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ATP-driven conformational dynamics reveal hidden intermediates in a heterodimeric ABC transporter

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eLife Assessment

The study presents **important** findings revealing previously unresolved conformational dynamics of the heterodimeric type IV ABC transporter TmrAB using single-molecule FRET. The evidence presented is **convincing**, integrating careful experimental design with computational approaches to uncover states that are typically masked and difficult to detect. The work will be of interest to scientists studying the molecular mechanisms of primary active transport processes.

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Abstract

ATP-binding cassette (ABC) transporters are essential molecular machines whose conformational dynamics have largely been inferred from ensemble-averaged measurements. Resolving dynamic heterogeneity and transient intermediates, however, requires single-molecule approaches. Here, we use single-molecule Förster resonance energy transfer (smFRET) to resolve ATP-driven conformational dynamics of the heterodimeric type IV ABC transporter TmrAB, a functional homolog of the human antigen transporter TAP, at the level of individual molecules. Fluorophores positioned at the nucleotide-binding domains and periplasmic gate were validated by accessible-volume simulations, fluorescence lifetimes, and ensemble FRET, demonstrating that these reporters reliably track conformational transitions. Single-molecule analysis distinguishes ATP-free and ATP-bound states and quantifies ATP-dependent population shifts from nucleotide-free to physiological ATP concentrations. Kinetic analysis further reveals an unexpectedly long ATP-bound dwell time of ~300 ms. Using complementary stabilization strategies, we directly resolve a previously hidden outward-facing open state that is kinetically masked under turnover conditions. These results provide the first single-molecule characterization of TmrAB and establish a quantitative single-molecule framework for dissecting ATP-coupled conformational dynamics in heterodimeric ABC transporters.

Impact Statement

ATP-driven single-molecule imaging uncovers hidden outward-facing intermediates and unexpectedly long-lived ATP-bound states in the heterodimeric ABC transporter TmrAB, revealing how conformational heterogeneity shapes transport dynamics.

Introduction

ATP-binding cassette (ABC) transporters constitute the largest family of primary active membrane transport systems, conserved across all domains of life¹⁻³. Despite considerable structural diversity, all ABC transporters share a modular architecture comprising two conserved nucleotide-binding domains (NBDs)—the defining hallmark of the family—and two transmembrane domains (TMDs) that form the substrate translocation pathway^{3,4}. Based on their TMD architecture, ABC transporters are classified into seven types that encompass importers, exporters, extractors, and

mechanotransmitters⁵. Substrate translocation is driven by large conformational changes that are chemo-mechanically coupled to ATP binding, hydrolysis, and phosphate/ADP release^{2,3}. ABC transporters play central roles in cellular homeostasis, nutrient uptake, waste removal, and toxin defense. Their dysfunction and misregulation are linked to numerous diseases and drug resistance⁶.

The heterodimeric type IV ABC transporter TmrAB from *Thermus thermophilus* has emerged as a powerful model system due to its exceptional thermal stability and functional homology to the transporter associated with antigen processing (TAP1/2), a key component of adaptive immunity⁷⁻⁹. Notably, TmrAB shares overlapping peptide specificity with TAP and can restore antigen presentation in TAP-deficient human cells¹⁰. Its inherent asymmetry, with one catalytically active (canonical) and one inactive (noncanonical) nucleotide-binding site (NBS), provides a unique opportunity to investigate functional specialization and asymmetry in ABC transport mechanisms.

Extensive structural studies, particularly using cryogenic electron microscopy (cryo-EM), have delineated the conformational landscape of TmrAB and yielded a detailed model of its translocation cycle^{11,12}. In this model, TmrAB fluctuates between inward-facing wide and narrow conformations (IF^{wide} and IF^{narrow}), characterized by a sealed periplasmic gate (PG) and well-separated NBDs, thereby permitting substrate access to the central binding cavity. ATP binding to both NBDs induces NBD dimerization and drives the transition into the outward-facing (OF) states, including an OF open (OF^{open}) conformation with an open PG that enables substrate release into the periplasm, as well as an OF occluded (OF^{occluded}) state characterized by a sealed PG and dimerized NBDs. Subsequent ATP hydrolysis and phosphate release lead to asymmetric unlocked return states (UR^{asym} and UR^{asym*}), before the transporter returns to the IF conformation. These UR states feature a sealed PG, a partially open ADP-bound canonical NBS, and a tightly ATP-occluded noncanonical NBS¹¹.

Single-turnover experiments have shown that ATP binding, rather than hydrolysis, drives the IF-to-OF transition, while phosphate release precedes the OF-to-IF switch¹²⁻¹⁴. Complementary ensemble approaches, including pulsed electron-electron double resonance (PELDOR/DEER) spectroscopy, have further characterized ATP-dependent conformational changes^{15,16}. However, ensemble averaging inherently masks molecular heterogeneity, obscures inactive or misfolded subpopulations, and limits access to kinetic information.

Single-molecule techniques overcome these limitations by resolving conformational dynamics at the level of individual molecules^{17,18}. In particular, single-molecule Förster resonance energy transfer (smFRET) enables real-time monitoring of protein conformational changes with nanometer precision¹⁹⁻²². Applied to ABC transporters, smFRET provides a unique opportunity to dissect transport cycles, resolve transient intermediates, and extract kinetic and mechanistic insights that remain inaccessible to ensemble-based measurement approaches²³⁻²⁵.

Here, we apply total internal reflection fluorescence (TIRF) microscopy combined with alternating laser excitation (ALEX)-based smFRET to detergent-solubilized heterodimeric ABC transporter TmrAB, providing the first single-molecule characterization of this system. By strategically positioning fluorophore pairs, we directly monitor ATP-dependent NBD dimerization and periplasmic gate (PG) opening, quantify conformational state occupancies across ATP concentrations ranging from nucleotide-free to physiological levels (3 mM), and uncover conformational dynamics previously masked by ensemble averaging. Using three orthogonal trapping strategies—(i) a slow-turnover mutant^{11,12}, (ii) Mg²⁺ depletion^{14,15}, and (iii) substrate trans-inhibition^{26,27}—we resolved a previously hidden outward-facing open (OF^{open}) state that rapidly exchanges with the outward-facing occluded (OF^{occluded}) state. Distance measurements derived from smFRET closely matched predictions from accessible-volume (AV) simulations, cryo-EM structures, and PELDOR/DEER spectroscopy, confirming that detergent-solubilized TmrAB retains a native-like conformational landscape. Together, these results provide the first single-molecule quantification of conformational state occupancies for a heterodimeric type IV ABC transporter and establish TmrAB as a versatile model for single-molecule studies of ABC transport systems.

Results

Design of FRET-labeled TmrAB variants to probe conformational dynamics

To monitor conformational changes in distinct regions of TmrAB, we engineered double-cysteine variants for smFRET targeting the nucleotide-binding domains (NBDs) and the periplasmic gate (PG). The NBDs undergo ATP-dependent dimerization and post-hydrolysis dissociation, whereas the PG opening and closing controls substrate release into the periplasm^{2,3,11}. Probing both regions provides complementary readouts of cytosolic and periplasmic coupling during transport.

Labeling positions were selected based on prior PELDOR/DEER studies¹⁶. The NBD reporter variant (TmrA^{C416B^{L458C}}, referred to as TmrAB^{NBD}) monitors the noncanonical nucleotide-binding site (NBS), while the PG reporter (TmrA^{C416A}, T61C^{B^{R56C}}, hereafter TmrAB^{PG}) reports PG opening. In TmrAB^{NBD}, the native single cysteine (C416) was retained for labeling, whereas in TmrAB^{PG} it was substituted by alanine to prevent off-target labeling. Using the noncanonical NBS prevents direct distinction between OF^{occluded} and asymmetric unlocked return states (UR^{asym} and UR^{asym*})¹¹, reducing the number of resolvable FRET states and simplifying data interpretation.

Both variants were labeled with photostable LD555/LD655 fluorophores containing a 1,3,5,7-cyclooctatetraene moiety to suppress photobleaching and blinking^{28,29}. Accessible-volume (AV) simulations³⁰ across nine cryo-EM structures¹¹ confirmed that donor–acceptor distances (R_{DA}) and simulated FRET efficiencies (E_{sim}) lie within the measurable range (Fig. 1 and Table 1). For TmrAB^{NBD}, E_{sim} shifts from 0.62 ± 0.02 (57.9 ± 0.7 Å, NBDs separated) to 0.84 ± 0.01 (45.2 ± 0.4 Å, NBDs dimerized), corresponding to predicted ΔE_{sim} of 0.22 ± 0.02 and ΔR_{DA} of 12.7 ± 0.8 Å. For TmrAB^{PG}, E_{sim} changes from 0.96 ± 0.02 (30.6 ± 0.4 Å, closed PG) to 0.69 ± 0.03 (53.8 ± 2.0 Å, open PG), corresponding to predicted ΔE_{sim} of 0.27 ± 0.04 and ΔR_{DA} of 23.2 ± 2.0 Å.

A slow-turnover TmrAB variant (TmrA^{C416A}, E523Q, T61C^{B^{R56C}}, hereafter TmrAB^{PG-EQ}) was also generated to kinetically stabilize ATP-bound conformations. This mutation reduces ATP hydrolysis by ~1000-fold, extending the catalytic half-life of ~25 min at 45 °C^{11,12,14,15}.

TmrAB variants are suitable for FRET studies

TmrAB variants were expressed in *E. coli* and purified by immobilized metal-affinity chromatography. SDS-PAGE and size-exclusion chromatography (SEC) confirmed purity and monodispersity of the samples (Fig. 1–Fig. S1a,b). ATPase assays showed that TmrAB^{wt} retained enzymatic activity after purification, yielding a Michaelis-Menten constant (K_m) of 0.97 ± 0.28 mM and turnover rate (k_{cat}) of 2.57 ± 0.38 s^{−1} at 40 °C (Fig. 1–Fig. S1c), consistent with previous reports^{9,10,12}.

Site-specific labeling yielded >90% labeling efficiency per cysteine, with ~40–50% per-site labeling efficiency for donor-only and acceptor-only populations (Fig. 1–Fig. S1d–f). Stochastic labeling of the TmrAB variants results in both homo (donor-donor, acceptor-acceptor) and hetero (donor-acceptor) labeled species. Only complexes with appropriate donor–acceptor stoichiometry were included in subsequent analyses.

Fluorescence lifetime (τ) analysis confirmed that the conjugated fluorophores retained their photophysical properties and sufficient rotational freedom for reliable FRET measurements. τ histograms of both conjugated and free fluorophores were fitted using a biexponential decay model, from which amplitude-weighted average lifetimes were calculated. For TmrAB^{NBD}, average τ values were 0.93 ± 0.02 ns (LD555) and 1.52 ± 0.01 ns (LD655), whereas TmrAB^{PG} exhibited average τ values of 0.95 ± 0.02 ns (LD555) and 1.65 ± 0.01 ns (LD655) (Fig. 1–Fig. S2a). By comparison, free dyes in buffer displayed lifetimes of 1.12 ± 0.02 ns (LD555) and 1.29 ± 0.01 ns (LD655). The similarity between free and conjugated dye lifetimes, together with comparable values across variants, indicates minimal protein-induced quenching and supports sufficient orientational averaging for quantitative smFRET analysis^{18,31–34}.

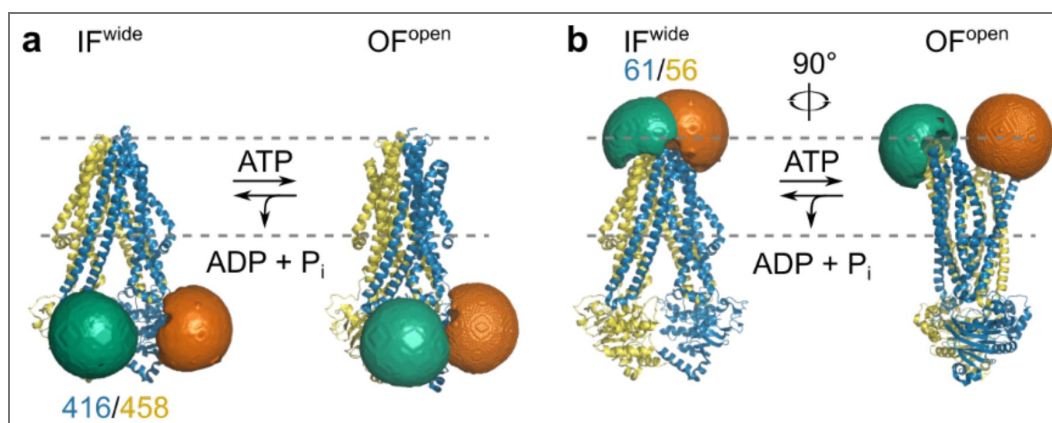
Table 1. Accessible-volume (AV) simulations of LD fluorophores on TmrAB variants.

AV simulations were performed for LD555 (donor) and LD655 (acceptor) fluorophores attached to the selected TmrAB labeling sites to assess whether donor-acceptor distances are suitable for smFRET measurements³⁰. AV simulations confirm that donor-acceptor distances (R_{DA}) remain within the FRET-sensitive range in both conformations, predicting measurable changes in simulated FRET efficiencies (E_{sim}). Cryo-EM structures of TmrAB reconstituted in lipid nanodiscs¹¹ were used as templates, including apo and ATP-bound (3 mM ATP) states. Outward-facing open (OF^{open}) and outward-facing occluded ($OF^{occluded}$) structures were obtained via orthovanadate trapping (V_i) or the slow-turnover mutant TmrA^{E523Q}B (EQ).

Conformation		PDB ID	TmrAB ^{NBD}		TmrAB ^{PG}	
			RDA (Å)	Esim	RDA (Å)	Esim
IF ^{narrow}	(turnover)	6RAF	58.6	0.60	33.0	0.95
IF ^{wide}	(turnover)	6RAG	57.2	0.63	30.2	0.96
IF ^{wide}	(apo)	6RAN	57.8	0.62	30.3	0.96
OF ^{open}	(V_i)	6RAJ	44.8	0.84	55.8	0.66
OF ^{open}	(EQ)	6RAH	44.9	0.84	51.8	0.72
OF ^{occluded}	(V_i)	6RAK	45.4	0.83	27.0	0.98
OF ^{occluded}	(EQ)	6RAI	45.5	0.83	32.4	0.95
UR ^{asym}	(turnover)	6RAL	45.9	0.83	31.2	0.96
UR ^{asym*}	(turnover)	6RAM	47.6	0.80	31.2	0.96

Figure 1. Accessible-volume (AV) simulations of LD fluorophores on TmrAB variants.

