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Dynamics of P-type ATPase transport cycle revealed by single-molecule FRET

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Abstract

P-type ATPases are ubiquitous primary transporters that pump cations across cell membranes through the formation and breakdown of a phosphoenzyme intermediate. Structural investigations suggest a transport mechanism defined by conformational changes in the cytoplasmic domains of the protein that are allosterically coupled to transmembrane helices so as to expose ion binding sites to alternate sides of the membrane. Here, we have employed single-molecule fluorescence resonance energy transfer (smFRET) to directly observe conformational changes associated with the functional transitions in the *Listeria monocytogenes* Ca²⁺-ATPase (LMCA1), an orthologue of eukaryotic Ca²⁺-ATPases. Using the smFRET approach we identify key intermediates with no known crystal structures, and our findings delineate reversible and an essentially irreversible step in the transport process wherein Ca²⁺ efflux by LMCA1 is rate limited by phosphoenzyme formation and resolved by ADP and Ca²⁺ release leading to an open E2P state.

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Author Contributions: P.N. conceived the study. M.D., with assistance from M.K., J.L.A. and C.R.K., performed site-directed mutagenesis, protein expression, purification, labeling, and ATPase activity experiments. M.D. and T.L.M.S., with guidance from J.P.A., performed and analyzed the phosphorylation experiments. D.S.T., with help from S.C.B. and R.B.A., conceived the single-molecule FRET experiments, performed them, and analyzed the data. P.N. and S.C.B. supervised the project. All authors wrote the manuscript.

Introduction

Phosphorylation-type (P-type) ATPases transport cations against their concentration gradients to maintain electrochemical potentials across cell membranes. In eukaryotes, Ca^{2+} -ATPases extrude or sequester Ca^{2+} into internal stores after calcium signaling events. This energetically uphill transport is powered by ATP hydrolysis *via* autocatalyzed formation and breakdown of a phosphoenzyme intermediate.

Biophysical and crystallographic studies of P-type ATPases outline a common reaction mechanism in which ATP-driven phosphorylation and subsequent dephosphorylation of the cytosolic phosphorylation (P) domain couples to the opening and closure of ion-binding sites within specific transmembrane segments^{1–9} (Fig. 1A–B; Extended Data Fig. 1). The transport cycle of a typical P-type ATPase is initiated by binding of Mg^{2+} -ATP and the transported substrate ion to an inward-facing E1 state, followed by phosphorylation of a conserved aspartate in the P domain to form an occluded E1P-ADP conformation. The subsequent transition to an outward-open E2P state, mediated by a rotation of the actuator (A) domain by $\sim 120^\circ$ relative to both the nucleotide binding (N) and P domains, induces extracellular release of substrate (in exchange for protons for Ca^{2+} -ATPases). The aspartate is then dephosphorylated, catalyzed by a conserved glutamate within the A domain, resulting in an occluded E2 state. Finally, an E2→E1 transition returns the system to an inward-oriented E1 state, reset for another transport cycle.

Kinetic features of P-type ATPase mediated transport have been defined through bulk experiments under conditions that isolate specific partial reactions. Such investigations have posited that the rate-limiting step for sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) is either the E1P→E2P transition^{10, 11} or the return of E2 to E1¹². By contrast, the rate-limiting feature of the sodium-potassium pump transport cycle is assumed to be counter-ion (K^+) release to the cytoplasm in the E2→E1 return step¹³ or, at high voltage, the extracellular release of Na^+ ¹⁴, whereas it is Cu^+ release and dephosphorylation for Cu(I)-ATPases¹⁵. These observed differences may reflect both diversity in P-type ATPase mechanisms and the specific experimental conditions imposed.

