/mL. The reconstitution efficiency was confirmed either by vesicle pelleting or carbonate crush (100 mM Na2CO3, pH 11.5) and the whole TmrAB reconstitution procedures. Dextran Alexa647-loaded TmrAB proteoliposomes were fitted with a model-free Tikhonov regularization to distance distributions. 

**Substrate Accumulation.** Substrate Accumulation.

**Phosphate Fluorescence Labeling.** The purified single-cysteine TmrA<sup>C416A</sup>/TmrB<sup>H246C</sup> complex was labeled with a 40-fold molar excess of ATTO473N-maleimide (Invitrogen) for 2 h at 20 °C in SEC buffer. Free fluorophore was removed by SEC purification. TmrA<sup>C416A</sup>/TmrB<sup>H246C</sup> was reconstituted as described above. Spin-labeled TmrAB displays the same transport activity as wild type. EPR experiments were performed without nucleotides (apo state) or with Mg-ATP, Mg-ADP, Mg-AMP-PNP, or Mg-ATP and the whole TmrAB reconstitution procedures. Dextran Alexa647-loaded TmrAB proteoliposomes were further washed with reconstitution buffer and a second harvesting step (270,000 × g, 30 min, 4 °C) was carried out to remove external dextran<sup>Alexa647</sup>. pH peptide labeling was performed in a total volume of 500 μL with proteoliposomes (50 μg protein) at a protein-to-lipid ratio of 1:20 (wt/wt) at 4 °C for 2 h in ARS transport buffer (20 mM Hepes, pH 7.5, 5% (vol/vol) glycerol, 0.1 mM MgSO<sub>4</sub>, 25 mM phosphocreatine sodium salt, and 3 mM MgSO<sub>4</sub>) adding 0.1, 1.0, or 10 μM of pH peptide. The reaction was started by adding ATP (3 mM final) and stopped after the indicated times by addition of 500 μL ice-cold stop buffer. Samples were transferred and treated as described for substrate transport. The amount of transported substrate was quantified by a fluorescence spectrometer (Cary Eclipse; Varian) at <i>λ<sub>ex</sub></i> = 492/517 nm (FL) in relation to the internal volume number of moles of fluorescent dextran at <i>λ<sub>ex</sub></i> = 650/668 μM (Alexa647). Samples without ATP were used as negative control and subtracted as background. Data are presented as means ± SD (n = 3).

**Substrate Binding.** Binding of fluorescently labeled substrates was analyzed by fluorescence polarization. A total sample volume of 20 μL in a 384-well format was chosen to minimize the protein consumption. pH peptide (50 nM) was titrated with increasing concentration of TmrAB in SEC buffer. The correlation coefficient of the amount of fluorescent dextran at <i>λ<sub>ex</sub></i> = 650/668 μM (Alexa647). Samples without ATP were used as negative control and subtracted as background. Data are presented as means ± SD (n = 3).

Supporting Information

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Movie S1. Structural interconversion between the inward- and outward-facing conformations of TmrAB. Using PyMOL, a morph was calculated between the inward-facing structure of TmrAB and a homology model based on the outward-facing state of Sav1866. Characteristic structural elements of the NBDs are highlighted in different colors: P-loop (Walker A), red; D-loop, green; signature motif, blue; Walker B, yellow; Q-loop, magenta; and H-switch, orange.

Other Supporting Information Files

SI Appendix (pdf)
Supplemental Information

Crystal structure and mechanistic basis of a functional homolog of the antigen transporter TAP

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\textsuperscript{1} A.N., C.T. and V.H. contributed equally to this work.

\textsuperscript{2} To whom correspondence should be addressed. Email: tampe@em.uni-frankfurt.de
**Supplementary Table**

**Table S1. Kinetic parameters of TmrAB for different substrates**

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<td>$K_m$&lt;sub&gt;substrate&lt;/sub&gt; (µM)</td>
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<td>$V_{max}$ (nmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>$k_{cat}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
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RRY<sup>FL</sup>CKSTEL: fluorescein-labeled peptide, RRY<sup>3H</sup>LKSTEL: tritiated peptide. The data are derived from measurements that were carried out at 68 °C for 3 min with 3 mM of Mg-ATP and 2.5 µg of TmrAB in 50 µg E. coli polar lipids/DOPC (7:3), buffer: 20 mM HEPES pH 7.5, 107 mM NaCl, 5% glycerol.

