Conformational Coupling and trans-Inhibition in the Human Antigen Transporter Ortholog TmrAB Resolved with Dipolar EPR Spectroscopy

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Supporting Information

ABSTRACT: ATP-binding cassette (ABC) exporters actively move chemically diverse substrates across biological membranes. Their malfunction leads to human diseases. Many ABC exporters encompass asymmetric nucleotide-binding sites (NBSs), and some of them are inhibited by the transported substrate. The functional relevance of the catalytic asymmetry or the mechanism for trans-inhibition remains elusive. Here, we investigated TmrAB, a functional homologue of the human antigen translocation complex TAP using advanced electron–electron double resonance spectroscopy. In the presence of ATP, the heterodimeric ABC exporter exists in a tunable equilibrium between inward- and outward-facing conformations. The two NBSs exhibit pronounced asymmetry in the open-to-close equilibrium. The closed conformation is more favored at the degenerate NBS, and closure of either of the NBS is sufficient to open the extracellular gate. We define the mechanistic basis for trans-inhibition, which operates by a reverse transition from the outward-facing state through an occluded conformation. These novel findings uncover the central role of reversible conformational equilibrium in the function and regulation of an ABC exporter and establish a mechanistic framework for future investigations on other medically important transporters with imprinted asymmetry. Also, this study demonstrates for the first-time the feasibility to resolve equilibrium populations at multiple domains and their interdependence for global conformational changes in a large membrane protein complex.

INTRODUCTION

The presence of asymmetric nucleotide-binding sites (NBSs) is a general blueprint among ABC exporters including 20 out of the 48 human ABC proteins. TAP1/2 translocates antigenic peptides into the endoplasmic reticulum (ER) for loading onto major histocompatibility complex I (MHC I) molecules. Peptide-MHC I complexes are subsequently displayed on the cell surface for selective recognition and elimination by CD8+ cytotoxic T-lymphocytes. Recently, TmrAB from Thermus thermophilus has been identified as a functional homologue of human TAP, which can restore antigen processing in TAP-deficient cells derived from Bare Lymphocyte Syndrome patients. In TmrAB, the conserved catalytic glutamate and the A-loop tyrosine at the consensus NBS are replaced to an aspartate and arginine, respectively, at the degenerate NBS. Beside their overlapping function and imprinted asymmetry in the two NBSs, TAP and TmrAB share a similar overall structure.

In catalytically asymmetric ABC exporters, the two NBSs may hydrolyze ATP in an asymmetric manner. However, the mechanism for conformational coupling between the NBSs and the transmembrane domains (TMDs) remains unknown. While crystal structures reveal either a globally open or closed conformation of the nucleotide-binding domains (NBDs), how closure of the individual NBSs is coupled to the TMDs is yet to be answered. Also, how the substrate regulates the transporter via trans-inhibition is least understood. In order to investigate the structural implications of the asymmetric NBSs and the trans-inhibition mechanism in TAP orthologs, we introduced spin-label pairs via double cysteine mutants at the NBSs and TMDs in TmrAB and followed the conformational changes and the equilibrium populations using pulsed electron–electron double resonance (PELDOR or DEER) spectroscopy.

RESULTS

Nucleotide-Induced Conformational Changes in TmrAB. All spin-labeled mutants are active in transport of an antigenic peptide (Figure S1). Distances between the spin

Received: November 23, 2017
Published: January 8, 2018

DOI: 10.1021/jacs.7b12409
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX
pairs engineered on the C-terminal zipper helices confirmed the partially closed conformation of the NBDs as observed in the structure3 (Figure S2a). To monitor the conformational dynamics of the consensus and degenerate NBSs, we designed the spin pairs 461-349 and 416-458, respectively (Figure 1a). At the TMDs, the periplasmic spin pairs 61-56 and 288-272 (called periplasmic gate I and II) are located on the short loops between TM1-TM2 and TM5-TM6 helices, respectively. The spin pair 112-97 at the cytoplasmic gate is located on TM2.

TmrAB was observed in several intermediate states of the transport cycle: The prehydrolysis intermediate state was prepared with ATP plus 0.5 mM of EDTA, ATP-BeF$_3$–Mg$^{2+}$, and AMP-PNP-Mg$^{2+}$. The transition state was populated by trapping with ortho-vanadate (ADP-Vi-Mg$^{2+}$) or with AlF$_4^−$ (ADP-AlF$_4^−$-Mg$^{2+}$), the nonhydrolyzable ATP analog AMP-PNP induced only minor changes, consistent with previous observations on CFTR and TM287/28815,16 (Figures S2–S5). Compared to ATP alone, hydrolyzing conditions somewhat enhanced the closure

Figure 1. Conformational cycle in TmrAB. (a) The investigated positions at the NBDs and TMDs are highlighted on the apo structure (Protein Data Bank (PDB) accession 5MKK). (b–f) PELDOR data after removal of the intermolecular contribution on the left and the corresponding normalized distance distributions on the right. The asterisks indicate distance peaks with uncertainty in the shape and width corresponding to the length of the dipolar evolution time detected. For 416-458 apo and 288-272 ATP-Vi-Mg$^{2+}$ samples, 7-pulse CP PELDOR was used. Simulations on the apo structure using MMM$^4$ are shown in light blue green. The apo and ATP-Vi-Mg$^{2+}$ data for 61-56 have been published and were overlaid for completeness. ATP binding or vanadate trapping closes the NBSs and switches the TMDs from the inward-facing (IF) to the outward-facing (OF) conformation. The actual modulation depth values and data analysis are provided in Figures S2–S5.