To directly measure large-scale dynamics of P-type ATPase transport, we employed single-molecule fluorescence resonance energy transfer (smFRET) imaging^{16–19} of LMCA1, a Ca^{2+} -ATPase of the pathogenic bacterium *Listeria monocytogenes*^{20–22}. LMCA1 belongs to a subclass of bacterial calcium pumps^{20, 23} associated with virulence and survival in infected host cells²⁴ and thus of microbiological and medical interest. LMCA1 shares 38% sequence identity with SERCA, and belongs to the same P2A-ATPase subfamily. Although it displays a higher apparent K_M for Ca^{2+} , a higher pH optimum, and transports one Ca^{2+} ion instead of two²⁰, both are predicted to follow structurally related transport mechanisms. In the present study, we directly observe the states transited by LMCA1 during the Ca^{2+} transport cycle, including intermediates associated with Ca^{2+} de-occlusion with no known crystal structure. Our findings delineate a kinetic framework in which steps leading to E1P state formation are rate-limiting for transport, and where ADP and Ca^{2+} release appears to be irreversible.

Intrinsic dynamics of labeled LMCA1

Conformational changes associated with Ca^{2+} transport were detected *via* time-dependent changes in energy transfer efficiency (FRET)¹⁸. To detect transitions between E1 and E2 states with a maximal FRET change (**Methods**), cysteine-labeling sites were introduced into the A and P domains of LMCA1 at positions T24 and A530, respectively, within an engineered LMCA1 construct, LMCA1_{TM}-A/P, with three out of five intrinsic cysteines removed²² (Fig. 1A; Extended Data Fig. 1). Based on the homology to SERCA, FRET is expected to be high in E1 and low in E2 states (Supplementary Table 1). Wild-type LMCA1 exhibits similar Ca^{2+} binding and ATPase activities in both non-ionic detergents and proteoliposomes²⁰. LMCA1_{TM}-A/P, hereafter denoted LMCA1, maintained ~33% of wild-type ATPase activity ($\sim 5.5 \text{ s}^{-1}$ vs. 17 s^{-1} at neutral pH and 10 mM Mg^{2+} ; Extended Data Fig. 2A). For smFRET, purified LMCA1 was labeled with self-healing fluorophores, critical for high spatial and temporal resolution imaging^{25, 26}. Near-quantitative fluorophore labeling (>90%) only modestly reduced ATPase activity ($\sim 3 \text{ s}^{-1}$; Extended Data Fig. 2B). Individual detergent-solubilized and immobilized LMCA1 molecules were imaged at 15 ms time resolution within passivated microfluidic devices using wide-field, total internal reflection fluorescence (TIRF) microscopy equipped with sCMOS cameras¹⁷ (Extended Data Fig. 3).

Ligands stabilize LMCA1 intermediates

In the absence of substrates (0.1 mM EGTA, pH 7.5), LMCA1 exhibited a single, broad FRET distribution with a mean of ~ 0.67 (Fig. 1C–E; Extended Data Fig. 4A). Anti-correlated fluctuations in donor and acceptor fluorescence traces suggest the width of this distribution may arise from rapid, poorly-resolved transitions between non-zero FRET states (Extended Data Fig. 4B). We quantified the presence of such dynamics using the Pearson correlation coefficient of the fluorescence traces (**Methods**; Fig. 1F), which should be negative only in the presence of FRET dynamics^{27, 28}. While the underlying rates could not be accurately determined, this analysis confirmed that the apo state is dynamic. Given the neutral pH and absence of substrates, we hypothesized that these dynamics report on reversible interconversion between Ca^{2+} -free, high-FRET E1 and low-FRET (H)E2 states, which reflect the protonation state of glutamates in the transmembrane Ca^{2+} binding site^{29, 30} (Fig. 1B). Consistent with this model, reduced pH lowered the population mean FRET value with a midpoint of 6.3 – similar to the estimated pK_a values of the glutamates in the SERCA Ca^{2+} binding site³⁰ (Fig. 1E; Supplementary Table 2).