**Table S2. Root-mean-square deviations of Cα positions between TmrAB and other ABC transporter structures**

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Fig. S1. Crystallographic features of TmrAB. (A) Crystals of TmrAB are characterized by an extremely long unit cell axis of 1044 Å. The unit cell is shown in grey with protein molecules depicting the symmetry elements of the space group. (B) Representative X-ray diffraction image of a TmrAB crystal. The long unit cell axis results in extreme narrowly spaced diffraction spots.
Fig. S2. Structural asymmetry in extended loops. (A) Superposition of nucleotide-binding regions of TmrA, TmrB, TM288, and TM287 (PDB code 4Q4A) highlighting the extended A-loop in TmrA. (B) ClustalW multiple sequence alignment of the A-loop region. The conserved A-loop Tyr, which is an arginine in the non-canonical nucleotide-binding site of TmrB, is shown in red, physicochemically conserved residues in yellow. (C) Structural comparison of the periplasmic loop 1 (PPL1) between TM1 and TM2 in TmrA, TmrB, TM288, and TM287. (D) Sequence alignment of the PPL1 region.
**Fig. S3. Conserved salt bridge between elbow helix and TM6.** (A, B) Conserved Arg-Asp salt bridge in TmrA and TmrB, respectively. Salt-bridge residues are shown with the corresponding section of the 2Fo-Fc map contoured at 0.8σ. (C) ClustalW multiple sequence alignment of different ABC transporters highlighting the invariant salt-bridge residues.

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**Fig. S4. Comparison of X-ray structure with cryo-EM model of TmrAB.** The X-ray structure and the cryo-EM model of TmrAB were superimposed and the Cα atomic distance for each residue was calculated. (A) Distance plot for TmrA. The secondary structural elements are shown schematically below the plot; characteristic sequence motifs are marked. (B) Distance plot for TmrB. (C) Putty cartoon representation of TmrAB depicting the Cα atomic distances; the color spectrum blue-white-red covers a distance range of 0.2-3 Å. The correlation between cartoon thickness and atomic distance is shown at the bottom.
Fig. S5. Sequence conservation between the TMDs of TmrAB and TAP1/2.