Figure 2. NBSs and TMDs exhibit distinct conformational equilibriums (see Figure 1 for location of labeled positions). (a–c) The conformational equilibriums at the consensus NBS, degenerate NBSs, and the periplasmic gate at varying ATP concentrations as observed from PELDOR. Data for the apo (0 mM) and ADP-Vi-Mg$^{2+}$ states are shown as the reference points. $P(r)$ was obtained with two Gaussian fitting corresponding to the open and closed states (Figures S6 and S7). (d) Dose–response curves for the consensus and degenerate NBSs (green and blue respectively) and the periplasmic gate (red). The fractional closure ($\theta$, defined as $P_{\text{closed}}/(P_{\text{closed}} + P_{\text{open}})$) of the consensus and degenerate NBS as well as the open probability ($P_o$, defined as $P_{\text{open}}/(P_{\text{open}} + P_{\text{closed}})$) for the periplasmic gate are plotted against ATP. The open symbols (in solid borders) indicate $\theta$ or $P_o$ under hydrolyzing conditions (Figures S2 and S3) or after ATP-Vi-Mg$^{2+}$ trapping (in dotted borders on top). The horizontal line indicates the 50% closure (for NBSs), and the vertical lines show the corresponding ATP concentrations. Overall, $\theta$ and $P_o$ varied 5% or less between independent measurements.
of the NBSs (Figures S2 and S3). The interspin distances corresponding to the open and closed conformations are rather identical between detergent micelles (β-DDM) and proteoliposomes (Escherichia coli polar lipids/DOPC, Figures S2a,b and S4), suggesting that the nature of the surrounding environment does not affect the conformation, at least for the tested positions. Therefore, we performed further measurements in detergent micelles. It also significantly reduced the measurement time due to the improved transversal relaxation time ($T_2^*$) and provided high quality data for reliable distance determination. Overall, the large amplitude conformational changes with ATP or vanadate, BeF$_3^-$, and AlF$_3^-$ trapping at all the positions reveal a wide open OF conformation of TmrAB.

ATP Modulates Conformational Equilibrium at the NBSs and TMDs in TmrAB. TmrAB has a turnover of 9 ATP per second with a $K_{m,ATP}$ value close to 0.9 mM. Next, we gradually increased the ATP concentration to monitor the conformational equilibriums at both NBSs and the TMDs. ATP binds to the open conformation predominantly through interaction with the A-loop, Walker A, and Walker B motifs. This may lead to an allosteric interaction between the NBSs and a cooperative movement along the conformational space toward the closed conformation. Eventually, the equilibrium populations at each NBSs must be determined by the interplay between ATP affinity and the relative energy differences between open and closed states.

Surprisingly, ATP gradually enhanced the closure (θ) of both NBSs, revealing characteristic dose–response curves fingerprinting the two NBSs. The consensus NBS exhibited a saturation response reaching a 1:1 (close-to-open) equilibrium populations at 25 mM ATP, while it was nearly completely locked into a closed conformation upon vanadate trapping (Figures 2a,d and S6). Thus, the ATP affinity is not the major limiting factor for the closure. The unvaried 1:1 equilibrium implies that open and closed states have a similar energy. Importantly, the presence of magnesium ions under hydrolyzing conditions did not enhance the 1:1 equilibrium (Figure 2, open symbols in solid line, and Figure S6, orange line). In contrast, the degenerate NBS closed in a linear response to increasing ATP, revealing a significantly enhanced closure compared to consensus NBS (Figures 2b,d and S6). Thus, the closed conformation is more stable at the degenerate NBS, and it achieved the 50% closure at a 2-fold lower ATP concentration as compared to the consensus NBS. Remarkably, the degenerate NBS closes independent of the consensus NBS (Figure 2d). The D-loop may play an important role for this added stability. The mutation of the catalytic glutamate at the consensus NBS (E523Q) in TmrA increased the closure of
both NBSs, but with a pronounced enhancement of the asymmetric behavior in the closure (Figures S2b and S3).

Consistent with the response of the NBSs, increasing concentrations of ATP gradually enhanced the opening of the periplasmic gate II at the TMDs. (Figures 2c,d and S7). Plotting the open probabilities ($P_o$) against ATP gave the dose–response curve with a saturation point close to 10 mM ATP. This gate exhibited a small population of the open conformation even in the absence of any ATP (Figure 2c, 0 mM). Surprisingly, it showed an earlier transition to the open state as compared to the closure ($\theta$) of either of the NBSs, which also appears to be the case with periplasmic gate I and the cytoplasmic gate (Figure 1b, ATP). At 10 mM ATP, the $P_o$ value is almost equal to the sum of the closure ($\theta$) of the two NBSs. Therefore, closure of a single NBS appears to be sufficient to open the periplasmic gate. At 25 and 50 mM ATP, the gate opening correlates with the combined probability for having one of the NBSs being closed. Between the apo state (0 mM) and 50 mM ATP, the $P_o$ increases from 0.12 to 0.7. Thus, the closure of the NBSs following ATP binding shifts the equilibrium at the periplasmic gate toward the open conformation. Interestingly, this behavior of TmrAB qualitatively resembles more of an allosteric regulation as in ligand-gated ion channels than to the strictly coupled alternating-access mechanism for ABC transporters. In CFT, ATP enhanced channel activation in a concentration-dependent (0 to 30 mM) manner, and the gating by ATP exhibits features of allosteric regulation.21 As the closed conformation is more favored at the degenerate NBS even under hydrolyzing conditions, it might have a greater role in periplasmic gate opening. Due to the isoenergetic nature between the open and the closed states, the consensus NBS exists in a 1:1 equilibrium, which also may partially explain why only the degenerate NBS and not the consensus NBS could be photobleached with 8-azido ATP in CFTR. Therefore, the consensus NBS may control the lifetime of the OF state, and hydrolysis at this site might open both NBSs and restore the transporter to the IF state.