AV simulations were performed for LD555 (donor) and LD655 (acceptor) fluorophores attached to the selected TmrAB labeling sites to assess whether donor-acceptor distances are suitable for smFRET measurements³⁰. **a**, TmrAB^{NBD} (TmrA^{C416A}B^{L458C}) and **b**, TmrAB^{PG} (TmrA^{C416A}, T61C^BR56C) in the inward-facing wide (IF^{wide}; PDB: 6RAN, left) and outward-facing open (OF^{open}; PDB: 6RAH, right) conformations. The approximate membrane position is indicated by the dashed grey line. TmrA is shown in blue with LD655 (orange) and TmrB in yellow with LD555 (green), with labeling positions indicated (416/458 for TmrAB^{NBD}; 61/56 for TmrAB^{PG}). For illustration, fluorophores are shown in a defined configuration; however, in experiments donor and acceptor dyes are stochastically attached, resulting in random distribution between subunits.



Ensemble ATP titration (0–10 mM ATP) showed ATP-dependent donor quenching and acceptor sensitization (Fig. 1–Fig. S2b–d). ATP-induced fluorescence changes provided a quantitative readout of conformational transitions and enabled estimation of apparent equilibrium dissociation constants for ATP binding ($K_{d, \text{ATP}}$) in labeled TmrAB variants (Fig. 1–Fig. S2e–g). The measured apparent $K_{d, \text{ATP}}$ values were $51 \pm 38 \mu\text{M}$ for TmrAB^{NBD}, $68 \pm 25 \mu\text{M}$ for TmrAB^{PG}, and $95 \pm 26 \mu\text{M}$ for the slow-turnover variant TmrAB^{PG-EQ}, consistent with prior biochemical measurements ($\sim 100 \mu\text{M}$ for TmrA^{E523Q}). Labeling did not measurably perturb ATP binding. Previous studies on spin-labeled TmrAB^{NBD} demonstrated transport activity comparable to wild-type TmrAB^{WT}, while AV simulations confirmed that fluorophores at these positions do not interfere with ATP- or substrate-binding sites. For labeled TmrAB^{PG} variant, no previous transport activity had been reported; we therefore performed transport assays (Fig. 1–Fig. S3) which confirmed that LD555/LD655 labeling at the PG does not impair function, as labeled and wild-type TmrAB showed indistinguishable substrate transport in single-liposome assays (Fig. 1–Fig. S3c).

ATP-induced conformational switching resolved by smFRET

TmrAB was immobilized on PEGylated surfaces using a conformation-independent, TmrB-specific nanobody (Nb9F10^{S63C}). Previous studies confirmed that Nb9F10^{S63C} does not perturb TmrAB transport^{11,14}, consistent with single-liposome transport assays performed in this study (Fig. 1–Fig. S3d). For surface immobilization, Nb9F10^{S63C} was conjugated to maleimide-PEG₁₁-biotin, serving as an anchor to attach TmrAB to surface-immobilized streptavidin (Fig. 2a). Donor and acceptor photons were recorded by a total-internal reflection fluorescence (TIRF) microscope using alternating laser excitation (ALEX; NanoImager) at 40 °C (Fig. 2b). Single-molecule localization, fluorescence-trajectory extraction, and background correction were performed using NanoImager software, followed by DeepFRET-based machine-learning trace classification and corrections for donor leakage, direct acceptor excitation, and differences in detection-efficiency^{18,35,36} (Fig. 2–Fig. S1 and Fig. 2–Fig. S2). FRET efficiency (E) and stoichiometry (S) were calculated from the corrected fluorescence trajectories (see Methods, Eq. 1 and Eq. 2), and 2D E versus S diagrams were constructed to verify that only complexes with appropriate donor–acceptor stoichiometry ($S \approx 0.5$) were included in the smFRET analysis (Fig. 2c).

FRET efficiency (E) histograms revealed two dominant Gaussian populations corresponding to apo and ATP-bound states (Fig. 2d,e). Low-amplitude features were excluded from quantitative analysis because they were not reproducibly observed across replicates, lacked structural support from cryo-EM and DEER/PELDOR data, introduced poorly constrained fitting parameters, and increased the risk of overfitting. In the absence of ATP, only the apo population was observed, whereas addition of 3 mM ATP induced the appearance of a second ATP-bound population. For TmrAB^{NBD}, the apo and ATP-bound populations exhibited mean E values of 0.58 and 0.88 ($\Delta E = 0.30$), respectively, with $\sim 77\%$ of molecules occupying the ATP-bound state. For TmrAB^{PG}, mean E values were 0.97 (apo) and 0.86 (ATP-bound) ($\Delta E = 0.11$), with $\sim 80\%$ of molecules in the ATP-bound state.

Distance estimates using a Förster radius of $R_0 = 63.5 \text{ \AA}$ (ref.³⁷) yielded apparent distances of 60.2 Å (apo) and 45.6 Å (ATP-bound) for TmrAB^{NBD}, and 35.6 Å (apo) and 46.9 Å (ATP-bound) for TmrAB^{PG}. For TmrAB^{NBD}, the experimentally derived ΔR of 14.6 Å closely agreed with the AV simulations. In contrast, the smaller ΔR of 11.4 Å observed for TmrAB^{PG} deviated from simulated values, suggesting that the ATP-bound population at this site represents a mixture of rapidly interconverting states rather than a single well-defined state.

ATP dependence of conformational equilibria

To assess the ATP sensitivity, we quantified conformational responses across a wide range of ATP concentrations, spanning well below the reported $K_{d, \text{ATP}}$ ($\sim 100 \mu\text{M}$ for TmrA^{E523Q})¹² up to physiologically relevant levels (3 mM ATP). ATP concentrations up to 3 mM were selected to approximate near-physiological conditions commonly used in in-vitro studies, where millimolar ATP levels ensure saturation of nucleotide-dependent conformational transitions and facilitate comparison with previous biochemical and structural analyses^{10–12,14}. smFRET measurements

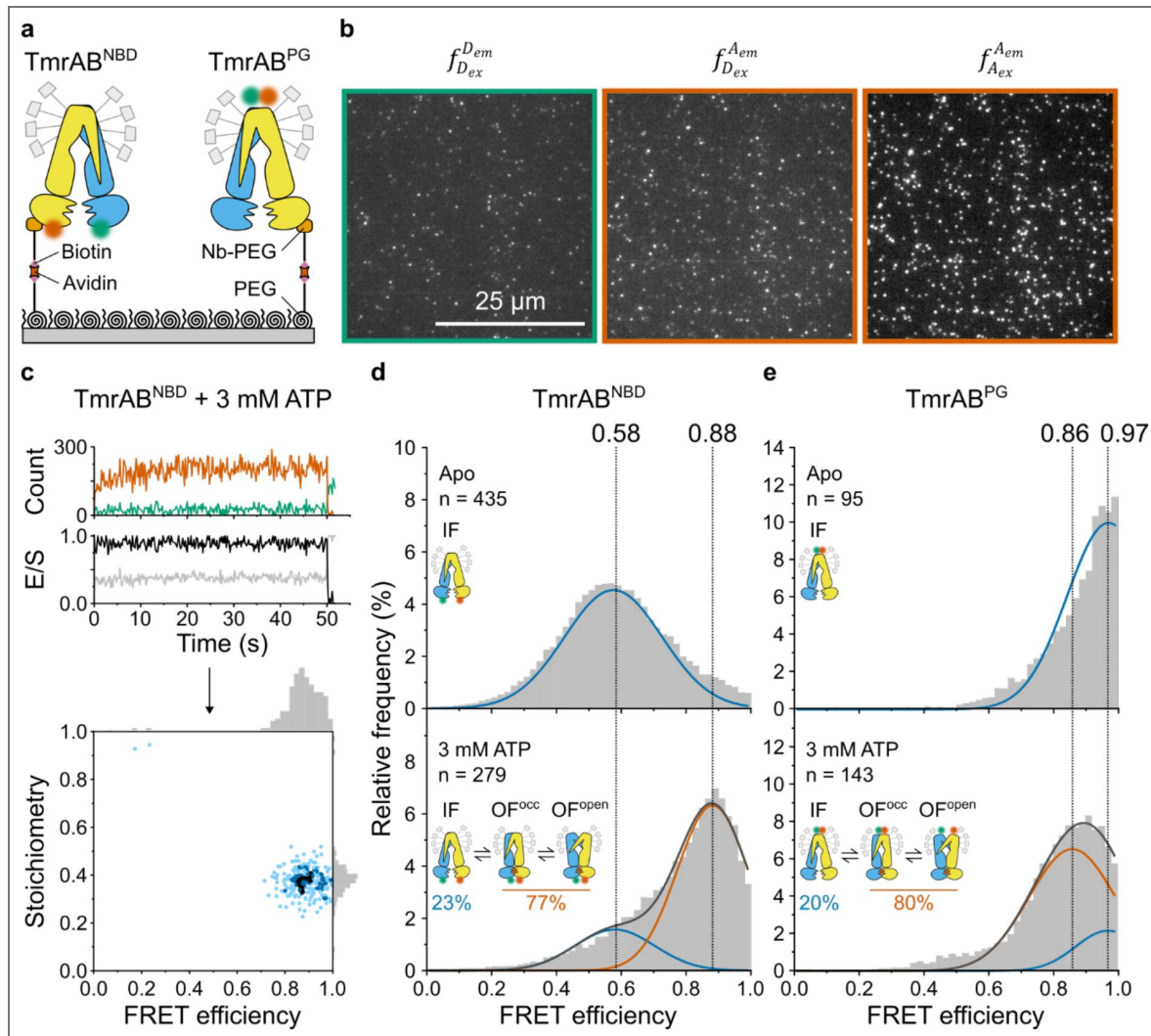


Figure 2. ATP-induced conformational changes of TmrAB analyzed by smFRET.

a, Experimental setup. TmrAB^{NBD} (left) and TmrAB^{PG} (right) were labeled with LD555/LD655 and immobilized on PEGylated coverslips via a biotinylated conformation-independent, TmrB-specific nanobody (Nb9F10^{S63C}). **b**, smFRET imaging was performed using total internal reflection fluorescence (TIRF) microscopy with alternating laser excitation (ALEX; donor: 532 nm; acceptor: 640 nm). Emission was collected in donor (498-620 nm) and acceptor (662-710 nm) channels. Donor emission upon donor excitation ($f_{D_{ex}}^{D_{em}}$), sensitized acceptor emission upon donor excitation ($f_{D_{ex}}^{A_{em}}$), and direct acceptor emission upon acceptor excitation ($f_{A_{ex}}^{A_{em}}$) were used to calculate FRET efficiency (E) and stoichiometry (S). Data were analyzed using DeepFRET³⁵. **c**, Representative TmrAB^{NBD} trace in ATP-bound state (top) and corresponding E/S plot (bottom). Donor emission upon donor excitation is shown in green (photon counts per frame), acceptor emission upon donor excitation in orange, FRET efficiency (E) in black, and stoichiometry (S) in grey. The initial increase in donor fluorescence reflects photophysical equilibration and instrumental stabilization and does not affect E or S , which are ratio-based and time-invariant¹⁸. **d,e**, Population analysis. FRET efficiency (E) histograms for **(d)** TmrAB^{NBD} and **(e)** TmrAB^{PG} are shown for apo (top) and ATP-bound (bottom; 3 mM ATP) conditions. Histograms were fitted with two Gaussian populations corresponding to the apo state (blue; defined from apo measurements) and ATP-bound state (orange; defined from saturating ATP fits). Dotted vertical lines indicate mean E values of each population, and relative fractions (Gaussian areas) are summarized schematically in each panel.

revealed dose-dependent population shifts: TmrAB^{NBD} shifted from a low-FRET apo state ($E = 0.58$) to a high-FRET ATP-bound state ($E = 0.88$), whereas TmrAB^{PG} shifted from a high-FRET apo state ($E = 0.97$) to a lower-FRET ATP-bound state ($E = 0.86$) (Fig. 3a,c). Langmuir isotherm fits yielded apparent $K_{d,ATP}$ values of $13 \pm 1 \mu\text{M}$ for TmrAB^{NBD} and $2 \pm 1 \mu\text{M}$ for TmrAB^{PG} (Fig. 3b,d), significantly lower than $K_{d,ATP}$ determined in ensemble FRET measurements (Fig. 1–Fig. S2e–g). This difference likely arises from the difficulty of deconvoluting overlapping FRET populations at sub- $K_{d,ATP}$ concentrations, particularly for TmrAB^{PG}, where state assignment is less well separated. Despite this quantitative offset, both approaches consistently indicate ATP saturation well below physiological concentrations and therefore support the same mechanistic conclusion that ATP binding drives conformational switching in TmrAB.

Trapping of TmrAB^{PG} reveals a previously hidden outward-facing open state

At 3 mM, ~80% of TmrAB^{PG} complexes populated the ATP-bound state ($E = 0.86$), closely matching the ATP-bound population (~77% NBD-dimerized) observed for TmrAB^{NBD} (Fig. 3). This agreement indicates that both labeling strategies consistently report ATP-dependent conformational changes. However, the ATP-induced shift observed for the periplasmic-gate reporter TmrAB^{PG} ($\Delta E = 0.11$, $\Delta R = 11.4 \text{ \AA}$; Fig. 3c) was substantially smaller than predicted by AV simulations ($\Delta E = 0.27$, $\Delta R = 23.2 \text{ \AA}$; Table 1). This discrepancy suggests that the ATP-bound population at $E = 0.86$ represents an unresolved ensemble, potentially comprising OF^{open} and OF^{occluded} conformations as well as the post-hydrolysis asymmetric unlocked return states (UR^{asym} and UR^{asym*}), which cannot be distinguished from OF^{occluded} within the current FRET geometry¹¹.

To determine whether these states are kinetically unresolved by smFRET, we applied three complementary strategies to arrest the OF^{open} conformation of TmrAB^{PG}: (i) a slow-turnover mutant (TmrAB^{PG-EQ}), (ii) Mg²⁺ depletion using EDTA, and (iii) trans-inhibition by high concentrations of the substrate peptide R9L (RRYQKSTEL) (Fig. 4). Slow-turnover variants have previously enabled structural separation of OF^{open} and OF^{occluded} states^{11,12,14,15}. Mg²⁺ depletion blocks ATP hydrolysis while preserving ATP binding, thereby enabling rapid and reversible trapping¹⁴. We further hypothesized that trans-inhibition by peptide binding sterically restricts PG closure and would therefore stabilize OF^{open} in a dose-dependent manner^{26,27}.

In the absence of ATP, either without nucleotide or in the presence of ADP (3 mM), the slow-turnover variant TmrAB^{PG-EQ} populated a single high-FRET state ($E = 0.97$) (Fig. 4a, top and middle). These results indicate that ADP binding alone is insufficient to promote either NBD dimerization or PG opening, which is consistent with previous biochemical and structural observations^{11,12}. Upon ATP addition (3 mM), however, the conformational landscape diverged sharply from that of TmrAB^{PG}. Instead of the two-state distribution observed for TmrAB^{PG} (apo: $E = 0.97$, ~20%; ATP-bound: $E = 0.86$, ~80%) (Fig. 2e, bottom), TmrAB^{PG-EQ} exhibited three well-resolved populations with mean E values of 0.97 (~14%), $E = 0.86$ (~55%), and $E = 0.63$ (~31%) (Fig. 4a, bottom).