At pH 7.5, the addition of Ca^{2+} to LMCA1 promoted a higher mean FRET value (~ 0.74 ; $p=2\text{E-}6$; Fig. 1C,D), a fluorescence correlation coefficient closer to zero ($p=0.006$; Fig. 1F; Extended Data Fig. 4C), and a narrower FRET distribution (Extended Data Fig. 4A) – all consistent with E1 state stabilization. The EC_{50} of this effect was $\sim 250 \mu\text{M}$ Ca^{2+} (Extended Data Fig. 4D), comparable to the concentration required for half maximal ATP turnover²⁰. At pH 6.0, the apparent Ca^{2+} affinity was reduced by an order of magnitude to 2.5 mM (Extended Data Fig. 4D), consistent with competition of Ca^{2+} and protons for the same binding site^{29, 30}.

Consistent with a partially independent binding mode, ATP also increased the mean FRET of the E1 state in the absence of Ca^{2+} ($p=6\text{E-}6$), albeit to a lesser extent than Ca^{2+} alone (Fig. 1C), and brought the fluorescence correlation coefficient closer to zero ($p=0.006$; Fig. 1F). We infer that both Ca^{2+} and ATP binding contribute to E1 state stabilization to prepare LMCA1 for autophosphorylation². These findings confirm that the cytoplasmic A and P domains are allosterically coupled to the transmembrane Ca^{2+} binding sites more than 50 Å away (Fig. 1A).

To identify and characterize the E2P state of LMCA1, we introduced BeF_x that coordinates the catalytic aspartate (D334) and mimics P domain phosphorylation⁵. As expected, BeF_x induced a stable, low-FRET LMCA1 conformation (~ 0.37 ; $p=8\text{E-}4$; Fig. 1C,F,G; Extended Data Fig. 4A,E). In agreement with the BeF_3^- -stabilized E2P state crystal structure of SERCA⁵ (Supplementary Table 1; Extended Data Fig. 1), the observed change in FRET is consistent with an increase in the distance between the A and P domains on the order of 15 Å for the E1P to E2P transition.

Transport cycles exhibit rapid dynamics

To directly monitor LMCA1 dynamics associated with repetitive Ca^{2+} transport cycles, we stopped-flow delivered saturating ATP (1 mM) to LMCA1 pre-loaded with Ca^{2+} (1 mM). In such experiments, we observed transient fluctuations to lower FRET states, each generally spanning only a single 15 ms frame (Fig. 1H). At the ensemble level, such transitions produced a lower mean FRET (Fig. 1C) and fluorescence correlation coefficient closer to zero (Fig. 1F; Extended Data Fig. 4F) compared to Ca^{2+} alone or Ca^{2+} with the slowly-hydrolyzing ATP analog ATP γS ($p<0.01$). Notably, dynamics of this kind were not evident in the presence of ATP γS , or ADP with AlF_4^- , i.e. conditions mimicking the E1-ATP pre-phosphorylation state or E1P-ADP transition state of phosphorylation (Fig. 1C,F; Extended Data Fig. 4G,H).

To resolve the rapid Ca^{2+} and ATP-induced FRET fluctuations, we repeated these experiments with three-fold faster time resolution (5 ms). Distinct dwells in low- and high-FRET states could be discerned clearly enough to enable state assignment (Fig. 1I,J). The higher-FRET state had a lifetime of ~ 200 ms, while the lower-FRET state had an upper bound of 5–10 ms (Extended Data Fig. 4I,J). The frequency of these events depended on ATP, Ca^{2+} , and Mg^{2+} concentrations (Fig. 1K), with a maximal rate of $\sim 6\text{ s}^{-1}$. The EC_{50} values were 19 μM , 160 μM , and 1.8 mM, respectively (Supplementary Table 2). The Hill coefficients were 1.0, consistent with the expected ligand stoichiometries. Ca^{2+} concentrations greater than 1 mM were inhibitory, likely by competitive, low affinity substitution of Ca^{2+} in the catalytic Mg^{2+} binding site³¹. We conclude that the FRET transitions observed in the presence of Ca^{2+} and ATP reflect repetitive transport cycles in which brief low-FRET, E2P states punctuate long-lived E1 states. This model posits that one or more conformational transitions prior to LMCA1's conversion to the E2P state may be rate-limiting to the overall transport cycle.