**Excess of the Peptide Substrate Reverses the Conformational Equilibrium to the Occluded State.** It has previously been shown that excess of the translocated substrate inhibits ATP hydrolysis and/or transport in human TAP and human lysosomal polypeptide transporter TAPL.20,23 However, the mechanism for this trans-inhibition in ABC exporters remains unknown. We employed a peptide substrate, which is transported by TmrAB and TAP with a $K_m$ value of 8.3 $\mu$M and 63 nM, respectively.3,20 The peptide had no influence on the conformational equilibrium when added at a 1:1 molar ratio with TmrAB (50 $\mu$M each, which is well above the $K_m$ value, Figure 3a, pink lines in 288-272). Thus, the substrate did not enhance transition to the OF conformation, which was determined rather by ATP binding at the NBSs. At 500 $\mu$M peptide, however, the OF population was significantly reduced at the periplasmic gates (61-56 and 288-272) with an increase of the IF population (Figures 3a and S8). Selective reduction of the OF population and the requirement for a nearly two orders higher concentration compared to the $K_m$ value reveal that the substrate interacts with the low-affinity binding site in the OF state.

With 500 $\mu$M peptide, the OF population was decreased at the cytoplasmic gate as well. Surprisingly, there was only a small increase of the IF population, and a novel closed conformation appeared as the major population (Figures 3a, 112-97). A simultaneously closed conformation of the periplasmic gates as well as the cytoplasmic gate is consistent with an occluded (OC) state as observed in McjD24 and PCAT25 structures (Figures 4b). At the periplasmic gates, the OC conformation is only poorly resolved due to the overlap of the distances with the IF conformation (Figure S8). Thus, excess of the peptide selectively reverses the OF transporters to the OC state. A small population of the transporters may reverse beyond the OC state to the IF state (see arrows on the OF population of 112-97). The closed conformation at the NBSs corresponds to either the OF19 or the OC state,24,25 and transition to the IF state opens the NBSs (Figure 5c). Excess of the peptide (500 $\mu$M) reduced the closed conformation with a corresponding increase of the open population at both NBSs (Figures 3b and S9). Overall, the IF population is rather small, which suggests that the OC state plays the central during trans-inhibition in TmrAB. The trans-inhibitory effect of the peptide was reduced under hydrolyzing conditions (Figure 3a,b, magenta lines). In line with the observations from the PELDOR experiments, the peptide inhibited ATPase activity in a concentration-dependent manner (Figures 3c and S10). Importantly, in ABC importers trans-inhibition operates via a different mechanism, where substrate binds to the regulatory C-terminal domains at the NBDs and stabilizes the transporter in the IF state.26,27

**DISCUSSION**

Asymmetric NBSs is a characteristic feature of heterodimeric ABC exporters as well as several human ABC exporters including CFTR, MRP1, and SUR1. TmrAB and TM287/288 structures revealed partially engaged NBSs,5,16 and our PELDOR data for the C-terminal zipper helices further validated this conformation, both in detergent micelles and proteoliposomes. However, BmrCD displays a wide-open conformation of the NBSs in the apo state and presence of nucleotides (AMP-PNP-Mg2+, ADP-Mg2+, or ATPyS-Mg2+) engaged the NBSs.28 Human CFTR, MRP1, and SUR1 (all single polypeptide exporters with asymmetric NBSs) also revealed disengaged NBSs, with SUR1 having the smallest
separation.\textsuperscript{20−31} In TmrAB, ATP fully engaged (closed) the NBSs, whereas addition of ADP-Mg\textsuperscript{2+} or AMP-PNP-Mg\textsuperscript{2+} had no visible effect. Moreover, titration experiments reveal that the closed conformation is more favored at the degenerate NBS, which was not observed in the EPR studies with BmrCD or TM287/288.\textsuperscript{16,28} Thus, ABC exporters with asymmetric NBSs display significant differences in the apo conformation as well as in the response of their NBSs to different nucleotides, which may have important roles for their function and regulation.

EPR studies with BmrCD and TM287/288 revealed a fundamental difference between them; it requires only ATP binding in TM287/288 whereas ATP hydrolysis in BmrCD for switching to the OF conformation.\textsuperscript{16,25} Though ATP binding is sufficient for IF-to-OF transition in TmrAB as well, modulation of this equilibrium in an allosteric manner by ATP reveals a novel feature of an ABC transporter. All the investigated positions on the TMDs suggested the presence of a small population of the OF conformation even in the absence of ATP (Figure 1). As observed with PgP and TM287/288,\textsuperscript{16,32} it might happen through an independent motion of the TMDs as either of the NBSs do not close without ATP. Moreover, ATP acted as a ligand to shift the IF-to-OF equilibrium and stabilized the latter conformation. These observations are in line with previous electrophysiology data for CFTR. ATP-dependent channel opening (with a low probability, \(P_\gamma\)) as well as ATP-dependent channel activation (in the mM range) has been observed in CFTR.\textsuperscript{15,23} This is analogous to the allosteric regulation in ligand-gated ion channels, where ligand binding biases the equilibrium to the open state. For CFTR, there is increasing evidence that ATP binding gates the channel activity in an allosteric manner.\textsuperscript{25} It appears that conformational switching in TmrAB and CFTR by ATP qualitatively resembles gating mechanism of ligand-gated ion channels.