As in TmrAB^{PG}, the high-FRET population ($E = 0.97$) corresponds to the IF state, whereas the intermediate-FRET population ($E = 0.86$) likely represents a dynamic equilibrium between OF^{open} and OF^{occluded} conformations, as suggested by cryo-EM analyses¹¹. Post-hydrolysis return states (UR^{asym} and UR^{asym*}) are expected to be minimally populated in TmrAB^{PG-EQ} because of its drastically reduced ATP hydrolysis rate¹². Notably, the low-FRET ATP-bound population ($E = 0.63$) was entirely absent in wild-type TmrAB^{PG}. The transition from $E = 0.97$ to $E = 0.63$ corresponds to $\Delta E = 0.34$ and $\Delta R = 22.5 \text{ \AA}$, in close agreement with the IF → OF^{open} distance predicted by AV simulations ($\Delta R = 23.2 \text{ \AA}$; Fig. 1 and Table 1), thereby supporting assignment of the $E = 0.63$ population to the OF^{open} conformation.

Mg²⁺ depletion independently reproduced this three-state landscape. In the absence of Mg²⁺, wild-type TmrAB^{PG} transitioned from a single apo population (without ATP; $E = 0.97$) (Fig. 4b, top) to three ATP-bound populations (3 mM ATP; $E = 0.97$, ~13%; $E = 0.86$, ~56%; $E = 0.63$, ~31%) (Fig.

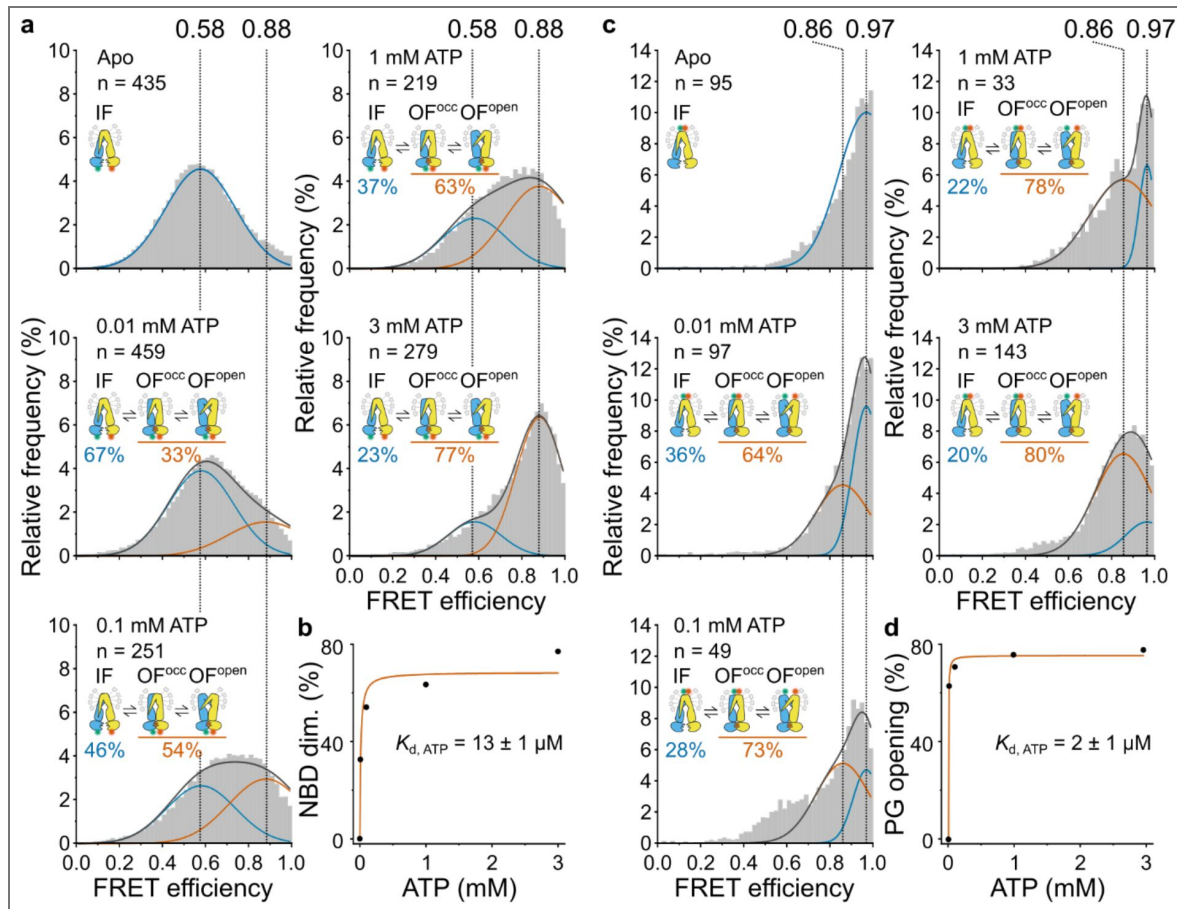


Figure 3. ATP-dependent shifts in smFRET populations of TmrAB.

a,c, Increasing ATP concentrations (0–3 mM) progressively shifted the population between apo and ATP-bound conformations for (a) TmrAB^{NBD} and (c) TmrAB^{PG}. FRET efficiency (E) histograms were fitted with two Gaussian populations corresponding to the ATP-free state (blue; defined from apo samples) and the ATP-bound state (orange; determined from saturating ATP conditions). Dotted vertical lines indicate the mean E values of each state, and relative fractions (Gaussian areas) are summarized schematically in each panel. **b,d**, ATP-binding curves obtained by plotting the fraction of molecules in the ATP-bound states as a function of ATP concentration for (b) TmrAB^{NBD} (reporting NBD dimerization) and (d) TmrAB^{PG} (reporting PG opening). Data were fitted with a Langmuir isotherm to determine the apparent dissociation constant $K_{d,ATP}$ of each variant.

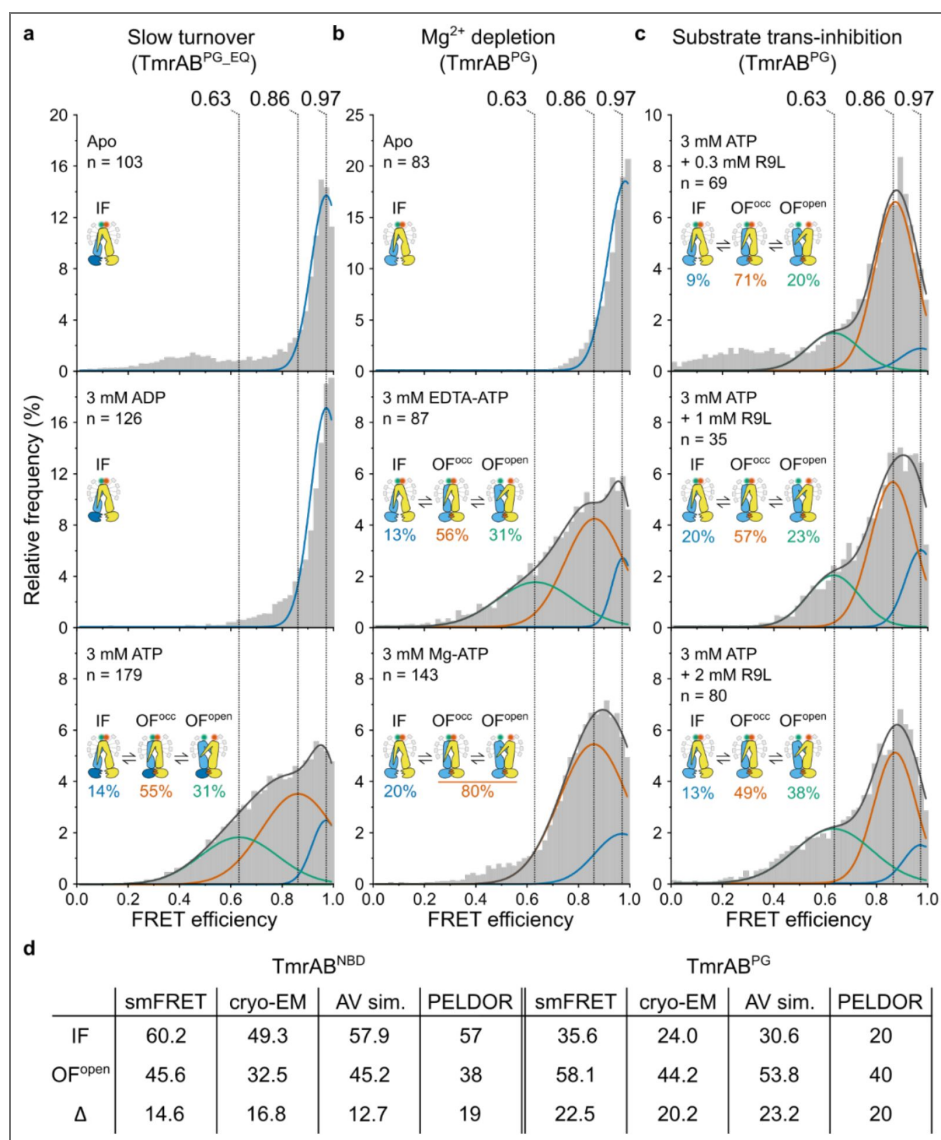


Figure 4. Identification of the outward-facing open (OF^{open}) conformation.

a–c, Three complementary approaches were used to resolve the OF^{open} state: **(a)** the slow-turnover variant TmrAB^{PG-EQ}, **(b)** imaging in Mg²⁺-free buffer supplemented with EDTA, and **(c)** stabilizing via trans-inhibition using high concentrations of peptide substrate R9L (0.3–2 mM). FRET efficiency (E) histograms were fitted with three Gaussian populations corresponding to the ATP-free state (blue), the ATP-bound state (orange), and the OF^{open} state (green). All three strategies reveal a distinct OF^{open} population. Dotted vertical lines indicate the mean E values of each state, and relative fractions (Gaussian areas) are summarized schematically in each panel. **d**, Comparison of inter-residue distances. Distances (Å) between selected residues on the NBDs and PG of TmrAB (C_β–C_β) were determined using smFRET (this study, detergent-solubilized TmrAB), cryo-EM structures of nanodisc-reconstituted TmrAB (PDB 6RAH, 6RAN)¹¹; accessible-volume (AV) simulations (this study), and PELDOR/DEER measurements of detergent-solubilized TmrAB¹⁶.

4b, middle), closely resembling those observed for the slow-turnover variant (Fig. 4a, bottom). Reintroducing Mg^{2+} abolished the $E = 0.63$ population and restored the wild-type TmrAB^{PG} two-state distribution (Fig. 4b, bottom). This reversibility confirmed that the ATP-bound OF^{open} state ($E = 0.63$) is selectively revealed only when ATP hydrolysis is prevented.

Finally, we tested whether periplasmic substrate binding shifts the conformational equilibrium of wild-type TmrAB^{PG} toward OF^{open}. In the presence of ATP (3 mM), increasing concentrations of peptide substrate (0.3–2 mM R9L) progressively enriched the $E = 0.63$ population from ~20% to ~38% (Fig. 4c). This dose-dependent stabilization mirrors the trans-inhibition behavior reported for human TAP1/2 and reflects the upper substrate-loading capacity of the transporter²⁶.

Distance changes derived from smFRET closely match AV simulations, cryo-EM structures (PDB 6RAH, 6RAN)¹¹, and DEER/PELDOR measurements¹⁵ (Fig. 4d), together validating assignment of the $E = 0.63$ population as the OF^{open} conformation.

Kinetics and thermodynamics of the transport cycle

ALEX-smFRET data were acquired with an effective temporal resolution of 200 ms (100 ms per excitation channel). Shorter integration times compromised the signal-to-noise ratio and precluded reliable FRET determination. To quantify conformational dynamics, we applied Hidden Markov Modeling (HMM) using MASH-FRET³⁸, classifying traces as either static (single FRET state) or dynamic (multiple states). Approximately 95% of traces in each condition were classified as static. The predominance of static traces indicates that most conformational transitions occur faster than the 200 ms observation window rather than reflecting conformational arrest.

Although individual transitions could not be directly resolved, population-based analysis (Fig. 3), combined with biochemical turnover measurements (Fig. 1–Fig. S1c), allowed estimation of ATP-bound dwell times. At saturating ATP conditions well above the apparent K_d , ATP (3 mM, 40 °C), wild-type TmrAB exhibited a catalytic turnover rate of $k_{cat} = 2.57 \pm 0.38 \text{ s}^{-1}$ (Fig. 1–Fig. S1c), corresponding to a full transport cycle time (τ_{cycle}) of $395 \pm 55 \text{ ms}$. ATP-bound dwell times (τ_d) were derived from population ratios obtained from Gaussian fits of the FRET efficiency histograms (Fig. 3; see Methods, Eq. 3 and Eq. 4). These analyses yielded ATP-bound dwell times of $304 \pm 43 \text{ ms}$ for TmrAB^{NBD} (~77% ATP-bound) and $316 \pm 44 \text{ ms}$ for TmrAB^{PG} (~80% ATP-bound), with the remaining ATP-free intervals (~20–23%) accounting for 91 ms and 79 ms of the cycle, respectively.

Together, these measurements establish a quantitative, single-molecule description of conformational state occupancies and dwell times throughout the catalytic cycle of a heterodimeric ABC transporter under active turnover conditions, providing a quantitative single-molecule framework for describing the dynamic landscape of its translocation cycle.

Discussion

smFRET has become an indispensable tool for dissecting conformational dynamics of membrane proteins, including receptors, ion channels, and transporters, by directly linking structural transitions to functional states. With the ABC transporter family, however, smFRET studies have largely focused on monomeric or homodimeric systems^{23–25}, leaving asymmetric heterodimeric transporters comparatively underexplored. Here, we extend single-molecule analysis to the heterodimeric type IV ABC transporter TmrAB, revealing dynamic features of the transport cycle that are inaccessible to ensemble-averaged approaches.

By positioning FRET reporters at the nucleotide-binding domains (NBDs) and periplasmic gate (PG), we directly monitored ATP-dependent coupling between chemical energy input and global conformational rearrangements. Importantly, NBD dimerization is not uniquely coupled to a single outward-facing state but can lead to either an outward-facing open (OF^{open}) or occluded (OF^{occluded}) conformation^{11,12}. This decoupling highlights the need to monitor both cytosolic and periplasmic regions to resolve the transport mechanism.

A limitation of the present study is the use of two FRET reporter pairs. While these positions were selected to probe key functional regions and validated by structural and spectroscopic benchmarks, additional labeling positions could in principle resolve further intermediates. However, increasing the number of states would also complicate quantitative analysis due to partially overlapping FRET populations, particularly for TmrAB^{PG}. The current design therefore balances mechanistic resolution with robust state assignment.

Labeling sites previously validated for PELDOR/DEER spectroscopy^{10,15,16} were adapted for smFRET and rigorously benchmarked using accessible-volume (AV) simulations³⁰, fluorescence lifetime analysis, and ensemble FRET titrations. This validation is essential because fluorophores impose stricter steric and rotational constraints than spin labels³². Collectively, these controls demonstrate that fluorophore attachment preserves native-like conformational behavior and ATP responsiveness. Fluorescence lifetime measurements further indicated efficient energy transfer without evidence of substantial protein-fluorophore quenching or restricted mobility¹⁸.