Invariant residues are shown in red, physicochemically conserved residues in yellow. (A) Ribbon representation of the TmrAB structure in different orientations (successive rotations starting at the top left corner of the panel); invariant residues are depicted as sticks, the periplasmic gate is shown at the center of the panel. Residues that are also invariant in TM288/287, Sav1866, and MsbA, are labeled. The cavity is shown in cyan. (B) Sequence alignment of the TMDs of TmrA and TAP2. (C) Sequence alignment of the TMDs of TmrB and TAP1.
Fig. S6. Substrate binding to TmrAB. (A) Oxidative cysteine cross-linking of cavity-residue positions with fluorescent peptide (RRYCKFL-STEI) containing a free cysteine; competitor: RRYCFLKSTEI. (B) Binding of FL peptide (50 nM) to TmrAB variants (10 µM) was followed by fluorescence polarization and normalized to the wild-type binding values. Substitutions were introduced in Cys-less TmrAB (C416A), which displays the same substrate binding properties as wild-type. X-link: C416A,L595C/E568C under reducing (red.) and oxidizing conditions (ox.); Cys-less: TmrAC416A/TmrB. (C) H246C was labeled with ATTO674N-maleimide as demonstrated by SDS-PAGE (Coomassie-stained) and in-gel fluorescence (λex/em 630/670 nm).
Fig. S7. MD-simulated lipid interactions with the lateral gate and periplasmic loop 1, and binding in the substrate cavity. (A) Interactions of a POPE molecule with the lateral gate in TmrB. In the overview, TmrAB is shown with its electrostatic surface charge. (B) Binding of a POPG molecule, whose anionic headgroup rigidifies the extended periplasmic loop 1 (X-ray structure shown in violet). (C, D) Two possible binding modes of lipid A in the substrate binding cavity of TmrAB. In most regions around the perimeter of the TMD, lipids exchanged readily on the μs timescale of the simulations. However, lipid interactions persisted in all four simulations, for POPE and POPG, with (i) the lateral gate, (ii) the nearby N-terminal arm, (iii) the symmetry-related cleft in TmrA, (iv) the TmrA N-terminal region, and (v) a cluster of arginines between TM2B/R81B and TM5A/R255A. Shown are representative snapshots of the MD simulations. TmrA in cartoon representation is shown in wheat, TmrB in cyan. Lipids are shown in yellow. Dashed lines indicate polar interactions.
Fig. S8. Cross-linking as a probe to identify conformational changes during the catalytic cycle of TmrAB. (A) Model of cross-linked C-terminal zipper helices of the C416A,L595C^A/E568C^B mutant in the inward-facing state. The model was prepared using the program PyMOL by mutating Leu595^A and E568^B in the X-ray structure to cysteine. (B) Coomassie-stained SDS-PAGE with samples of the vanadate-trapping experiment. Cross-linking of the C-terminal zipper helices in C416A,L595C^A/E568C^B is less efficient when the mutant is vanadate-trapped in the presence of ATP, indicating that the zipper helices separate during NBD closure and nucleotide occlusion.
Fig. S9. Important polar interactions mediate the allosteric crosstalk within TmrAB. (A) Contacts between the intracellular loop 2 (ICL2) of TmrB and the NBD of TmrA. (B) Interactions between residues of the TmrA-ICL2 region and the NBD of TmrB. (C) Contacts between residues of the TmrA-TMD-NBD loop, TmrA-ICL1 and TmrB-ICL2, respectively, and the NBD of TmrA.
Fig. S10. Selenium-edge anomalous density and final 2Fo-Fc density map.
Selenomethionine (SeMet)-substituted protein was used to obtain phases and to guide model building. (A) The Se-edge anomalous density map contoured at 4σ is shown in surface representation (red) together with the model of TmrAB. (B) Close-up view of a model region; methionine side chains are shown as sticks, the anomalous density map is shown as red mesh. (C) Final 2Fo-Fc electron density map of TmrAB (contour level: 1.0σ).
Movie S1. Structural interconversion between the inward- and outward-facing conformations of TmrAB. Using PyMOL, a morph was calculated between the inward-facing structure of TmrAB and a homology model based on the outward-facing state of Sav1866. Characteristic structural elements of the NBDs are highlighted in different colors: P-loop: red; D-loop: green; signature motif: blue; Walker B: yellow; Q-loop: magenta; H-switch: orange.
Supplemental Experimental Procedures

Cross-linking of substrate with cavity residues
Purified and solubilized single-cysteine TmrAB mutants at a concentration of 1 µM were incubated in reaction buffer (20 mM HEPES pH 6.8, 150 mM NaCl, 1 mM β-DDM) at room temperature for 2 min with 5 µM fluorescein-labeled peptide containing a free cysteine (RRYCKFLSTEL). Control reactions were pre-incubated for 15 min with 500 µM of a fluorescein-labeled competitor peptide that lacks a free cysteine (RRYCFLKSTEL). Subsequently, 12.5 µM copper phenanthroline was added and the reactions were incubated for an additional 2 min to increase cross-linking efficiency. 5 mM N-ethylmaleimide (NEM) was added to stop the reaction. The samples were supplemented with non-reducing SDS-loading buffer and analyzed by SDS-PAGE. Bands were visualized by fluorescein fluorescence using an ImageQuant LAS 4000 (GE Healthcare) and by Coomassie staining.