Each NBD monomer consists of a RecA-like subdomain connected to an \(\alpha\)-helical subdomain through a flexible bilobal hinge.\textsuperscript{18} The A-loop, Walker A and Walker B motifs, and Q- and H-loops of the RecA domain interact with the ABC signature motif and the D-loop of the \(\alpha\)-helical subdomain of the opposite NBD to form two ATP binding sites (NBSs). ATP binding induces a rotation of the \(\alpha\)-helical subdomain toward the opposite NBD and \(\alpha\)-helical subdomain of the RecA-like subdomain.\textsuperscript{34} While the two NBSs may open or close independently in the presence of ATP,\textsuperscript{35−37} how that is coupled to the TMD conformation remains unknown. We addressed this question by individually observing the NBSs and the TMDs in the presence of ATP. While a closed conformation is more favored at the degenerate NBS, the consensus NBS exhibits an isoenergetic nature (1:1 equilibrium) between open and closed states. The E-to-Q mutation at the consensus site further enhanced this asymmetric response. The value for \(\theta\) (partial closure) increased from 0.27 to 0.50 and 0.38 to 0.90 for the consensus and degenerate NBSs, respectively, at 10 mM ATP (Figures S2 and S3), most likely due to an increase in ATP affinity.\textsuperscript{16} Surprisingly, closure of either of the NBSs appears to be sufficient to open the periplasmic gate located on the TMDs. This response enabled a more efficient switching of the TMDs to the OF conformation. Despite the low fractional closure of both NBSs, their combined effect (together with intrinsic opening without ATP) opened the periplasmic gate in a much larger fraction of the transporters, even at low ATP concentrations (Figure 2d).

Although TAP is an active transporter, peptide transport is inhibited by a high luminal peptide concentration.\textsuperscript{35} This phenomenon called trans-inhibition might protect the ER from stress response and the unfolded protein response at high peptide concentration inside the lumen. Surprisingly, excess of the peptide reversed OF transporters into the OC conformation (Figure 3). Thus, trans-inhibition involves the interaction of the peptide with a second low-affinity binding site in the OF conformation. Comparison of the interspin distances for the OC state with simulations on the corresponding homology model suggests a smaller cavity in TmrAB as compared to the antibacterial peptide exporter McjD\textsuperscript{24} (Figure 3a). The absence of substrate in the McjD structure or the smaller size of the peptide (9 vs 21 amino acids for TmrAB and McjD, respectively)\textsuperscript{24} might account for this difference. Only a small fraction of the transporters are further reversed to the IF conformation, which suggests a higher energy barrier for the OC-to-IF transition. Though the occluded conformation has been observed in the McjD and PCAT crystal structures, its relevance in the conformational cycle has remained elusive. Our results validate this conformation in the presence of substrate and also establish its central role for trans-inhibition in TmrAB.

The data presented here can be framed into a mechanistic model for transport and trans-inhibition (Figure 4). In a transport cycle, substrate must bind exclusively to the apo state as closure of either of the NBSs leads to the OC conformation. Subsequent ATP binding to one (or both) of the NBSs can spontaneously switch the transporter to the OC state. Under transporting conditions, OC state must be transient and immediately switch to the OF state as we observed it only under trans-inhibition conditions. The low-affinity binding pocket in the OF state acts as a release as well as a trans-inhibition site. Under transporting conditions, the substrate diffuses out from this pocket, whereas substrate binding in trans-inhibition reverses the conformational changes, mostly to the OC state. The reversibility reveals that ATP hydrolysis may follow substrate translocation, which is further supported by ATP-ase inhibition under trans-inhibition conditions (Figures 3c and S10). Also, hydrolysis might change the course of the pathway for the OF to the IF transition and make the transport irreversible. In agreement, the trans-inhibition was decreased under hydrolysing conditions (Figure 3a,b, magenta lines). ATP hydrolysis at the consensus site might dissociate both NBSs and restore the transporter to the IF conformation as recently suggested for CFTR.\textsuperscript{38}

In ABC exporters with catalytic asymmetry, ATP hydrolysis takes place mostly, if not exclusively, at the consensus NBS,\textsuperscript{2,17,22} and how this energy is coupled to transport is not well understood. In view of the existing biochemical data,\textsuperscript{15,21,39} the mechanistic basis for NBSs-TMD coupling, which we presented here, could be valid for CFTR as well. As the human TAP\textsuperscript{30} and TmrAB are inhibited by the same peptide (also the human TAP-L is inhibited by a similar peptide),\textsuperscript{31} the trans-inhibition mechanism might be conserved among these transporters. Further, our results may be relevant to other ABC exporters with catalytic asymmetry such as SUR1 or MRPI, all of which are implicated in human health and diseases. An understanding of the mechanistic basis of trans-inhibition in an ABC exporter presents an opportunity to develop new small therapeutic molecules to modulate the activity of these medically important transporters. Finally, a single mechanism may not fit for all,\textsuperscript{1,22} and each ABC exporter needs to be individually investigated to explore the specific details of its mechanism.
CONCLUSION

In summary, we uncovered the molecular mechanism for the conformational coupling between NBSs and TMDs as well as the structural basis for trans-inhibition in TmrAB, an ortholog of the human antigen transporter complex. We show that closure of either of the NBSs is sufficient to open the periplasmic gate located on the TMDs. During trans-inhibition, the peptide molecules interact with the OF transporters and populate the OC state. Thus, a reversible conformational equilibrium lies at the heart of the function and regulation in TmrAB. Moreover, our results demonstrate that dipolar EPR spectroscopy is a powerful tool to resolve conformational equilibriums at the individual domains within a large molecular complex. This allowed us to resolve the interdependence of those equilibriums for the global conformational change in TmrAB, for the first-time to our knowledge, in a large membrane protein complex.

METHODS

Cloning and Expression. Wild-type TmrAB was expressed and purified in Escherichia coli BL21 (DE3) cells as described previously.3,4 Cysteine mutants were prepared by site-directed mutagenesis.