Single-molecule measurements resolved two dominant FRET populations corresponding to apo and ATP-bound states for both TmrAB^{NBD} and TmrAB^{PG}. ATP titrations from sub- $K_{d,ATP}$ to physiological concentrations (3 mM ATP) produced gradual, concentration-dependent redistribution between these states, demonstrating high sensitivity of both reporters. Apparent $K_{d,ATP}$ values derived from smFRET (2–13 μ M) were lower than those obtained from ensemble FRET (50–100 μ M). Importantly, both approaches agree that TmrAB reaches conformational saturation well below physiological ATP concentrations and retains Michaelis–Menten behavior.

Using three independent strategies—slow-turnover catalysis, Mg^{2+} depletion, and substrate trans-inhibition—we resolved this previously hidden OF^{open} conformation. The observed distance changes agree with cryo-EM structures¹¹, PELDOR/DEER data^{10,15,16}, and AV-based predictions, indicating that detergent-solubilized TmrAB samples a largely native-like conformational landscape.

Although detergent and lipid nanodisc environments yield similar global states, fluorophore behavior may still be influenced by membrane proximity in lipid systems^{20,39,40}. AV simulations suggest that dyes near the periplasmic gate may experience steric constraints due to partial membrane overlap. While negligible under detergent conditions, this effect should be considered in future studies of membrane-reconstituted systems.

Substrate addition shifted the conformational equilibrium in a concentration-dependent manner, progressively stabilizing the OF^{open} state. This behavior is consistent with trans-inhibition observed in other ABC transporters, including TAP1/2²⁶ and ABCC1²⁷, and reflects finite substrate loading capacity²⁶.

Quantitative deconvolution of FRET populations enabled estimation of conformational state occupancies under physiological ATP concentrations. During active turnover, TmrAB populates the IF state (~20%), OF^{open} (~25%), and OF^{occluded}/post-hydrolysis states (UR^{asym} and UR^{asym*}) (~55%; Fig. 5 [C](#)). The current labeling scheme does not allow direct separation of OF^{occluded} from post-hydrolysis states due to fluorophore placement at the noncanonical NBS. However, post-hydrolysis contributions are expected to be reduced in the slow-turnover mutant TmrAB^{PG-EQ}. To our knowledge, this represents the first single-molecule quantification of conformational equilibria in a heterodimeric ABC transporter under catalytic conditions. While IF substructure resolution is lower than in cryo-EM classifications^{11,14}, smFRET uniquely captures ATP-bound intermediates that interconvert too rapidly for structural resolution.

All experiments were performed at 40 °C for consistency with prior biochemical work and fluorophore stability. At the physiological temperature of *T. thermophilus* (68 °C), absolute rates are expected to increase, although relative state distributions may be preserved if the free-energy landscape remains unchanged. Despite a substantial fraction of static trajectories, ATP-dependent shifts indicate that TmrAB operates near the temporal resolution of the measurements. Combining state occupancies with enzymatic turnover yields an ATP-bound dwell time of ~300 ms, consistent with previous biochemical estimates^{9,12}.

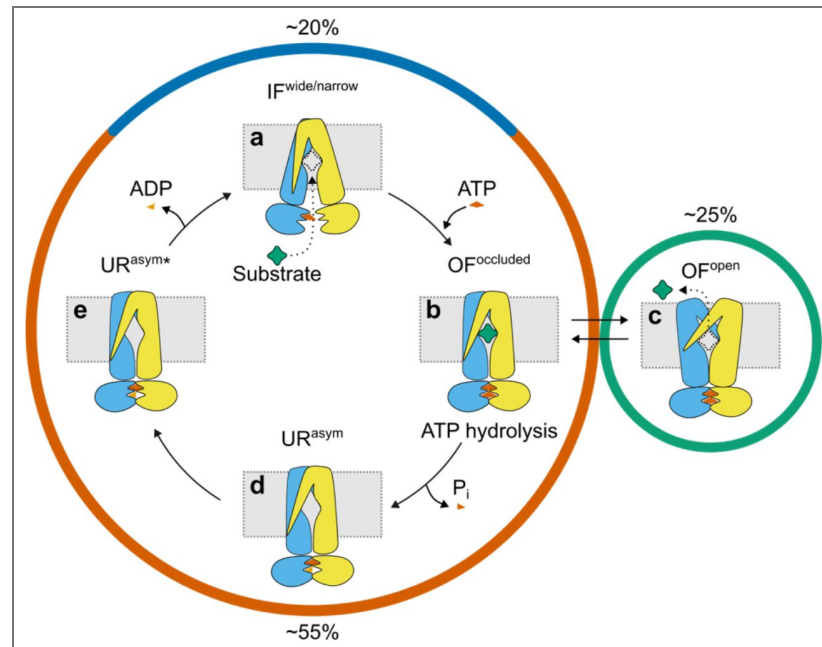


Figure 5. Conformational state distribution and catalytic cycle of TmrAB under active turnover.

Schematic of the TmrAB transport cycle summarizing major conformational states and their estimated population distributions under physiological ATP concentrations (3 mM, 40 °C). **a**, The inward-facing apo state (IF^{narrow} and IF^{wide} ; blue arc) accounts for ~20% of molecules and is characterized by separated NBDs and a cytosol-accessible substrate-binding cavity. Substrate binding stabilizes the IF^{wide} conformation¹¹. ATP binding induces NBD dimerization and formation of the ATP-bound ensemble. **b,c**, Under substrate-bound turnover conditions, TmrAB proceeds via **(b)** $OF^{occluded}$ and **(c)** OF^{open} states in which substrate release occurs. In steady-state turnover, the ATP-bound ensemble rapidly interconverts between $OF^{occluded}$ and OF^{open} , accounting for ~25% of the ATP-bound population (green circle). These transitions occur faster than the ~200 ms temporal resolution of smFRET measurements, resulting in an averaged signal under turnover conditions. $OF^{occluded}$ is proposed as an obligate intermediate between IF and OF^{open} , preventing substrate backflow by maintaining an occluded binding cavity during rearrangements of the PG and NBDs. Although a substrate-bound $OF^{occluded}$ state has not been directly observed for TmrAB, its existence is supported by structures of the homodimeric type IV transporter BmrA⁴⁷. Reduced ATP hydrolysis or substrate trans-inhibition enables trapping of the OF^{open} state. **d,e**, ATP hydrolysis and phosphate (P_i) release generate post-hydrolysis return states **(d)** UR^{asym} and **(e)** UR^{asym*} . Subsequent ADP release restores the apo IF conformation, completing the transport cycle. Overall, the ATP-bound phase (**b–e**) represents ~55% occupancy (orange arc) with an estimated dwell time of ~310 ms, whereas the apo/ATP-rebinding phase (**a**) lasts ~90 ms, yielding a total cycle time of ~400 ms ($k_{cat} = 2.57 \text{ s}^{-1}$). TmrA is shown in blue, TmrB in yellow, substrate as a green diamond, and nucleotides as orange symbols. Dotted grey boxes indicate the approximate position of the NBD dimer interface.

Emerging microsecond-resolution smFRET approaches could directly resolve short-lived intermediates⁴¹, and multi-color FRET strategies^{42,43} may enable simultaneous monitoring of NBDs, PG, and nucleotide sites. These advances would allow direct detection of the O_F^{occluded} states and post-hydrolysis dynamics.

Overall, the consistency between reporter positions, ensemble measurements, and structural data support the conclusion that the observed conformational states reflect native TmrAB behavior. While certain intermediates remain kinetically unresolved under turnover conditions, they are strongly supported by independent trapping approaches, reinforcing the robustness of the mechanistic model. Additional conditions, including nucleotide-state asymmetry and substrate-only binding, represent promising directions for future work.

In summary, this work establishes smFRET as a powerful approach for mapping the dynamic landscape of asymmetric ABC transporters. By quantitatively linking ATP binding, conformational equilibria, and kinetics at the single-molecule level, we define how chemical energy is converted into directional transport in heterodimeric ABC systems.

Methods

Expression, purification, and labeling of TmrAB

His₁₀-tagged TmrAB variants were expressed in *E. coli* BL21(DE3) (Thermo Fisher Scientific) as described previously¹². Cells were grown in high-salt LB media (Carl Roth) supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin (PAA Laboratories) at 37 °C. At an OD₆₀₀ of 0.5, expression was induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG; Carl Roth), and cultures were incubated for 3 h at 37 °C. Cells were harvested by centrifugation (4,500 $\times g$, 4 °C, 15 min) and stored at –80 °C.

For purification, cell pellets were resuspended in lysis buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 50 $\mu\text{g ml}^{-1}$ lysozyme, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) and lysed by sonication. Cell debris was removed by centrifugation (18,000 $\times g$, 4 °C, 35 min), and membranes were collected by ultracentrifugation (100,000 $\times g$, 4 °C, 30 min). Membranes were solubilized for 2 h at 4 °C in purification buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl) containing 20 mM n-dodecyl β -D-maltoside (β -DDM; Carl Roth). After ultracentrifugation (100,000 $\times g$, 30 min, 4 °C), the supernatant was incubated with Ni-NTA agarose (Bio-Rad) for 1 h at 4 °C. The resin was washed with 20 column volumes of wash buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 1 mM β -DDM) containing 50 mM imidazole, and TmrAB was eluted with elution buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 1 mM β -DDM, 300 mM imidazole).

For fluorophore labeling, TmrAB variants were conjugated via maleimide chemistry using LD555 and LD655 (Lumidyne Technologies). Labeling was carried out at a 1:10:10 molar ratio of protein to each dye in elution buffer for 3 h at 4 °C. Excess dye was quenched with 2 mM β -mercaptoethanol (Sigma-Aldrich), and the labeled protein was buffer-exchanged into size-exclusion chromatography (SEC) buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM β -DDM) using a Zeba Spin Desalting Column (Thermo Fisher Scientific). Unreacted fluorophores were removed by SEC on a Superdex 200 Increase 10/300 GL column (Cytiva). Labeling efficiency was determined by analytical SEC (Superdex 200 Increase 3.2/300; Cytiva) by monitoring absorbance at 280, 555, and 655 nm. To preserve sample integrity for smFRET measurements, TmrAB was purified and labeled within a single day, stored on ice, and imaged over the following two days.

Time-correlated single-photon counting (TCSPC)

Fluorescence lifetime measurements were performed using a FluoTime 100 spectrometer (PicoQuant) equipped for time-correlated single-photon counting (TCSPC). Experiments were carried out on labeled TmrAB in SEC buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM β -DDM). LD555 and LD655 were excited at 510 nm and 610 nm, respectively. Emission was collected using a 620/60 nm bandpass filter for LD555 and a BG4 700 nm long-pass filter for LD655. Photon arrival times were accumulated until the TCSPC histogram reached a peak count of 50,000.

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain (<i>Escherichia coli</i>)	BL21(DE3)	Thermo Fisher Scientific	Cat. -Nr.: EC0114	Expression of His ₁₀ -tagged TmrAB variants and nanobody Nb9F10 ^{S63C}
Gene (<i>Thermus thermophilus</i>)	TmrAB ^{wt}	Ref. ¹¹	Plasmid ID: AT30	Wild-type TmrAB
Gene (<i>Thermus thermophilus</i>)	TmrAB ^{NBD} (TmrA ^{C416A} B ^{L458C})	Ref. ¹⁵	Plasmid ID: AT08	TmrAB variant
Gene (<i>Thermus thermophilus</i>)	TmrAB ^{PG} (TmrA ^{C416A} , T61C B ^{R56C})	Ref. ¹⁵	Plasmid ID: AT27	TmrAB variant
Gene (<i>Thermus thermophilus</i>)	TmrAB ^{PG_EQ} (TmrA ^{C416A} , E523Q, T61C B ^{R56C})	Ref. ¹⁵	Plasmid ID: AT01	Slow-turnover TmrAB variant
Gene (<i>Vicugna pacos</i>)	Nb9F10 ^{S63C} (nanobody)	Ref. ¹¹	Plasmid ID: BH16	TmrB-specific, conformation-independent nanobody
Peptide	R9L	This study	N/A	RRYQKSTEL
Peptide	C4F	This study	N/A	RRYCFKSTEL; ^F , fluorescein
Chemical compound	LD555 (maleimide)	Lumidyne Technologies	CAT #4	FRET donor
Chemical compound	LD655 (maleimide)	Lumidyne Technologies	CAT #8	FRET acceptor
Chemical compound	ATP (adenosine 5'-triphosphate)	Sigma-Aldrich	CAS Nr.: 74804-12-9	
Chemical compound	ADP (adenosine 5'-diphosphate)	Sigma-Aldrich	CAS Nr.: 20398-34-9	
Chemical compound	EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich	CAS Nr.: 6381-92-6	
Chemical compound	n-Dodecyl β-D-maltoside (β-DDM)	Carl Roth	CAS Nr.: 69227-93-6	

Key resources table.

Chemical compound	Trolox	Sigma-Aldrich	CAS Nr.: 53188-07-1	
Chemical compound	Streptavidin	Sigma-Aldrich	CAS Nr.: 9013-20-1	
Chemical compound	Biotin-PEG ₁₁ -maleimide	Sigma-Aldrich	Cat. -Nr.: QBD10195	
Chemical compound	<i>E. coli</i> polar lipids	Avanti Polar Lipids	CAS Nr.: 1240502-50-4	
Chemical compound	dioleoylphosphatidylcholine (DOPC)	Avanti Polar Lipids	CAS Nr.: 4235-95-4	
Commercial assay or kit	Ni-NTA Agarose	Bio-Rad	Cat. -Nr.: 30250	IMAC purification of His10-tagged TmrAB
Software	Nanolmager software	ONI	v1.19.16.20250510 165527-1aaf735	Microscope software
Software	DeepFRET	Ref. ³⁵	v2.0.5	Machine-learning FRET trace classification
Software	MASH-FRET	Ref. ³⁸	v1.3.4	Hidden Markov Modeling of smFRET traces
Software	FluoFit	PicoQuant	v4.6.6 (discontinued)	Fluorescence decay analysis
Software	OriginPro 2024	OriginLab	v10.1.0.178	Data analysis
Software	FlowJo 10.6.1	Waters Biosciences	RRID: SCR_008520	Flow cytometry data analysis
Software	AV simulation toolkit	Ref. ³⁰	Zenodo: 3517287	Accessible-volume simulations

Key resources table. (continued)

photons. Fluorescence decay curves were analyzed by fitting mono- or bi-exponential decay models using FluoFit software (PicoQuant) and amplitude-weighted average of fluorescence lifetime was calculated.