Trapping phosphotransfer intermediates

To better resolve the kinetics of transitions, we introduced mutations in LMCA1 that isolate partial transport reactions by blocking phosphorylation or dephosphorylation. To study events leading up to enzymatic phosphotransfer and E1P formation, we introduced a D334N mutation blocking phosphorylation³² (Fig. 2A; Extended Data Fig. 1). As for LMCA1, Ca^{2+} stabilized a high-FRET (~ 0.72), E1-like state of D334N-LMCA1 (Fig. 2B). Consistent with potent inhibition of phosphotransfer and E2P state formation, the E1 state was further stabilized by ATP (0.75 ± 0.003 vs. 0.72 ± 0.005 ; mean $\pm$ SD, $n=3$; $p=0.008$; Extended Data Fig. 5A; Fig. 2B), in line with the (Ca)E1-ATP γ S state observed for LMCA1 (Extended Data Fig. 4A,G). Stopped-flow delivery of ATP and Ca^{2+} to D334N-LMCA1 revealed that the rate of (Ca)E1-ATP formation was faster than the imaging time resolution ($>67 \text{ s}^{-1}$; Extended Data Fig. 5B–C).

To examine steps subsequent to phosphotransfer, we introduced an E167Q mutation to remove the glutamate that mediates dephosphorylation³³ (Fig. 2A). In the absence of ATP, E167Q-LMCA1 behaved similarly to wild-type LMCA1 (Fig. 2C). However, in stark contrast to LMCA1 and D334N-LMCA1, the addition of ATP in the presence of Ca^{2+} completely converted the E167Q-LMCA1 mutant into a stable, low-FRET, E2P state (Fig. 2C,D). As for the BeF_x -trapped LMCA1 (Fig. 1G), high-FRET E1 recurrences were not observed once the E2P state was achieved (Fig. 2D).

Achieving E1P sets the transport rate

To investigate the rate-limiting features of the first half of the transport cycle (E1 $\rightarrow$ E2P; Fig. 1B), we quantified the time between ligand delivery to E167Q-LMCA1 and formation of the E2P state (Fig. 2D) as a function of ATP, Ca^{2+} or Mg^{2+} concentrations (Fig. 2E–G). In all cases, the rate of achieving the E2P state followed a single exponential process that was well fit to a dose-response function with a Hill slope of unity, consistent with the expected ligand stoichiometry. The concentration dependence of the observed E1 $\rightarrow$ E2P transition in E167Q-LMCA1 was in line with affinity measurements made on SERCA³⁴, and similar to those deduced from transition rates of wild-type LMCA1 (Fig. 1; Supplementary Table 2). Notably, the apparent rate of E2P formation was $\sim 6 \text{ s}^{-1}$ (Fig. 2G), at least 10 times slower than substrate binding (Extended Data Fig. 5C).

On path to E2P formation, LMCA1 is expected to transit a [Ca]E1P-ADP phosphoenzyme intermediate (Fig. 1B), which in SERCA is approximated by the $[\text{Ca}_2]\text{E1-AlF}_4^-$ -ADP and $[\text{Ca}_2]\text{E1P-AMPPN}$ structures^{2, 5} (Extended Data Fig. 1). Given the aforementioned observation that Ca^{2+} and ATP bind rapidly ($>67 \text{ s}^{-1}$; Extended Data Fig. 5C), the transport cycle appears rate-limited by either formation of the [Ca]E1P state or the subsequent conformational transition to the occluded [Ca]E2P state (Fig. 1B). To determine if the latter transition is rate-limiting, we imaged E167Q-LMCA1 at saturating ATP (1 mM) and sub-saturating Ca^{2+} (10 μM) concentrations. We hypothesized that if the conformational change from [Ca]E1P to [Ca]E2P were rate-limiting, we would expect transient accumulation of high-FRET [Ca]E1P prior to formation of low-FRET E2P states. However, although rare instances of single-frame dwells of this nature were evidenced (Extended Data Fig. 6A),