Cross-linking of C-terminal helices in vanadate-trapped TmrAB
Purified and solubilized TmrA\textsuperscript{C416A,L595C}/TmrB\textsuperscript{E568C} was stored in reducing buffer (20 mM HEPES pH 6.8, 150 mM NaCl, 1 mM β-DDM, 30 mM TCEP) to avoid cross-linking. To initiate vanadate trapping, 0.25 mg/ml protein was supplemented with 10 mM vanadate, 10 mM MgCl\textsubscript{2}, and 10 mM ATP, ADP, or H\textsubscript{2}O. The solution was incubated for 5 min at 68 °C and the reducing buffer was directly exchanged to non-reducing buffer (20 mM HEPES pH 6.8, 150 mM NaCl, 1 mM β-DDM) via a Zeba Spin Desalting Column 7K (Thermo Fisher Scientific). To induce cross-linking, CuSO\textsubscript{4} was added (10 mM final) and incubated for 1 min before the reaction was stopped by addition of 8 mM N-ethylmaleimide (NEM). The samples were analyzed by non-reducing SDS-PAGE (Coomassie stain).

Molecular dynamics simulations
We conducted extensive (5 µs) all-atom explicit solvent molecular dynamics (MD) simulations of lipid-membrane embedded TmrAB starting from the X-ray structure. According to MCCE (1) electrostatics calculations for neutral pH, all Asp, Glu, Arg, and Lys residues were charged. All histidine residues were neutral, protonated at either their N\text{ð} or N\text{æ} atoms according to the network of hydrogen bonds, except the
charged H100B. Buried water molecules were added using DOWSER (2). To explore lipid effects, TmrAB was embedded (3) in a bilayer of 491 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) lipids (system 1) and in a bilayer of 518 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) lipids (system 2). Both systems were hydrated with 150 mM NaCl electrolyte in a simulation box of ~13×13×16.5 nm³. Consistently in all simulations, we used the all-atom CHARMM36 force field for TmrAB, lipids, and ions, together with TIP3P water (4-6). The parameters for lipid A were taken from Wu et al. (7). The MD trajectories were analyzed with Visual Molecular Dynamics (VMD) (8).

Simulations were performed using the NAMD 2.9 (9) and GROMACS 4.6 (10) simulation packages, taking advantage of their different functionalities and computational speeds. A first set of MD simulations of the two systems was performed using NAMD 2.9. After 10,000 steps of conjugate gradient energy minimization, 10 ns of MD simulation was carried out in which all non-hydrogen atoms of the protein were constrained to their initial positions using springs with progressively smaller force constants, starting at 15 kcal·mol⁻¹·Å². Analysis was carried out on unconstrained simulations, each 0.5 μs long. Periodic boundary conditions were used, with particle-mesh Ewald electrostatics, a 1.2 nm non-bonded cutoff, a time step of 2 fs, SHAKE constraints on all bond lengths, and a constant temperature of 310 K maintained by a Langevin thermostat with a coupling coefficient of 1.0 ps⁻¹. A Nosé–Hoover Langevin barostat was used to apply constant pressure of 1 bar normal to the membrane plane. The box shape in the x-y membrane plane was kept fixed. A second set of simulations was performed using GROMACS 4.6. The starting systems were minimized for 50,000 steps with steepest descent energy minimization and equilibrated for 100 ps in the NVT ensemble, then 10 ns of MD simulation in the NPT ensemble was carried out in which all non-hydrogen atoms of the protein were restrained to the fixed reference positions with progressively smaller force constants, starting at 4000 kJ·mol⁻¹·nm². Analysis was carried out on unconstrained simulations, each 2 μs long. Periodic boundary conditions were used. Particle-mesh Ewald with cubic interpolation and a 0.16 nm grid spacing was used to treat long-range electrostatic interactions. The time step was 2 fs. The LINCS algorithm was used to fix all bond lengths. Constant temperature (310 K) was set with a Nosé-Hoover thermostat, with a coupling constant of 0.5 ps. A semi-isotropic
Parrinello-Rahman barostat was used to maintain a pressure of 1 bar. All simulations with lipid A bound to TmrAB were performed using NAMD 2.9 following a similar protocol as described above. A lipid A molecule was placed in the substrate-binding cavity in several random orientations and after the equilibration runs, each run was extended for 30-50 ns. After identifying two distinct binding modes, two snapshots from the initial runs were used as the starting points for new runs of 120 ns duration. In the binding mode with the headgroup pointing upward, both H246\(_B\) and H87\(_A\) were assigned to be charged.

**Supplementary References**


    algorithms for highly efficient, load-balanced, and scalable molecular simulation. 