Nanobody production and purification

The nanobody Nb9F10^{S63C} was expressed and purified as described previously¹¹. Briefly, Nb9F10^{S63C} was produced in *E. coli* BL21(DE3) cells grown in Terrific Broth (TB; Carl Roth) supplemented with 100 µg ml⁻¹ ampicillin at 37 °C. At an OD₆₀₀ of 0.6, expression was induced with 1 mM IPTG, followed by overnight incubation at 28 °C. Cells were harvested by centrifugation (4,500 × *g*, 4 °C, 15 min) and stored at -80 °C. For purification, cell pellets were resuspended in nanobody lysis buffer (25 mM HEPES-NaOH pH 7.4, 300 mM NaCl, 15 mM imidazole, 0.5 mM PMSF) and disrupted by sonication. Cell debris was removed by centrifugation (18,000 × *g*, 4 °C, 35 min), and the clarified lysate was applied to Ni-NTA agarose equilibrated in potassium phosphate (KP_i) buffer (25 mM KP_i pH 6.5, 100 mM KCl, and 0.5 mM tris(2-carboxyethyl) phosphine (TCEP)). Bound nanobody was washed with 10 column volumes (CV) of KP_i buffer and eluted with 8 CV of elution buffer (25 mM KP_i pH 6.0, 20 mM KCl, 300 mM imidazole, 0.5 mM TCEP). Eluted fractions were pooled and further purified by cation exchange chromatography (CEX) on a HiTrap SP column (Cytiva) using a linear gradient from low-salt buffer (25 mM KP_i pH 6.0, 20 mM KCl, 0.5 mM TCEP) to high-salt buffer (25 mM KP_i pH 6.0, 500 mM KCl, 0.5 mM TCEP). The purified nanobody was concentrated and buffer-exchanged into nanobody SEC buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl) using Zeba spin desalting columns, followed by SEC (Superdex 200 Increase 10/300 GL; Cytiva). For site-specific conjugation, Nb9F10^{S63C} was incubated with a biotin-PEG₁₁-maleimide linker (Sigma-Aldrich) at a 1.2:1 molar ratio of protein to linker in the presence of 0.5 mM TCEP for 2 h at 4 °C. Excess linker was removed by desalting on Zeba Spin Desalting Columns, followed by a final SEC step (Superdex 200 Increase 10/300 GL).

SDS-PAGE

The purity of TmrAB samples was assessed by SDS-PAGE. Resolving gels (10%) were prepared using 10% (w/v) acrylamide, 0.5 M Tris-HCl (pH 8.8), 0.13% (w/v) SDS, 0.05% (w/v) ammonium persulphate (APS), and 0.25% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED). Stacking gels contained 4.3% (w/v) acrylamide, 0.5 M Tris/HCl (pH 6.8), 0.09% (w/v) SDS, 0.09% (w/v) APS, and 0.33% (v/v) TEMED. Gels were used immediately or stored at 4 °C for up to 4 weeks. Protein samples were mixed with 4× SDS loading buffer containing dithiothreitol (DTT; Sigma-Aldrich) and heated at 90 °C for 5 min before loading. Electrophoresis was performed at a constant voltage of 120 V using 1× SDS running buffer (25 mM Tris-HCl pH 8.8, 192 mM glycine, 0.1% SDS). Proteins were visualized by staining with InstantBlue™ Protein Stain (Abcam) for 1 h at room temperature with gentle agitation and imaged using a Fusion FX system (Vilber).

ATPase activity assay

The ATPase activity of β-DDM-solubilized TmrAB^{wt} was quantified using a Malachite Green-based colorimetric assay as described previously⁴⁴. Detergent-solubilized TmrAB (60 nM) was incubated in ATPase buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM β-DDM) containing 3 mM ATP (Sigma-Aldrich) at 40 °C for 7 min. Autohydrolysis controls were prepared by incubation of ATP in ATPase buffer without protein. Reactions were quenched by adding 20 mM H₂SO₄, followed by incubation with 3 mM Malachite Green (Thermo Fisher Scientific), 0.2% (v/v) Tween20 (Carl Roth), and 1.5% (w/v) ammonium molybdate (Carl Roth) for 10 min at room temperature. The absorbance at 620 nm was recorded on a CLARIOstar v.5.20 R5 plate reader (BMG LABTECH).

Ensemble FRET measurements

The ATP binding and FRET characteristics of selected TmrAB variants were assessed by ensemble FRET. Labeled TmrAB (100 nM) was incubated with increasing concentrations of ATP at 42 °C for 5 min. Donor-excited emission was recorded from 550–700 nm with an excitation wavelength of 520

nm using a CLARIOstar v.5.20 R5 plate reader (BMG LABTECH). Acceptor emission intensities at 675 nm were plotted against ATP concentration and fitted with a hyperbolic function to determine the apparent dissociation constant ($K_{d, \text{ATP}}$) for each variant.

Reconstitution of TmrAB

TmrAB variants were reconstituted into proteoliposomes composed of *E. coli* polar lipids and dioleoylphosphatidylcholine (DOPC) at a molar ratio of 7:3. A protein-to-lipid ratio of 1:20 (w/w) was used, corresponding to approximately 50 TmrAB complexes per liposome. Extruded large unilamellar vesicles (LUVs) were destabilized by incubation with 0.3% (v/v) Triton X-100 for 30 min. TmrAB was then added to the detergent-destabilized liposomes and incubated for 30 min at 8 °C under gentle rotation. Detergent was removed gradually using sequential addition of polystyrene Bio-Beads SM-2 (Bio-Rad): two incubations at 40 mg ml⁻¹ (1 h and overnight), followed by two incubations at 80 mg ml⁻¹ (1 h each). Proteoliposomes were subsequently harvested by ultracentrifugation (100,000 × g, 30 min, 4 °C) and resuspended in the appropriate buffer.

Single liposome transport assay

Single liposome transport assay was performed as described previously^{12,13}. Wild-type TmrAB or LD555/LD655-labeled TmrAB^{PG} (0.6 μM each), reconstituted into liposomes (~50 TmrAB per liposome), was mixed with fluorescently labeled peptide RRYC^FKSTEL (30 μM C4F; ^F, fluorescein), 3 mM ATP or ADP, and 5 mM MgCl₂ in liposome buffer (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5% (v/v) glycerol). Where indicated, nanobody Nb9F10^{S63C} (0.6 μM) was added. Samples were incubated for 5 min at 40 °C (unless specified otherwise), followed by addition of ice-cold ethylenediaminetetraacetic acid (EDTA; 10 mM). Proteoliposomes were then washed twice by centrifugation (270,000 × g, 30 min) to remove non-transported peptide. Mean fluorescence intensities (MFIs) of translocated substrates were quantified by flow cytometry using a FACSMelody (Waters Biosciences) and analyzed in FlowJo 10.6.1 (Waters Biosciences). The gating strategy used to determine fluorescence intensities of single liposomes from raw flow cytometry data consisted of three steps: First, forward scatter area versus side scatter area (FSC-A vs SSC-A) was used to separate populations based on size and granularity. Second, forward scatter height versus forward scatter area (FSC-H vs FSC-A) was used to exclude aggregates and retain singlet events. Finally, fluorescence intensities of the gated singlet population were quantified at the wavelength of interest (527 nm), and the resulting values were used to calculate mean fluorescence intensity (MFI) of the proteoliposome population.

Solid-phase peptide synthesis

The peptides used in this study were synthesized and labeled as described previously^{14,45}. The 9-mer peptides RRYQKSTEL (R9L) and RRYC^FKSTEL (C4F; ^F, fluorescein) were synthesized on preloaded Fmoc-L-Leu resin using a Liberty microwave-assisted peptide synthesizer (CEM) according to a standard protocol (54 W, 3 min, 75 °C). Each coupling step was performed twice using 0.2 M fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, 0.5 M O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluoro-phosphate (HBTU), and 1-hydroxybenzotriazole hydrate (HOBt H₂O). Fmoc deprotection was carried out with 20% (v/v) piperidine and 0.1 M HOBt H₂O in dimethylformamide (DMF). Peptides were cleaved from the resin using a cleavage cocktail containing 92.5% (v/v) trifluoroacetic acid (TFA), 2.5% (v/v) H₂O, 4.5% (v/v) thioanisole, and 0.5% (v/v) 1,2-ethanedithiol (EDT) for 1.5 h at room temperature. Cleaved peptides were precipitated in ice-cold diethyl ether (Et₂O), pelleted, dissolved in tert-butanol (tBuOH)/water (4:1, v/v), and lyophilized. Peptides were purified by reverse-phase (RP) C18 HPLC (PerfectSil C18 column; MZ-Analysentechnik) using buffer A (Milli-Q water containing 0.05% (v/v) TFA) and buffer B (acetonitrile containing 0.05% (v/v) TFA). Peptide identity and purity were confirmed by LC-MS. For fluorescence labeling, the peptide RRYC^FKSTEL was dissolved in 33% (v/v) DMF in PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and incubated for 1 h at room temperature with a 1.3-fold molar excess of 5-iodoacetamido fluorescein (Merck).

Functionalization of glass slides for single-molecule FRET analysis

Glass coverslips used for TmrAB immobilization in smFRET experiments were functionalized by PEGylation as described previously⁴⁶. Coverslips (Carl Roth) were cleaned by sequential sonication in Milli-Q water and analytical-grade acetone (>99.9%; VWR International), followed by oxygen plasma treatment (0.3 mbar, 80% power, 15 min) using a Zepto plasma cleaner (Diener) and a 10 min incubation in methanol (Avantor, Gliwice, PL). Coverslips were then silanized by incubation for 30 min in a solution of 100 ml methanol, 5 ml acetic acid, and 3 ml 3-aminopropyltrimethoxysilane (APTES; Tokyo Chemical Industry). After silanization, slides were rinsed four times with methanol and dried under a nitrogen stream. Surface functionalization was achieved using a mixture of biotinylated-PEG (4 mol%) and nonbiotinylated-PEG (96 mol%; Rapp Polymer). The PEG solution was sandwiched between two coverslips and incubated overnight in a humidity chamber. Coverslips were then rinsed thoroughly with Milli-Q water and dried under nitrogen. To enhance passivation, a second PEGylation step was performed using 25 mM CH₃-PEG-NHS (333 Da; Thermo Fisher Scientific) under the same conditions. Finally, slides were rinsed with Milli-Q water, dried under nitrogen, and stored at -20 °C under argon until use.

Single-molecule FRET imaging

Single-molecule FRET (smFRET) experiments were performed using a flow chamber system (Ibidi). Chambers were assembled by placing a biotin-PEG-functionalized glass slide onto a μ -Slide I Luer Family flow channel (Ibidi), with the functionalized surface facing inward. All buffers and Milli-Q water were filtered through 0.2 μ m filters (Sigma-Aldrich). Chambers were washed with 1 ml Milli-Q water and incubated with 0.2 mg ml⁻¹ streptavidin (Sigma-Aldrich) at 4 °C for 30 min to allow binding to the biotin-PEG surface. Unbound streptavidin was removed by washing with 1 ml Milli-Q water. The surface was then treated with 0.3 mg ml⁻¹ biotinylated-PEG₁₁-Nb9F10^{S63C} at 4 °C for 45 min, followed by flushing with 2 ml SEC buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM β -DDM). Detergent-solubilized, fluorophore-labeled TmrAB (100 nM) was added and incubated at 4 °C for 1 h. Unbound protein was removed by five washes with 1 ml TmrAB-SEC buffer. Chambers were equilibrated with 1 ml of imaging buffer containing 25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 3 mM MgCl₂, 50 mM glucose, 5 mM Trolox, 7.5 U ml⁻¹ pyranose oxidase, and 1 kU ml⁻¹ catalase, supplemented with the desired ATP concentration. For EDTA trapping experiments, MgCl₂ was omitted and replaced with 3 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich). smFRET data were acquired at 40 °C using alternating laser excitation (ALEX) on a total internal reflection fluorescence (TIRF) microscope (NanoImager S, ONI, Oxford, UK). Typically, 600 frames were recorded per region of interest (ROI) with 100 ms exposure time. Laser powers were 0.8 mW cm⁻² (532 nm) and 0.9 mW cm⁻² (640 nm). Data were recorded in 1-min intervals, except for TmrAB^{NBD} variant in apo and 3 mM ATP conditions, where 3-min intervals were used to confirm that conformational transitions do not occur on timescales longer than one minute due to the reduced temperature.

Single-molecule FRET data analysis

Single-molecule FRET (smFRET) measurements were performed using alternating laser excitation (ALEX), allowing assignment of detected photons based on both excitation and emission wavelengths. Photon counts for each molecule were extracted using NanoImager software (ONI NanoImager, Development Build) and classified into three detection channels: donor excitation with donor emission ($f_{D_{ex}}^{D_{em}}$), donor excitation with acceptor emission ($f_{D_{ex}}^{A_{em}}$), and acceptor excitation with acceptor emission ($f_{A_{ex}}^{A_{em}}$). Traces were analyzed with DeepFRET³⁵ and manually curated. To minimize bias, a second researcher independently curated traces from both ATP-free and 3 mM ATP samples, yielding 98% overlap between curations. FRET efficiency (E) and stoichiometry (S) were calculated as:

$$E = \frac{f_{D_{ex}}^{A_{em}}}{f_{D_{ex}}^{D_{em}} + f_{A_{ex}}^{A_{em}}} \quad (1)$$

$$S = \frac{f_{D_{ex}}^{D_{em}} + f_{D_{ex}}^{A_{em}}}{f_{D_{ex}}^{D_{em}} + f_{D_{ex}}^{A_{em}} + f_{A_{ex}}^{A_{em}}} \quad (2)$$

Population analysis was performed by constructing one-dimensional histograms of FRET efficiency (E) and stoichiometry (S) using OriginPro 2024 (OriginLab). Histograms were fitted with two Gaussian distributions corresponding to the ATP-free state (defined from apo samples) and the ATP-bound state (defined by a two-component fit at saturating ATP). Hidden Markov Modeling (HMM) of individual traces was performed using MASH-FRET³⁸ to distinguish dynamic from static molecules within each sample.

ATP-bound dwell times and distribution of conformational states

The ATP-bound dwell time (τ_d) was estimated as:

$$\tau_d = \tau_{\text{cycle}} \times f_{\text{ATP-bound}} \quad (3)$$

where $f_{\text{ATP-bound}}$ is the fraction of ATP-bound molecules derived from Gaussian fits of the FRET efficiency histograms (Fig. 3 [4](#)). This approach assumes (i) ATP hydrolysis occurs exclusively at the canonical NBS, with negligible contribution from the noncanonical site^{9,11,15}, and (ii) the majority of molecules are catalytically competent and continuously cycling.

The distribution of conformational states within ATP-bound FRET population ($E = 0.86$) of TmrAB^{PG} under turnover conditions (3 mM ATP) was determined using a two-state model:

$$E = f_{\text{open}} \times E_{\text{open}} + f_{\text{other}} \times E_{\text{other}} \quad (4)$$

where f_{open} is the fraction of OF^{open} state ($E_{\text{open}} = 0.63$) readily resolved under trapping conditions (Fig. 4 [4](#)), and f_{other} represents the combined fraction of PG-closed states (OF^{occluded}/UR^{asym}/UR^{asym*}, $E_{\text{other}} = 0.97$).