Protein Purification. E. coli cells were resuspended in lysis buffer (20 mM HEPES pH 7.5, 300 mM NaCl) containing lysozyme (Sigma) and DNase I (AppliChem). Cells were disrupted using an automated French press (1.0 kbar, Basic Z; Constant Systems). Cell debris was removed by centrifugation at 10,000g for 15 min. Then the supernatant was centrifuged at 200,000g for 1 h to pellet the membranes. The membranes were suspended in solubilization buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 20 mM n-dodecyl-β-D-maltopyranoside (β-DDM; Roth) and incubated for 1 h at 4 °C. Afterward, the detergent-solubilized protein was separated by centrifugation at 200,000g for 30 min. His-tagged TmrAB was separated using Ni-NTA agarose (Qiagen) pre-equilibrated with the concentration of 10 mg mL−1. After incubation for 30 min at 4 °C, the detergent was removed stepwise over a period of 20 h using Bio-Beads (Bio-Rad). Proteoliposomes (1 mg mL−1 lipid) were incubated with 3 mM MgCl2, 1 mM ATP (supplemented with tracer amounts of [γ-32P]-ATP) with or without the peptide (RYYCFLKSTEL) in SEC buffer. After incubation at 68 °C, 1 μL of the reaction mixture was withdrawn at periodic intervals (5, 10, and 15 min) and applied onto polyethylenimine cellulose plates, and 32Pi was separated by thin-layer chromatography in 0.8 M LiCl and 0.8 M acetic acid mixture and quantified using autoradiography. The percent ATP hydrolysis was calculated from the comparison of the 32Pi intensity with the initial reaction mixture and for each peptide concentration, and the values are displayed as the average of the turnovers at the three different time points.

Spin-Labeling and Continuous-Wave EPR Spectroscopy. Cysteine variants of TmrAB were labeled with a 40-fold molar excess of methanethiosulfonate spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl, MTSSL) in SEC buffer for 30 min. Free spin probes were removed by Micro Bio-Spin 6 (Bio Rad) centrifugation. A labeling efficiency of 80–100% was determined for all the variants. Continuous wave (cw) EPR measurements were performed at X-band frequency (9.4 GHz) using a Bruker E500 spectrometer with a TE102 or SHQE cavity with the following experimental parameters: 100 kHz modulation frequency, 1.5 G modulation amplitude, 0.6 mW microwave power, 20.48 ms time constant, 81.92 ms conversion time, 1024 points, and 20 mT sweep width.

PELDOR/DEER Spectroscopy. EPR experiments were performed without nucleotides (apo state) or with Mg2+-ATP, Mg2+-ADP, Mg2+-AMP-PNP, or ATP alone (10 mM each). If not stated different, 0.5 mM EDTA was always added with the ATP sample, which diminished the ATPase activity below detection. To prepare samples under hydrolyzing condition, EDTA was omitted, and Mg2+-ATP (10 mM) was added. ADP-V-Mg2+ trapping was performed with Mg2+-ATP and orthovanadate (10 mM each). For ATP-BeF2−-Mg2+ trapping, 10 mM Mg2+-ATP with 16 mM sodium fluoride and 4 mM beryllium sulfate was used. For ADP-AlF4−-Mg2+ trapping, the latter was replaced with aluminum chloride. The typical protein concentration ranged between 30 and 60 μM. For the ATP titration experiments, samples contained 50 μM protein and 0.5 mM EDTA, and the ATP concentration was varied between 0.5 mM to 50 mM. For plotting the dose–response curves of the NBS, fractional closure θ was defined as (Pclosed/Popen + Popen)−1, where Popen stands for the calculated probability/population of the closed or open states in the presence of ATP. Similarly, for the periplasmic gate, the open probability Popen was defined as (Popen/Pclosed + Popen). The data for θ and Popen were fitted to the Hill equation y = (axθ)/(bx + xθ) to give the best fit (R2 = 0.99) with a Hill coefficient (n) of 1. Experiments with the peptide (RYYCFLKSTEL) contained 50 μM protein, 10 mM ATP, and 0.5 mM EDTA (which was omitted for the sample under hydrolyzing condition). All the samples were incubated at 68 °C for 5 min, followed by addition of 15–20% (v/v) deuterated glycerol. The equilibrium populations in the presence of ATP were found to be the same when the sample was frozen in liquid nitrogen straight from 68 °C or after brining to the room temperature. For PELDOR measurements, 10 μL of the sample was transferred into a 1.6 mm outer diameter quartz EPR tube (Suprasil, Wilmad LabGlass) and immediately frozen in liquid nitrogen.

All PELDOR data were recorded on an ELEXSYS ES800 EPR spectrometer (Bruker) equipped with a PELDOR unit (ES80–400L, Bruker), a continuous-flow helium cryostat (CF935, Oxford Instruments), an arbitrary waveform generator (SpinJet-AWG), and a temperature control system (ITC 502, Oxford Instruments). Pulsed experiments were performed at Q-band frequencies (33.7 GHz) using an ELEXSYS SuperQFT accessory unit and a Bruker AmpQ 10 or 150 W TWT (Applied Systems Engineering Inc.) amplifier with a Bruker ENS107D2 cavity at 50 K. With 4-pulse PELDOR (DEER), a dead-time free four-pulse sequence with phase-cycled π/2-pulse was

buffer. The liposomes were lysed with lysis buffer (1× PBS supplemented with 0.1% of SDS) for 10 min. The samples were transferred into a microtiter plate, and fluorescence was quantified using an ELISA reader (FULOstar Galaxy BMG) (λex/em 485/520 nm). The fluorescent peptide was synthesized and labeled as previously described.