high-FRET states did not accumulate (Extended Data Fig. 6B–C), indicating that the [Ca]E1P state is generally much shorter-lived than the integration time of imaging (15 ms). Notably, the addition of ADP (1 mM), which rapidly reacts with ADP sensitive [Ca]E1P states after phosphotransfer³⁵, displayed no significant effect (Extended Data Fig. 6D). Hence, we conclude that steps between substrate binding and phosphoenzyme formation, and not the following structural transition from high to low FRET states, are rate limiting for transport in LMCA1.

We used [γ -³²P]ATP phosphorylation experiments to independently measure the rate of phosphoenzyme formation. Under steady-state conditions the phosphorylation level of LMCA1_{WT} was ~20% of the dephosphorylation incompetent E167Q-LMCA1_{WT} mutant, consistent with a slow phosphorylation step (Extended Data Fig. 7A). Quenched-flow experiments triggered by addition of [γ -³²P]ATP to E167Q-LMCA1_{WT} displayed a rate of ~13 s⁻¹ when pre-incubated with Ca²⁺ (Fig. 2H), and ~11 s⁻¹ upon simultaneous addition of Ca²⁺ and [γ -³²P]ATP (Extended Data Fig. 7B). These rates are considerably slower than those determined for SERCA1a under similar conditions¹⁰. The rate of phosphoenzyme formation was approximately 8.2 s⁻¹ for labeled LMCA1_{TM}-A/P pre-loaded with Ca²⁺ (Fig. 2H), consistent with a slight decrease due to fluorophore labeling, but similar to the rate of E2P formation estimated from our smFRET results (~6 s⁻¹), and close to the maximal rate of phosphorylation (V_{max}) obtained from a Michaelis-Menten analysis (~15 s⁻¹; Extended Data Fig. 7C). As the labeled LMCA1_{TM}-A/P protein rapidly binds Ca²⁺ and ATP (>67 s⁻¹; Extended Data Fig. 5), these data provide supporting evidence that E1P formation subsequent to substrate binding is rate-limiting to the overall transport cycle.

Characterization of the [Ca]E2P state

We next sought to slow dephosphorylation to examine steps preceding outward release of Ca²⁺ (Fig. 1B). This was accomplished by inserting four glycines between residues 44 and 45 in the A/M1 linker connecting the A domain and transmembrane M1 helix (G₄-LMCA1; Fig. 3A), which in SERCA reduces the rate of transport by slowing Ca²⁺ release and dephosphorylation³⁶. The G₄ insertion reduced the rate of ATP turnover ~15-fold to ~0.35 s⁻¹ (Extended Data Fig. 2A), while having little impact on E1 conformations or ligand responsiveness (Extended Data Figs. 8A and 4A). In the presence of 1 mM Ca²⁺, G₄-LMCA1 exhibited only brief excursions to lower-FRET states, which were diminished in the presence of ATP γ S (Extended Data Fig. 8A–C). We therefore infer that G₄-LMCA1 is only modestly altered with respect to substrate binding, while exhibiting weaker allosteric coupling between the A and TM domains.

In striking contrast to LMCA1, stopped-flow addition of ATP (1 mM) in the presence of Ca²⁺ (1 mM) to G₄-LMCA1 resulted in repeated, clearly defined cycles between high- and low-FRET states (Fig. 3B; Extended Data Fig. 8A,D). Notably, the lower state had a distinctly higher FRET efficiency than the BeF_x-stabilized state (0.36±0.005 vs. 0.29±0.01, mean ± SD, *n*=3; *p*=5E-5; Extended Data Fig. 8A). This finding suggests that the G₄ insertion prevents efficient E2P-state formation from which Ca²⁺ is normally released (see further below). We therefore infer that the G₄-LMCA1 mutant reversibly samples an

intermediate state prior to Ca^{2+} site de-occlusion in which the A domain is not completely rotated – the putative and structurally unknown [Ca]E2P state³⁶.