Data availability

Source data are provided with this paper: DOI: <https://doi.org/10.25716/gude.0jbq-k1q6> [4](#).

Figure supplements

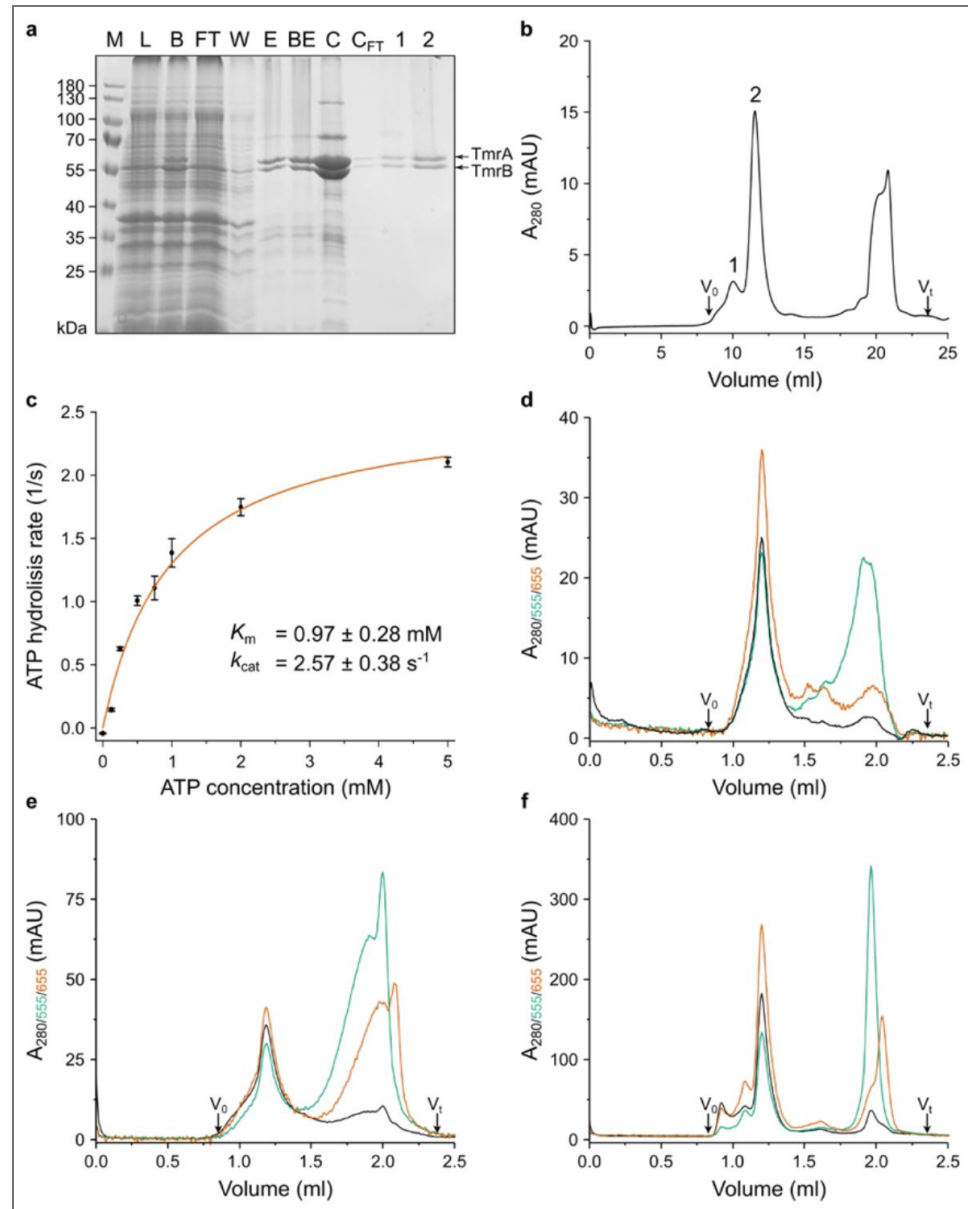


Figure 1-Figure supplement 1. Quality of TmrAB purification and fluorophore labeling. **a**, SDS-PAGE analysis (10%, reducing gel, Coomassie staining) of successive purification steps: M, molecular weight marker; L, cell lysate; B, Ni-NTA beads after incubation with lysate; FT, flow-through; W, wash; E, eluted TmrAB; BE, buffer-exchanged sample; C, concentrated protein; CFT, concentrator flow-through; 1 and 2, first and second peaks eluted from size-exclusion chromatography (SEC). Only the second peak was used for subsequent FRET experiments. **b**, SEC (Superdex 200 increase 10/300 GL) showing monodisperse labeled TmrAB and efficient removal of free fluorophores. A representative chromatogram of TmrAB^{PG} is shown. **c**, ATP hydrolysis activity of purified wild-type TmrAB (60 nM TmrAB^{WT}) measured at 40 °C for 7 min using the Malachite Green assay. Released inorganic phosphate (P_i) was quantified and data fitted to Michaelis-Menten kinetics, yielding $K_m = 0.97 \pm 0.28$ mM and $k_{cat} = 2.57 \pm 0.38$ s⁻¹. **d-f**, Analytical SEC (Superdex 200 increase 3.2/300) used to determine fluorophore labeling efficiencies of **(d)** TmrAB^{NBD}, **(e)** TmrAB^{PG}, and **(f)** TmrAB^{PG-EQ}. LD555 and LD655 labeling efficiencies were ~55 and ~53% (TmrAB^{NBD}), ~43 and ~52% (TmrAB^{PG}), and ~42% and ~52% (TmrAB^{PG-EQ}), respectively. Total cysteine occupancy exceeds 90% in all variants, reflecting near-complete dual-labeling with equimolar fluorophores.

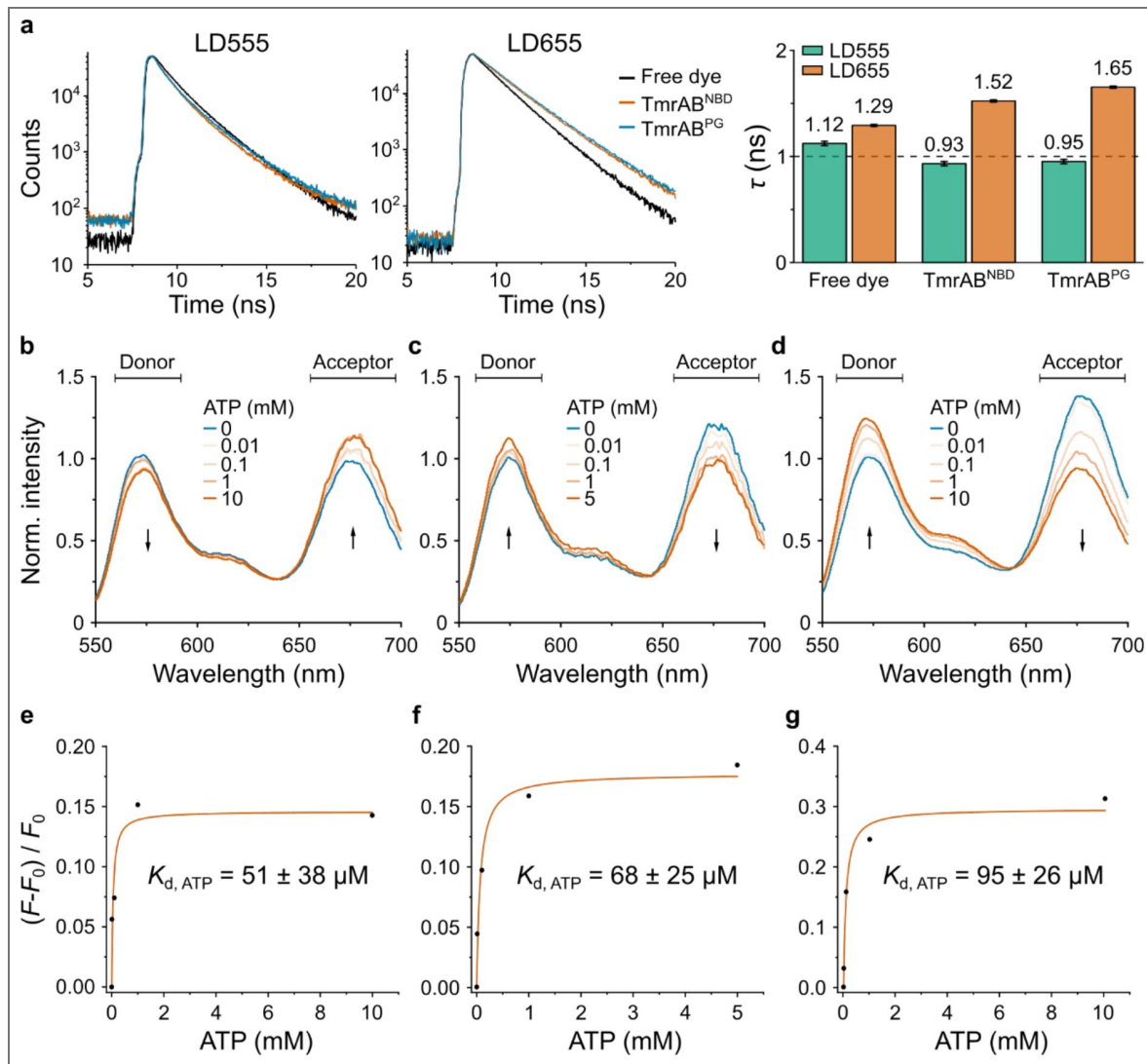


Figure 1-Figure supplement 2. FRET properties of labeled TmrAB variants.

a, Time-correlated single-photon counting histograms of LD555 (left) and LD655 (middle) measured for free dye in buffer (black), LD555/LD655-labeled TmrAB^{NBD} (orange), and LD555/LD655-labeled TmrAB^{PG} (blue). Amplitude-weighted average fluorescence lifetimes (τ) are summarized in the histogram (right), confirming sufficient fluorophore mobility for reliable FRET measurements. **b–d**, Donor-excited ensemble emission spectra (550–700 nm, excitation 520 nm) of stochastic LD555/LD655-labeled (b) TmrAB^{NBD}, (c) TmrAB^{PG}, and (d) the slow-turnover variant TmrAB^{PG,EQ} measured at increasing ATP concentrations. Spectra were normalized to donor intensity in the apo state. ATP-dependent donor quenching and acceptor sensitization demonstrate that all variants retain FRET capability. **e–g**, Fractional fluorescence changes, $(F-F_0)/F_0$, plotted as a function of ATP concentration for (e) TmrAB^{NBD}, (f) TmrAB^{PG}, and (g) TmrAB^{PG,EQ}, where F is the acceptor emission intensity and F_0 the intensity in the apo state. Data were fitted with a hyperbolic (Langmuir-type) binding model to determine apparent $K_{d,ATP}$ values, which are consistent with ensemble FRET measurements of ATP binding.

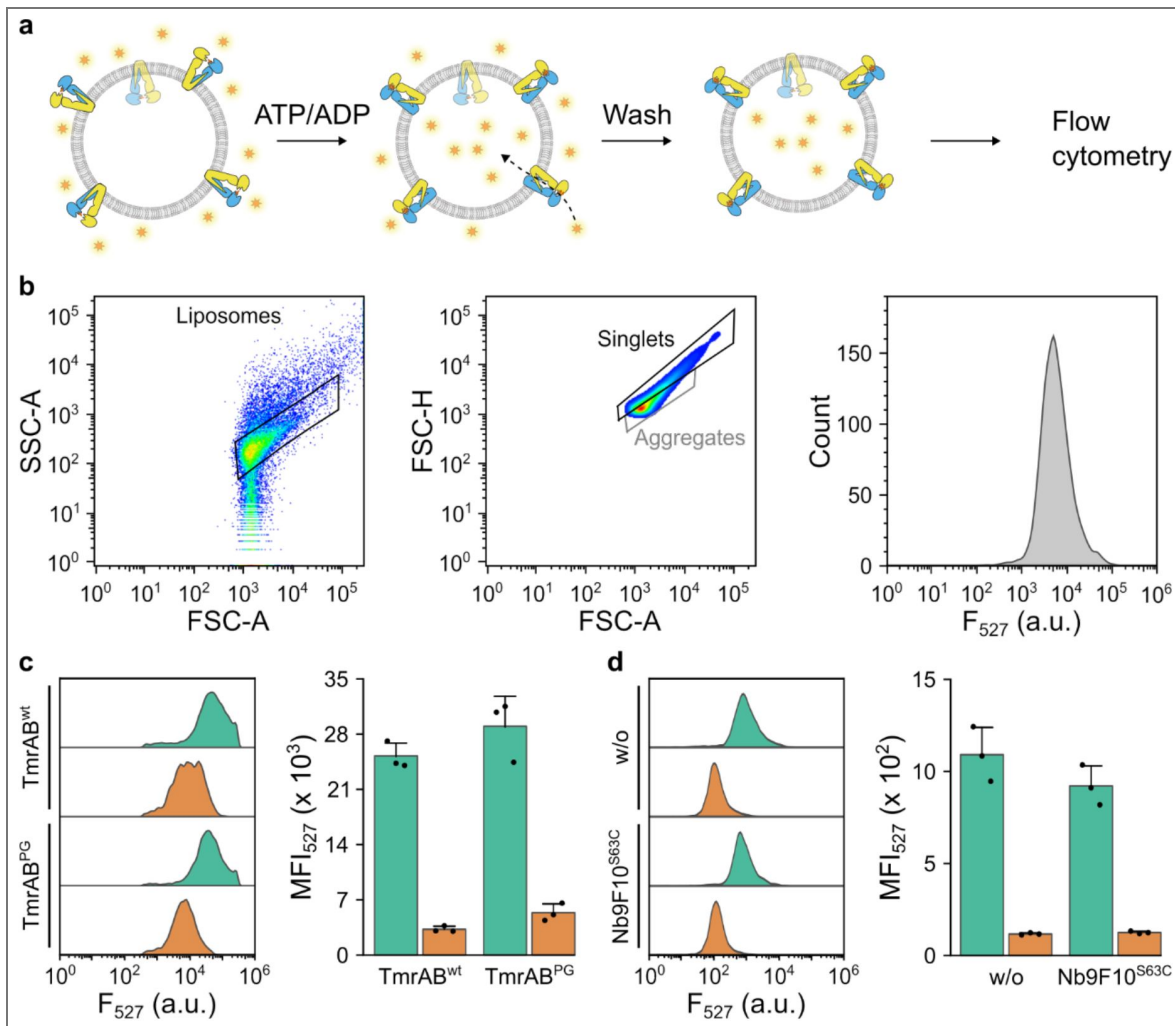


Figure 1-Figure supplement 3. Effect of fluorophore labeling and nanobody binding on TmrAB transport activity.