ATPase Assay. 200 nM of TmrAB was incubated with 3 mM MgCl2, 1 mM ATP (supplemented with tracer amounts of [γ-32P]-ATP) with or without the peptide (RYYCFLKSTEL) in SEC buffer. After incubation at 68 °C, 1 μL of the reaction mixture was withdrawn at periodic intervals (5, 10, and 15 min) and applied onto polyethylenimine cellulose plates, and 32Pi was separated by thin-layer chromatography in 0.8 M LiCl and 0.8 M acetic acid mixture and quantified using autoradiography. The percent ATP hydrolysis was calculated from the comparison of the 32Pi intensity with the initial reaction mixture and for each peptide concentration, and the values are displayed as the average of the turnovers at the three different time points.

Transport Assay. Proteoliposomes (1 mg mL−1 lipid final) were incubated with 3 mM MgCl2, 3 mM ATP, and 3 μM fluorescent peptide (RYYCFLKSTEL) in transport buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 5% (v/v) glycerol) at 68 °C for 5 min. Reactions were stopped by adding four volumes of ice-cold stop buffer (10 mM Na2HPO4 1.8 mM KH2PO4 pH 7.3, 140 mM NaCl, 2.7 mM KCl (1× PBS), and 10 mM EDTA). Samples were transferred to a polyethylenimine-coated Multi Screen Filter Plate (Durapore membrane 0.65 μm; Millipore) and washed twice with five volumes of stop buffer. The liposomes were lysed with lysis buffer (1× PBS supplemented with 0.1% of SDS) for 10 min. The samples were transferred into a microtiter plate, and fluorescence was quantified using an ELISA reader (FULOstar Galaxy BMG) (λex/em 485/520 nm). The fluorescent peptide was synthesized and labeled as previously described.
used. The typical pulse lengths for the observer pulses were 16 and 32 ns (for π/2 and π pulses, respectively) and 12 ns (π pulse with 150 W) or 20 ns (π pulse with 10 W) for the pump pulse, which was set to the maximum of the echo-detected field sweep spectrum, and the observer pulses were set at 80 MHz lower. Deuterium modulations were averaged by increasing of the first interpulse delay by 16 ns for eight steps. For 7-pulse Carr-Purcell PELDOR, typical pulse lengths were 32 ns (π/2 and π) for the observer pulses and 400 ns (π) for the sech/tanh pump pulses. The interpulse delays τm, τp, and τr were chosen to be as same as possible while avoiding overlap with any of the unwanted echoes created by the four observer pulses. The second pump pulse was kept fixed in time, while the first and the third pump pulses were moved in equal but opposite time increments of 24 ns. The third pump pulse was reversed in time to avoid signal distortion from ringing. Following data acquisition, the form factor was obtained after background correction and artifact removal using a home-written MATLAB routine. DeerAnalysis software was used for determination of distance distributions. The normalized primary PELDOR data V(t)/V(0) were processed to remove the intermolecular contribution and the resulting form factors F(t)/F(0) were fitted with a model-free Tikhonov regularization or Gaussian function(s) to distance distributions. The MATLAB based MMM software was used for simulation of interspin distances based on the crystal structures. Homology models were generated with SWISS-MODEL using Sali1866-AMP-PNP (PDB 2ONJ) or the McJd (PDB 4PLD) structures as the templates against the amino acid sequences of TmrA and TmrB.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b12409.

Details of protein expression, purification, spin labeling, transport assays, ATPase assays, PELDOR/DEER measurements, and data analysis are presented (PDF).

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**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the German Research Foundation Grants SFB 807 – Membrane Transport and Communication and Cluster of Excellence EXC 115 – Dynamics of Macromolecular Complexes (to R.T., T.F.P., and B.J.).

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Conformational coupling and trans-inhibition in the human antigen transporter ortholog TmrAB resolved with dipolar EPR spectroscopy