The transition frequency in $\text{G}_4\text{-LMCA1}$ increased as ATP, Ca^{2+} , and Mg^{2+} substrates approached the millimolar range (Fig. 3C; Supplementary Table 2), with EC_{50} values congruent with those observed with the E167Q mutant (Fig. 2G). This increase in transition rate arose from shorter high-FRET state dwells (Extended Data Fig. 9A–D), which converged to ~90 ms, while low-FRET state dwells remained relatively constant at ~250 ms (Extended Data Fig. 9E–H). The net result of increasing substrate concentration was thus an accumulation in low-FRET, [Ca]E2P states (Extended Data Fig. 8E). Consistent with previous studies^{20, 31, 37, 38}, and as described above, Ca^{2+} concentrations above 1 mM (Fig. 3C), and low pH (Fig. 3D), slowed the overall transition rates by prolonging the dwell time in high-FRET, E1 states (Extended Data Fig. 9B,D).

At saturating substrate concentrations (1 mM ATP, 1 mM Ca^{2+} , 10 mM Mg^{2+} ; pH 7.5), $\text{G}_4\text{-LMCA1}$ transitioned between states at approximately 3 s^{-1} (Fig. 3C) – over 8 times faster than ATP hydrolysis (0.35 s^{-1} ; Extended Data Fig. 2A). This finding suggests that the dynamics observed in the mutant principally reflect non-productive transitions. All considered, the Ca^{2+} concentration dependence of transition rates in $\text{G}_4\text{-LMCA1}$ suggests that the observed dynamics are dominated by reversible transitions between E1 and [Ca]E2P intermediate states in which the phosphoenzyme is formed *via* phosphotransfer from ATP and broken down *via* ATP synthesis from ADP that remains bound in the [Ca]E2P intermediate. In support of LMCA1 returning to apo E1 states in these cycles, rapid buffer exchange to remove ATP or Ca^{2+} , or to replace ATP with ATP γS , halted dynamics within ~380 ms (Extended Data Fig. 8G–I) – on the order of one conformational cycle. In support of ADP remaining bound during the [Ca]E2P state dwells, addition of ADP had no effect on state occupancy or rates of dynamics (Extended Data Fig. 8J).

To better define the low-FRET state visited during these reversible sampling events, we next combined the A/M1 insertion and dephosphorylation mutation to create $\text{G}_4\text{-E167Q-LMCA1}$. Like E167Q-LMCA1, the combined mutant showed a rapid drop in FRET following ATP addition in the presence of Ca^{2+} (Fig. 3E; Extended Data Fig. 10). Unlike E167Q, however, we observed an essentially irreversible, multi-step process wherein the E2P state was achieved via a short-lived intermediate (~550 ms). This state exhibited a modestly higher FRET value, consistent with the [Ca]E2P state sampled by $\text{G}_4\text{-LMCA1}$. No such dwell was observed in E167Q-LMCA1 (Fig. 3F), suggesting that this intermediate is transient (<15 ms) during the unimpeded transition to the E2P state. Unlike $\text{G}_4\text{-LMCA1}$, reverse transitions from intermediate FRET to E1 states, however, were only rarely observed in the $\text{G}_4\text{-E167Q-LMCA1}$ mutant. E167 may therefore play a role in the reverse transition or the mutation accelerates de-occlusion. Consistent with the latter, the [Ca]E2P to E2P transition rate in $\text{G}_4\text{-E167Q-LMCA1}$ was $\sim 2 \text{ s}^{-1}$ (Fig. 3G) – nearly an order of magnitude faster than ATP turnover in $\text{G}_4\text{-LMCA1}$ (Extended Data Fig. 2A).