Single-liposome transport assays were performed by flow cytometry using mean fluorescence intensity at 527 nm (MFI_{527}) as a readout of peptide transport. **a**, TmrAB (0.6 μ M) reconstituted into liposomes (~50 transporters per liposome) was incubated with C4F peptide (30 μ M; RRYC^FKSTEL, ^F, fluorescein) in the presence of 3 mM ATP (green) or 3 mM ADP (orange) at 40 °C for 5 min. After extensive washing, proteoliposomes were analyzed by flow cytometry. **b**, Flow-cytometry gating strategy. Forward scatter area (FSC-A) versus side scatter area (SSC-A; left) was used to identify homogeneous proteoliposomes, forward scatter height (FSC-H) versus FSC-A (middle) to select singlets, and fluorescence intensity at 527 nm (F_{527} ; right) to determine the mean fluorescence intensity (MFI) values of the gated population. **c**, Transport activity of unlabeled wild-type TmrAB ($TmrAB^{WT}$) compared with LD555/LD655-labeled TmrAB^{PG}. **d**, Effect of nanobody binding on $TmrAB^{WT}$ transport activity. Assays were performed in the absence or presence of Nb9F10^{S63C} at a 1:1 molar ratio (0.6 μ M each). Fluorophore labeling at the periplasmic gate and nanobody binding did not measurably affect TmrAB transport activity.

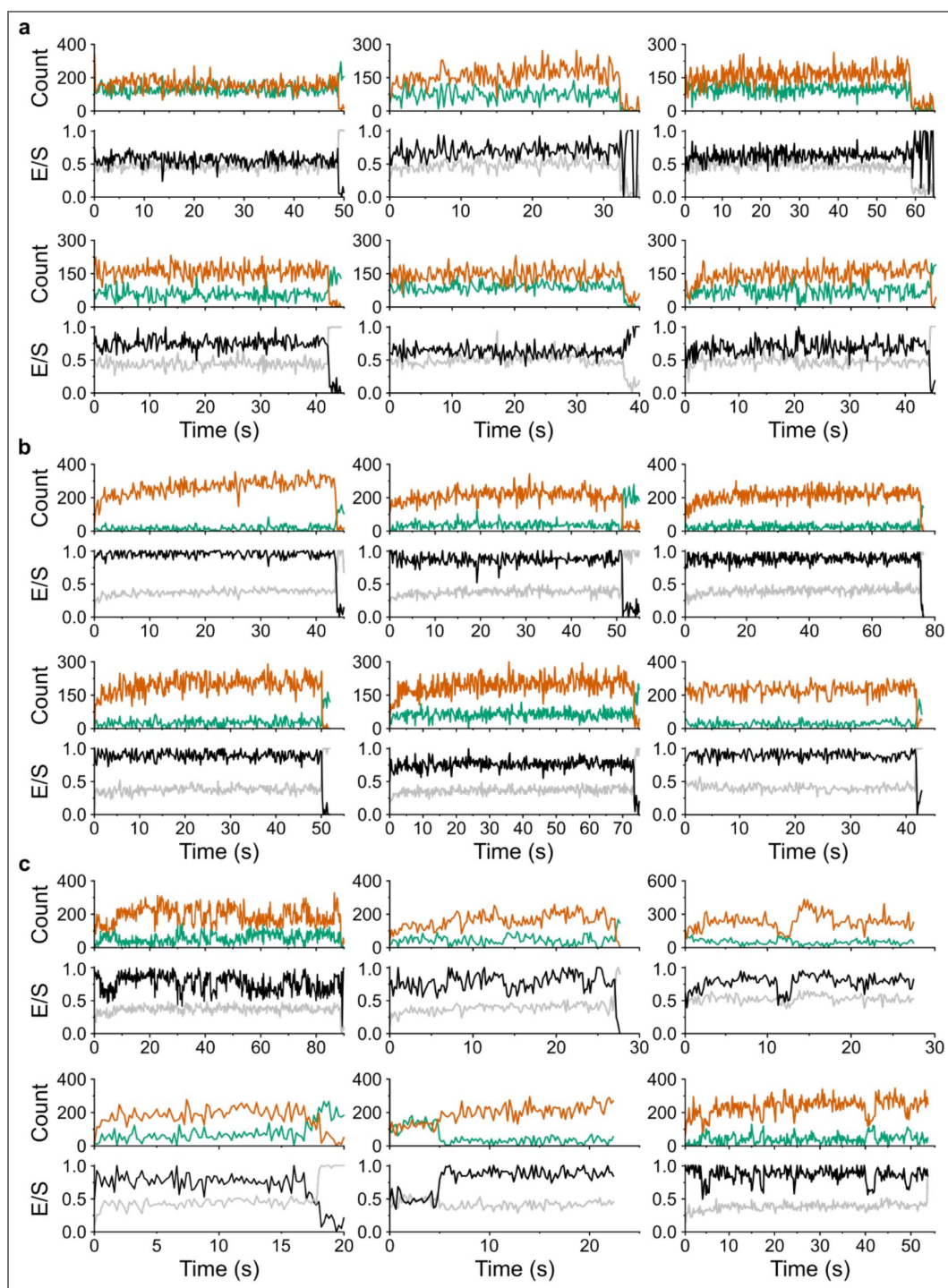


Figure 2-Figure supplement 1. Representative smFRET traces of TmrAB^{NBD}.

Representative single-molecule FRET (smFRET) traces of TmrAB^{NBD} recorded (a) in the absence of ATP and (b, c) in the presence of 3 mM ATP. Hidden Markov modeling (HMM) classified ATP-bound traces as (b) static or (c) dynamic based on the absence or presence of transitions between ATP-free and ATP-bound conformational states. Donor fluorescence upon donor excitation is shown in green, acceptor fluorescence upon donor excitation in orange, FRET efficiency (E) in black, and stoichiometry (S) in grey.

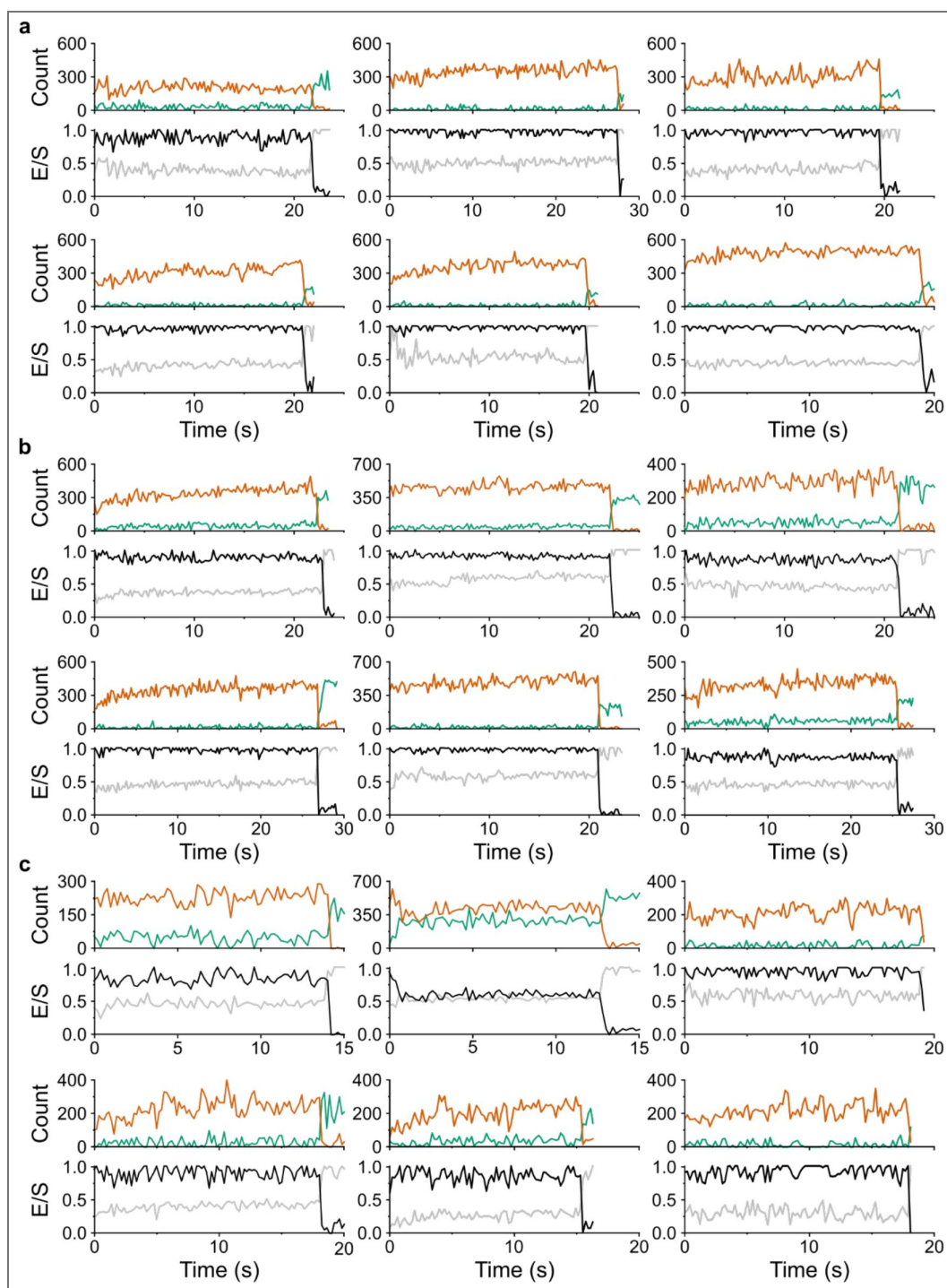


Figure 2-Figure supplement 2. Representative smFRET traces of TmrAB^{PG}.

Representative single-molecule FRET (smFRET) traces of TmrAB^{PG} recorded (a) in the absence of ATP and (b, c) in the presence of 3 mM ATP. Hidden Markov modeling (HMM) classified ATP-bound traces as (b) static or (c) dynamic based on the absence or presence of transitions between ATP-free and ATP-bound conformational states. Donor fluorescence upon donor excitation is shown in green, acceptor fluorescence upon donor excitation in orange, FRET efficiency (E) in black, and stoichiometry (S) in grey.

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Additional information

Author contributions

M.P. prepared all TmrAB samples and carried out the experiments for this study. M.P. performed data analysis. Curated traces were independently checked by C.N. to avoid human bias. M.P. and C.N. prepared functionalized glass slides for single-molecule FRET. M.P. and R.T. wrote the manuscript. R.T. conceived and supervised the work.

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Peer reviews

Reviewer #1 (Public review):

Summary:

Pecak et al have deciphered the conformational dynamics of a heterodimeric model ABC transporter, TmrAB, a functional homolog of the human antigen transporter TAP, using single molecule Forster resonance energy and fluorophores attached to residues at either nucleotide binding domains or periplasmic gate. The analysis not only differentiated ATP-free and bound states, but also enabled the real time monitoring of protein conformational changes precisely dissecting transport cycles and resolving transient intermediates. This study is absolutely significant in providing and establishing a general pipeline delineating the conformational dynamics in heterodimeric ABC transporters.

Strengths:

The scientific study is very well documented for experimental design, results and conclusions supported by the experimental data. Authors have determined the conformational dynamics of TmrAB across different ATP concentrations including physiological ones and resolved an outward open state and other conformational states consistent with previous cryoEM and DEER studies. Authors have also mentioned limitations in the study.

Comments on revised version.

Authors have worked on most of the revisions stated in previous feedback and included in the newer version, which has been significantly improved. Other comments have been described to be out of scope from this study.

<https://doi.org/10.7554/eLife.110967.2.sa2>

Reviewer #2 (Public review):

In their manuscript entitled 'ATP-driven conformational dynamics reveal hidden intermediates in a heterodimeric ABC transporter', Pečak et al. use elegant single-molecule FRET experiments in detergent to investigate the heterodimeric ABC transporter TmrAB. By combining simulations of the transporter's accessible volume with elegant trapping strategies, the authors identify an unresolved outward-facing open state and conclude that it is usually obscured by a rapidly interconverting ATP-bound ensemble. Overall, the study demonstrates that smFRET can resolve the short-lived intermediate states of TmrAB and potentially other ABC transporters that are obscured in ensemble measurements.

It is a very interesting study that highlights the power of combining high-resolution structural information with spectroscopic approaches. I had three major concerns with the original version, all of which have been addressed by the authors in this revised version.

<https://doi.org/10.7554/eLife.110967.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

Pecak et al have deciphered the conformational dynamics of a heterodimeric model ABC transporter, TmrAB, a functional homolog of the human antigen transporter TAP, using single-molecule Forster resonance energy and fluorophores attached to residues at either nucleotide binding domains or periplasmic gate. The analysis not only differentiated ATP-free and bound states but also enabled the real-time monitoring of protein conformational changes, precisely dissecting transport cycles and resolving transient intermediates. This study is absolutely significant in providing and establishing a general pipeline delineating the conformational dynamics in heterodimeric ABC transporters.

We thank the reviewer for this accurate and thoughtful summary of our work and its broader significance. We agree that the combination of single-molecule FRET with orthogonal validation approaches enables mechanistic resolution of conformational states and transitions that are not accessible by ensemble measurements. In particular, this framework allows direct discrimination of ATP-free and ATP-bound conformations, real-time tracking of transport cycle progression, and identification of transient intermediates in the heterodimeric ABC transporter TmrAB. We further agree that these capabilities support a generalizable strategy for dissecting conformation dynamics in related ABC transporters.

Strengths:

The scientific study is very well documented for experimental design, results, and conclusions supported by the experimental data. The authors have determined the conformational dynamics of TmrAB across different ATP concentrations, including physiological ones, and resolved an outward open state and other conformational states consistent with previous cryoEM and DEER studies.

Weaknesses:

The scientific study needs a bit of in-depth analysis with respect to consistency in K_d and its implications on the mechanism.

The apparent $K_{d,ATP}$ values were determined using two complementary approaches that report on different aspects of the system. Ensemble FRET measurements yielded values of $51 \pm 38 \mu\text{M}$ (TmrAB^{NBD}), $68 \pm 25 \mu\text{M}$ (TmrAB^{PG}), and $95 \pm 26 \mu\text{M}$ (TmrAB^{PG-EQ}), which are in good agreement with previously reported biochemical estimates ($\sim 100 \mu\text{M}$ for TmrAB^{EQ}) (Stefan et al, 2020). The slightly elevated value observed for the E – Q variant may reflect modest perturbation of nucleotide handling in this slow-turnover background. Notably, the close agreement between labeled and unlabeled variants indicates that fluorophore attachment does not measurably affect ATP binding.

In contrast, smFRET-derived $K_{d,ATP}$ values ($13 \pm 1 \mu\text{M}$ for TmrAB^{NBD} and $2 \pm 1 \mu\text{M}$ for TmrAB^{PG}) are systematically lower. This difference likely arises from the difficulty of deconvoluting overlapping FRET populations at sub- $K_{d,ATP}$ concentrations, particularly for TmrAB^{PG}, where state assignment is less well separated. Despite this quantitative offset, both approaches consistently indicate ATP saturation well below physiological concentrations and

therefore support the same mechanistic conclusion that ATP binding drives conformational switching in TmrAB.