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**Figure S1.** Transport and ATPase activity of TmrAB. (a) The transport activity of spin labeled TmrAB is given as percentage of the wildtype, which was 5 nmol min\(^{-1}\) mg\(^{-1}\) protein. The transport activity for the periplasmic gate I variant (61-56) has been previously published.\(^1\) The mutation of the catalytic glutamate (E523Q) in TmrA abolished the transport activity. (b) The ATPase activity of TmrAB is inhibited by 0.5 mM EDTA. The values are given as the percentage of the activity at 5 mM ATP. All the values are given as mean ± SD (\(n = 3\)).
Figure S2. The NBDs exhibit a partially closed conformation. (a) The global conformation of the NBDs was monitored by measuring distances between spin labels placed on the C-terminal zipper helices of TmrAB solubilized in β-DDM. Corresponding simulations on the TmrAB apo structure (PDB 5MKK) is shown in grey. Original PELDOR data are shown in the left panels and the background corrected data in the middle and the corresponding distance distributions on the right. The asterisks indicate distance peaks with uncertainty in the shape and width corresponding to the length of the dipolar evolution time detected. Data analysis is performed with DeerAnalysis software. The error bars on 578-555 distance distribution indicate the maximum variation of the P(r) when the
beginning of the background function (ending at 3.9 µs) was systematically varied between 1 to 3 µs. For both positions, a part of the experimental distances agrees with the simulations and reveal a more dynamic conformation than observed in the structure. However, overall the results support the partially closed conformation of the NBDs. (b) Conformational cycle at the consensus NBS. The mean interspin distance decreases by about 1 nm during the open-to-close transition upon ortho-vanadate, AlF₄⁻ or BeF₃⁻ trapping. ATP alone populates an equilibrium and AMP-PNP-Mg²⁺ or ADP-Mg²⁺ fails to close the NBS. The mutation of the catalytic glutamate (E523Q) in TmrA or ATP under hydrolyzing conditions enhanced the closure, for the latter a two Gaussian fitting as described below is overlaid. The distance distributions were obtained with Tikhonov regularization. A similar open-to-close transition is observed in proteoliposomes (E. coli polar lipids/DOPC). The distributions in proteoliposomes were obtained from Gaussian fitting with 3.1 ± 0.4 nm and 3.9 ± 0.5 nm corresponding to the open and closed states (shown in green) and the results from Tikhonov regularization are overlaid (grey dotted lines). The vertical lines indicate the most probable distance in the closed (red) and open (black) states.
Figure S3. Conformational changes at the degenerate NBSs. The apo data was obtained using 7-pulse CP PELDOR and the middle panel shows the background corrected data with the artifact (grey), the computed artifact (blue), and the artifact subtracted data (green) with the fitting from Tikhonov regularization (black dotted lines). The artifact comes from the non-ideal behavior of the broadband sec/tanh inversion pump pulses. The corresponding 4-pulse PELDOR data is shown below for a comparison, which was background corrected to fit the two Gaussian distributions corresponding to the open and closed states (apo and ADP-V_i-Mg^{2+} samples respectively). The ADP-V_i-Mg^{2+} data (which contains a fraction of the apo state due to incomplete reaction) was also fitted with Gaussians functions after fixing 5.7 ± 0.3 nm corresponding to the apo state as obtained from the Tikhonov regularization (first panel). This gave 3.8 ± 0.4 nm for the closed conformation, identical to the result from Tikhonov regularization (black dotted line). The AMP-PNP-Mg^{2+} data is fitted with a single Gaussian corresponding to the apo state and the rest of the data were fitted with two Gaussians corresponding to the open and closed states (5.7 ± 0.3 nm and 3.8 ± 0.4 nm respectively). The mean interspin distance decreases by about 2 nm during open-to-close transition upon ortho-vanadate or AlF<sub>4</sub> trapping. ATP alone populates an equilibrium and AMP-PNP-Mg^{2+} or ADP-Mg^{2+} fails to close the NBS. The E-to-Q mutation or ATP under hydrolyzing conditions enhanced the closure. The original data in the apo state for the E-to-Q mutation (black, marked as E-to-Q - apo) is overlaid with the wild-type data (grey dotted, marked as apo) to show the similarity.
Figure S4. Conformational changes at the periplasmic gate II. The ADP-V_i-Mg^{2+} data was obtained using 7-pulse CP PELDOR as described for the degenerate NBS (Figure S3). The fitting from Tikhonov regularization (black dotted lines) is overlaid on the two Gaussian fitting (green line) with 5.2 ± 0.3 nm (open, ADP-V_i-Mg^{2+} state) and 3.4 ± 0.3 nm (closed, apo state, top panel). The corresponding 4-pulse PELDOR data is shown below for a comparison, which was background corrected to fit the two Gaussians corresponding to the open and closed states. For the rest of the data, ADP-Mg^{2+} was fitted with Tikhonov regularization and the others with the two Gaussians as described above. The mean interspin distance increases by about 2 nm during IF-to-OF transition upon ortho-vanadate, AlF_4^−, or BeF_3^− trapping. Similar conformational changes are observed in proteoliposomes as well. In line with the observations for the NBSs, ATP alone populates an equilibrium between open and closed states.
Figure S5. Conformational changes at the cytoplasmic and the periplasmic gates. The distance distributions were obtained with Tikhonov regularization. When the traces are not sufficiently long for precise distance determination, the original data (left) are overlaid to reveal the similarity. (a) Data for the cytoplasmic gate. For the apo data, the error bars indicate full variation of the probability when the beginning of the background function (ending at 4.8 µs) was systematically varied between 1 to 3 µs.

b, Data for the periplasmic gate I. The asterisks indicate distance peaks with uncertainty in the shape and width corresponding to the length of the dipolar evolution time detected. The apo, ADP-V-Mg\textsuperscript{2+}, ADP-Mg\textsuperscript{2+}, and AMP-PNP-Mg\textsuperscript{2+} data has been previously published. During the IF-to-OF transition, the mean interspin distance decrease by more than 1 nm at the cytoplasmic gate, whereas the periplasmic gate I opens by 2 nm. AMP-PNP-Mg\textsuperscript{2+} or ADP-Mg\textsuperscript{2+} did not induce such changes and ATP alone populates an equilibrium between open and closed states.
Figure S6. ATP modulates the conformational equilibriums at the NBSs. (a, b) PELDOR data were acquired under varying ATP concentrations and at a fixed protein concentration. For the apo (0 mM) and the ADP-V_i-Mg^{2+} reference samples, fitting from Tikhonov regularization and Gaussian fitting are indicated in dotted and solid lines respectively. (a) Titration data for the consensus NBS. The open conformation (0 mM) has a mean distance at 3.9 ± 0.5 nm and the closed conformation (ADP-V_i-Mg^{2+}) has a mean distance at 3.1 ± 0.4 nm. The titration data sets were fitted as a linear combination of those two conformations. The dotted lines (for 0.5 to 50 mM) show fitting from Tikhonov regularization. The consensus NBS exhibited a saturation response at 25 mM ATP (see also Figure 2d). (b) Titration data for the degenerate NBS. The open (0 mM) state was determined with 7-pulse CP PELDOR (Figure S3 for data analysis and corresponding 4-pulse PELDOR). The open and closed states are centered at 5.7 ± 0.3 and 3.8 ± 0.4 nm respectively (see Figure S3) and the titration data sets were fitted as a linear combination of those two states. The degenerate NBS closed in a linear response to increasing ATP. For both NBSs, the original data under hydrolyzing conditions (in orange, taken from Figure S1-2) are overlaid with 50 mM ATP.
Figure S7. ATP modulates conformational equilibria at the periplasmic gate. The ADP-Vi-Mg\textsuperscript{2+} state was determined with 7-pulse CP PELDOR (see Figure S4 for data analysis and corresponding 4-pulse PELDOR). The open (0 mM) and the closed states are centered at 5.2 ± 0.3 nm and 3.4 ± 0.3 nm respectively. The titration data sets were fitted as a linear combination of those two states (solid lines). Similar to P-gp (ABCB1)\textsuperscript{4} and TM287/288\textsuperscript{5}, this gate exhibited a small population of the open conformation (~12%) even in the absence of any nucleotides, as revealed from both Tikhonov regularization and Gaussian fitting. The top panel in the middle shows a zoomed-in view of the comparison of the original data (black) with the simulation (green) in absence of the open population, which further validates the presence of this small population in the time-domain data. Consistent with the response at the NBSs, increasing concentrations of ATP enhanced the opening of the periplasmic gate II.
Figure S8. Excess of the peptide substrate reverses the conformational equilibria at the periplasmic and the cytoplasmic gates. PELDOR data were acquired with 50 µM of protein and 10 mM of ATP. 0 µM (green), 50 µM (pink) and 500 µM (orange) peptide or the latter under hydrolyzing conditions (purple) was tested to explore the effect of the substrate on the conformational equilibrium. (a) Data for the periplasmic gate I. \( P(r) \) was obtained using Tikhonov regularization. The asterisks indicate distance peaks with uncertainty in the shape and width corresponding to the length of the dipolar evolution time detected. (b) Data for the periplasmic gate II. \( P(r) \) was obtained with two Gaussians fitting corresponding to the open and closed states, which were independently determined (see Figure S4). Fitting from Tikhonov regularization is overlaid for the sample containing 500 µM of peptide. At a 1:1 ratio with protein (50 µM each), the substrate does not have any visible influence on the equilibrium (288-272, pink line). However, at a 1:10 molar ratio (500 µM peptide), the substrate selectively reduces the OF conformation and increases the population of the IF (closed) conformation.
at both gates. c, Data for the cytoplasmic gate. P(r) was obtained using Tikhonov regularization. The long distances in the IF conformation are well defined in the ATP-EDTA sample (green), which also facilitated an unambiguous background correction for the other data in presence of the substrate. At 500 µM peptide the OF population decreases, but with a rather small increase of the IF conformation and a novel closed conformation appears to be the major population. A closed conformation at all the gates suggest an occluded (OC) state of the TMDs. Due to the similarity, the IF and the OC states cannot be resolved at the periplasmic gates, whereas all the three conformations are well resolved at the cytoplasmic gate (also, see Figure 3). The simulations for the corresponding positions on the homology model for TmrAB based on the McjD (PDB 4PL0) occluded structure is overlaid (red dotted lines). A rather good agreement is observed at the periplasmic gate I, whereas the periplasmic gate II (288-272) and the cytoplasmic gate (112-97) conformations show large differences (see the comparison of the homology models at the bottom). This could be explained by the presence of the substrate (which is absent in the structure) in the PELDOR experiment as well as the smaller size of the substrate for TmrAB (9 vs. 21 amino acids). Hydrolyzing conditions partially restored the effect of excess (500 µM) peptide at all the gates. (d) Relative positions of the gates in the occluded (OC, red), the OF (green) homology models (based on McjD (PDB 4PL0) and Sav1866 AMP-PNP (PDB 2ONJ) structures respectively), or in TmrAB apo structure (PDB 5MKK, in grey with the helices carrying the spin labels in blue) are highlighted with the corresponding Cα atoms in ball representation. For each position, the distance between those Cα atoms in the three conformations are also indicated.
Figure S9. Excess of peptide substrate reverses the conformational equilibria at the NBSs. (a, b) PELDOR data acquisition is as described in Figure S8. The original data are overlaid on the top left panels for each NBS to provide a direct comparison of the changes in the equilibrium populations in the time-domain data. For the consensus NBS (461-349), data was fitted using Tikhonov regularization. The asterisks indicate distance peaks with uncertainty in the shape and width corresponding to the length of the dipolar evolution time detected. The degenerate NBS (416-458) data were fitted with two Gaussians corresponding to the open and closed states, which were independently determined (see Figure S3). As revealed from the crystal structures of homodimeric ABC exporters (Sav1866-AMP-PNP, PDB 2ONJ; McjD, PDB 4PL0), the NBSs are closed both in the outward-facing (OF) and the occluded (OC) states (see the comparison between the conformations at the bottom). Excess of the substrate selectively reduces the OF population at the TMDs as shown in Figure S8. Further, the data for the cytoplasmic gate reveal that a small fraction of the transporters are reversed to the IF conformation passing the OC state (Figure S8c), which would require the opening of the NBSs in those transporters. In agreement, the population of the closed conformation at both NBSs is partially reduced at 500 µM peptide. (c) The TmrAB apo structure (PDB 5MKK) and the homology models for the OC and OF conformations based on the McjD and Sav1866 structures respectively. The structures were overlaid and then copied from the same frame for a direct comparison. While the TMDs transition through three distinct conformations, overall the NBSs stay either in the open or the closed conformations.
Figure S10. Excess of the peptide substrate inhibits ATPase activity in TmrAB. The ATPase activity decreased upon increasing the substrate concentration. These results are in line with the observations from the PELDOR experiments. ATP alone populates an equilibrium between IF and OF states (Figure 1). Presence of an excess of the substrate (with ATP) selectively reverses the OF conformation, mostly to the OC conformation (Figure 3 and Figures S8-9), which might be accompanied with a partial reduction of the ATPase activity. Trans-inhibition is observed under hydrolyzing conditions as well, though less pronounced as compared to ATP alone, which also might explain the requirement for a higher peptide concentration to inhibit the ATPase activity. In summary, results from ATP titration (PELDOR) combined with the ATPase assay reveal that the substrate trans-inhibits TmrAB in a concentration-dependent manner.

References