Reviewer #2 (Public review):

In their manuscript entitled 'ATP-driven conformational dynamics reveal hidden intermediates in a heterodimeric ABC transporter', Pečák et al. use elegant single-molecule FRET experiments in detergent to investigate the heterodimeric ABC transporter TmrAB. By combining simulations of the transporter's accessible volume with elegant trapping strategies, the authors identify an unresolved outward-facing open state and conclude that it is usually obscured by a rapidly interconverting ATP-bound ensemble. Overall, the study demonstrates that smFRET can resolve the short-lived intermediate states of TmrAB and potentially other ABC transporters that are obscured in ensemble measurements.

It is a very interesting study that highlights the power of combining high-resolution structural information with spectroscopic approaches. I have three major points and a few minor criticisms.

We thank the reviewer for the thoughtful and constructive evaluation of our manuscript and for highlighting the strength of combining structural and single-molecule approaches. We have addressed all major and minor points in detail below and revised the manuscript where appropriate to clarify limitations, justify analysis choices, and improve transparency.

Major points:

(1) The main weakness is that the authors base their conclusions on a very limited set of FRET pairs. While TmrAB has been extensively studied in terms of its structure, the authors should at least acknowledge this limitation more clearly.

We agree that our conclusions are based on a limited number of FRET reporter pairs, and we now explicitly state this limitation in the revised manuscript. The chosen labeling positions were selected to probe two functionally critical regions—the nucleotide-binding domains and the periplasmic gate—based on prior structural and spectroscopic evidence. While this represents sparse sampling of the full conformational space, it is consistent with typical smFRET studies of membrane transporters, where experimental constraints generally limit the number of simultaneously accessible labeling positions (Asher et al, 2021; Asher et al, 2022; Levring et al, 2023; Wang et al, 2020).

Importantly, both independent reporter variants yield consistent ATP-dependent population shifts, supporting the robustness of the observed trends. We further clarify that additional labeling sites could, in principle, resolve finer structural sub-states; however, given the already limited population separation in the current variants, such extensions would likely provide diminishing returns in state resolvability under the present experimental conditions. This trade-off is now explicitly discussed.

(2) Most smFRET distributions were fitted with one, two, or three Gaussians. However, in several cases, additional populations with noticeable amplitudes appear to be present (e.g., Figure 3c at 0.1 mM and 3 mM ATP; Figure 4a, apo; Figure 4c, 0.3 mM R9L). Could the authors clarify why these populations were not included in the analysis?

We thank the reviewer for this careful observation. Low-amplitude sub-populations are occasionally detected in individual histograms; however, they were not included in the quantitative model because they do not meet criteria for reproducibility, amplitude robustness, or structural assignability. Specifically, these features vary between replicates, contribute minimally to total population, and cannot be mapped to structurally or

biochemically defined states based on available cryo-EM (Hofmann et al, 2019), DEER/PELDOR (Barth et al, 2018; Barth et al, 2020), or accessible-volume simulations.

Similar minor subpopulations have been reported in smFRET studies and often attributed to photophysical or labeling heterogeneity effects (Asher et al, 2022; Husada et al, 2018). To avoid over-parameterization, we therefore restricted analysis to reproducible, structurally supported states. This rationale is now clarified in the revised manuscript.

(3) Figure 3c (3 mM ATP): Is it truly possible to distinguish the two states in this distribution?

We agree that state separation in the TmrAB^{PG} variant is limited ($\Delta E = 0.11$), and we now explicitly acknowledge this constraint in the manuscript. To improve robustness under these conditions, we used a constrained fitting strategy in which the apo-state distribution was fixed from nucleotide-free measurement, reducing parameter degeneracy during fitting of ATP-bound datasets.

While single-molecule trajectory-based approaches such as Hidden Markov Modeling would be ideal for resolving dynamic interconversion, this was not feasible due to the low fraction of dynamic traces at the available temporal resolution. We therefore rely on population-level analysis, which remains consistent across replicates and reporter variants.

Notably, independent measurements from two reporter positions (TmrAB^{NBD} and TmrAB^{PG}) yield similar ATP-bound population fractions at saturating ATP concentrations (~77% vs. ~80%), supporting the robustness of the inferred state distribution despite partial overlap.

We have revised the manuscript to more clearly articulate methodological limitations, strengthen the justification of our analytical approaches, and improve the clarity of data presentation. These revisions enhance the transparency and robustness of the study and address the reviewer's concerns.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

Here are a few comments that can help to improve the study.

(1) Line 115: The authors have checked the purity and monodispersity of the protein sample using SDS-Gel and size exclusion chromatography; however, additional characterization using negative stain electron microscopy, which clearly shows the monodispersity, will be useful.

We agree that negative stain EM can provide an additional assessment of sample homogeneity. Given the extensive prior structural characterization (Hofmann et al, 2019; Nocker et al, 2026; Nöll et al, 2017) and the SEC profiles presented here, we believe that additional negative stain EM would unlikely provide substantial new information regarding sample homogeneity. We have clarified this point in the manuscript by explicitly referencing the relevant cryo-EM studies.

(2) Line 116: The authors have mentioned that the enzymatic activity of TmrAB was retained after purification. Although smFRET results showing conformational dynamics of TmrAB confirm its ATPase activity, a comment on the effect of labelling on ATPase activity will be useful.

We appreciate this important point. Previous studies on spin-labeled TmrAB^{NBD} demonstrated transport activity comparable to wild-type TmrAB, indicating that cysteine substitution and label conjugation do not substantially perturb this variant (Barth et al, 2018). In addition, AV simulations showed that fluorophores at the TmrAB^{NBD} labeling positions do

not interfere with ATP- or substrate-binding sites, supporting the conclusion that FRET labeling does not affect ATP binding, hydrolysis, or transport. For TmrAB^{PG}, however, equivalent transport data were not available, and AV simulations suggested interference of fluorophores with periplasmic gate dynamics. We therefore directly compared the transport activity of LD555/LD655-labeled TmrAB^{PG} and unlabeled wild-type TmrAB using a single-liposome transport assay with the fluorescein-labeled peptide C4F (RRYC^FKSTEL) (F, fluorescein; Fig. 1– Fig. S3a). Both variants showed indistinguishable transport activity, demonstrating that fluorophore conjugation at the periplasmic gate preserves transport function.

(3) Line 117 and Figure S1c. Please add the reference for consistency of ATPase activity with previous studies on TmrAB.

We have added a reference to previous biochemical studies reporting comparable ATPase activity and kinetic parameters for TmrAB to support the consistency of our measurements.

(4) Line 119: It mentions that "Cysteine-maleimide labeling of detergent-solubilized TmrAB achieved site-specific labeling efficiencies exceeding 90%". The legend of Figure S1d mentions about labeling efficiency in the range of 40-50%. A clarification will be helpful for the reader. Also, calculations can be extended to the ratio of LD555 and LD655 labels on the molecule, which can be considered in analyzing results.

We apologize for the lack of clarity. The reported >90% labeling efficiency refers to the site-specific cysteine labeling efficiency per accessible site, as determined by dye incorporation. In contrast, the 40–50% values shown in Fig.1–Fig. S1d reflect the per-site efficiency for donor-lonely and acceptor-only populations respectively, which together account for the >90% overall labeling efficiency. We have revised the main text and figure legend to clearly distinguish between per-cysteine labeling efficiency and the fraction of correctly double-labeled molecules. We also clarify that only complexes with appropriate donor– acceptor stoichiometry were included in the smFRET analysis.

(5) Figure 1: Line 627: This line mentions "For all simulations, TmrA is shown in blue with LD655 (orange) and TmrB in yellow with LD555 (green)." Is it (which label on which subunit) known for the experimental setup?

We thank the reviewer for pointing out this potential source of confusion. In the experimental system, fluorophore attachment occurs stochastically. Therefore, the assignment of donor and acceptor dyes to specific subunits is random. The representation shown in Figure 1 reflects one possible configuration for visualization purposes only. We have clarified this explicitly in the figure legend to avoid misinterpretation.

(6) Figure S1-2a. Tau value can be better represented in a graph for visual readers instead of in the form of a table, and a dotted line with the threshold (~1 ns) will give a better representation of no change. Values can be included in the graph as well.

We appreciate this helpful suggestion. We have revised Figure S1-2a to include a graphical representation of fluorescence life times, including a reference line around ~1 ns to facilitate visual comparison. Numerical values are retained alongside the plot for completeness.

(7) Figure 2a: Each component of the assembly has been pointed with an arrow, which can mix two components and confuse readers. It would be good to make a legend column on the left or right and depict or indicate each component of the assembly clearly.

We have changed the labeling in Figure 2a to improve clarity by separating the components and introducing a clearer legend layout, ensuring that each element of the assembly is unambiguously labeled.

(8) *The physiological concentration of ATP can range up to 5-10 mM. A comment on choosing the ATP concentration specifically to be 3 mM would be useful for the readers.*

We appreciate this suggestion. While intracellular ATP concentrations can reach up to 5–10 mM, values around 3 mM are commonly used as physiologically relevant conditions in *in vitro* biochemical and biophysical studies. We selected 3 mM ATP as a representative near physiological concentration that ensures saturation of ATP-dependent conformational transitions while remaining comparable to previous studies on TmrAB (Hofmann et al, 2019; Nocker et al, 2026; Nöll et al, 2017; Stefan et al, 2020). We have clarified this rationale in the manuscript.

(9) *Figure 2c is not cited in the text.*

We thank the reviewer for noting this oversight. Figure 2c is now explicitly cited in the main text.

(10) *Results in Figure 2 and 3 have been analyzed using 2 and 3 Gaussian distributions, respectively. It would be good to explain the rationale for it.*

We appreciate that this important point was brought to our attention. The number of Gaussian components was determined based on the minimal model required to describe reproducible and structurally supported populations. For ATP titration experiments (Figure 2 and Figure 3), two populations (apo and ATP-bound) were sufficient and consistent across replicates. In contrast, three populations were required under trapping conditions (Figure 4), where an additional state (OFF^{open}) becomes kinetically stabilized and clearly resolved. We have clarified this rationale in the manuscript.

(11) *Figure 3b: data points do not seem to be saturated with respect to ATP concentration. It needs more points beyond 3 mM. Different K_d at different sites in the structure could represent differential local dynamics over the structure.*

Previous structural studies demonstrated that 1 mM ATP is sufficient to saturate both nucleotide-binding sites under trapping conditions (Hofmann et al, 2019), indicating that the concentration range used here is adequate. Consistent with this, both ensemble and smFRET measurements approach saturation by 3 mM ATP, a near-physiological condition commonly used in biochemical studies. While additional data points above 3 mM could further define the plateau, they are unlikely to alter the mechanistic conclusion. We have clarified this point in the manuscript.

(12) *Figure 3 and Figure 1 - S1 have two different K_d values with respect to ATP concentration; both of these graphs measure conformational changes using smFRET. A comment specifying these K_d values based on single molecule verses ensemble measurement from will be helpful for readers.*

We appreciate this important point and have clarified it in the manuscript and the response to Reviewer #1 above. The $K_{d,ATP}$ values in Fig. 1–Fig. S1 are derived from ensemble FRET measurements, whereas those in Fig. 3 are obtained from smFRET population analysis. This difference likely arises from the difficulty of deconvoluting overlapping FRET populations at sub- $K_{d,ATP}$ concentrations, particularly for TmrAB^{PG}, where state assignment is less well separated. Despite this quantitative offset, both approaches consistently indicate ATP saturation well below physiological concentrations and therefore support the same mechanistic conclusion that ATP binding drives conformational switching in TmrAB. We now explicitly distinguish these methods and their interpretation in the manuscript.

(13) *Figure 4: Slow-turnover TmrAB mutant has been employed in cysteine mutant on the PG opening side, but not towards the NBD side. Either experimental data or a comment*

on not pursuing it would be helpful for the reader. Similarly, experiments in the presence of peptide and in the absence of ATP, which can help to understand the role of substrate in conformational dynamics in the absence of ATP, are not pursued in this study. Along similar lines, experiments with wild type, in the presence of MgADP +/- substrate, are not shown in this study.

We thank the reviewer for these insightful suggestions. The slow-turnover variant was specifically applied to the periplasmic gate reporter (TmrAB^{PG}) because this construct provides direct sensitivity to outward-facing conformations, which are central to resolving the OF^{open} state. In contrast, the NBD reporter primarily monitors nucleotide-binding domain (NBD) dimerization and is less suitable for distinguishing periplasmic conformational differences.

Experiments in the absence of ATP but in the presence of peptide, as well as MgADP ± substrate, would indeed be valuable for further dissecting substrate effects. However, these conditions are beyond the scope of the current study, which focuses on ATP-driven conformational dynamics and the identification of kinetically hidden intermediates. We have added a statement in the Discussion to acknowledge these possibilities as directions for future work.

(14) Figure 4, peptide concentration has been varied in the right panel. The result can also be presented as the % of OFopen and OFoccluded state with increasing concentration of peptide.

We thank the reviewer for this suggestion. While such a plot would indeed be informative and could improve our understanding of substrate binding and substrate-induced trans-inhibition, the current dataset does not contain sufficient data points to construct a reliable concentration-dependent curve, particularly given that peptide saturation was not reached in our experiments. The characterization of substrate binding is further complicated by the presence of two distinct substrate-binding sites one in the outward-facing and one in the inward-facing state with likely completely different K_d values and would require a more complex binding model. We have therefore decided against including this plot in the current manuscript. We do acknowledge, however, that future smFRET studies with improved temporal resolution are particularly well suited to investigating substrate binding to TmrAB and its effects on conformational equilibrium, and we have noted this in the Discussion.

Reviewer #2 (Recommendations for the authors):

(1) In all figures, can you please label the transporter schematics with the conformational states they represent?

We thank the reviewer for this suggestion. All transporter schematics in the main and supplementary figures have been updated to include clear labels indicating the corresponding conformational states, thereby improving clarity and consistency.

(2) As a suggestion, it may improve clarity to include the labelling positions (residue numbers) directly in Figure 1a and b, even though they are provided in the legend.

We appreciate this suggestion. Residue numbers corresponding to labeling positions have now been added directly to Figure 1a and b to improve readability and facilitate interpretation.

(3) Lines 183-188: This is a key point. It would be helpful to include a reference line for the expected state (0.63). Interestingly, this value coincides with the shoulder observed in Fig. 3c (0.1 mM ATP). Is there an explanation for this (see also point 2)?

We thank the reviewer for highlighting this point. We considered adding a reference line at 0.63 to the plot; however, we decided against it. While a subpopulation does appear at ~0.63—consistent with the expected FRET efficiency of the OF^{open} conformation—it is only present in a single condition (0.1 mM ATP) and is not observed across other ATP concentrations for this TmrAB variant. It more likely reflects a minor non-reproducible subpopulation or photophysical artefact, in line with our response to Point 2 of the public review (Reviewer #2).

(4) The final section of the Results section seems like an afterthought, especially since the heading suggests a broader scope.

We appreciate this comment. We have revised the final section of the Results to improve its structure and ensure that the scope indicated by the heading is fully reflected in the content. This section now more clearly integrates kinetic and thermodynamic aspects of the transport cycle.

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