Discussion

The present findings support the E1/E2 Post-Albers paradigm^{39, 40} and is congruent with established models of the general P-type ATPase mechanism derived from crystallographic and biochemical studies of partial reactions⁵. Using smFRET, we quantified structural and dynamical features of complete pumping cycles in a single experiment to reveal the kinetic mechanism of transport (Fig. 4, Supplementary Video 1). Although additional advancements in time resolution will be needed to fully resolve each step in the transport cycle, our present findings reveal that steps just prior to E1P formation are rate-limiting to transport in LMCA1. A reduced rate of phosphoenzyme formation has also been described for the secretory pathway Ca^{2+} -ATPase (SPCA)⁴¹ and intriguingly LMCA1 and SPCA both transport only a single calcium ion per catalytic cycle; the reduced rate of phosphoenzyme formation may therefore be related to the transport stoichiometry. Conversely, in LMCA1 we do not observe a steady-state accumulation of the E1P state as was reported for SPCA⁴¹ and SERCA¹⁰.

A key feature of the proposed transport mechanism is that under physiological conditions formation of the de-occluded E2P conformation is essentially irreversible, while ligand binding, phosphorylation of D334, and subsequent transition to an [Ca]E2P state are reversible. Hence, Ca^{2+} release represents a “point of no return” that ensures tight coupling between ion translocation and ATP usage as well as unidirectional Ca^{2+} transport against a steep ion concentration gradient. Our findings also provide the first structural information regarding the [Ca]E2P intermediate state, whose distinct FRET value indicates that the A domain is largely, but not completely, rotated prior to Ca^{2+} de-occlusion³⁶, and whose reaction kinetics suggest that the ADP product of phosphorylation remains bound. While the mutations investigated here enabled a nearly 50-fold stabilization of this normally transient intermediate, in-depth structural investigations require further stabilization.

smFRET provide a potentially powerful means to survey P-type ATPase mechanisms across a wide spectrum of subtypes to determine their conserved and divergent features and to define the underlying mechanistic principles of ATP-driven ion pumps. Such investigations will be greatly aided by imaging transport from multiple structural perspectives to resolve how the motions of each individual cytoplasmic domain couple to the transmembrane bundle. A powerful extension would be simultaneous measurements of substrate transport tracked also at a single-molecule scale⁴². Such pursuits will be greatly aided by the continued development of chemical strategies enabling the site-specific labeling of native, cysteine-containing integral membrane proteins as well as robust strategies for incorporating ligand-specific fluorescence sensors within the lumen of reconstituted proteoliposomes⁴³.

METHODS

Site-directed mutagenesis, protein expression, purification and labeling

A pET-22b:LMCA1–10×His construct was used as a template for the introduction of cysteines for labeling purposes as described previously²². The protocol for LMCA1 expression and purification was adapted from²⁰. Briefly, solubilized membranes were loaded on a 5 ml HisTrap HP column (GE Healthcare) equilibrated in buffer C (20 mM Tris-HCl

pH 7.6, 200 mM KCl, 20% v/v glycerol, 1 mM MgCl₂, 5 mM β-mercaptanol, 0.25 mg/ml C₁₂E₈) with 50 mM imidazole. After washing the column with the same buffer, bound protein was eluted with buffer C containing 150 mM imidazole. For smFRET experiments, protein samples at 20 μM were labeled with a mixture of maleimide-activated LD550 and LD650 dyes (Lumidine Technologies) at concentrations of 320 and 400 μM, respectively, for 60 min at room temperature. Labeled proteins were purified from the excess dyes by size exclusion chromatography.

Determination of ATPase activity

The ATPase activity of LMCA1 was measured by determining the liberation of inorganic phosphate by the Baginski method⁴⁴, as described previously²². Briefly, 0.1–5 μg of purified LMCA1 was added to a final volume of 50 μl reaction buffer (50 mM Tris-HCl pH 7.6, 200 mM KCl, 10% v/v glycerol, 10 mM MgCl₂, 1 mM CaCl₂, 1 mM BME, 0.25 mg/ml C₁₂E₈) at room temperature. Reactions were initiated by addition of 3 mM ATP and stopped after 1–30 minutes by addition of 50 μl ascorbic acid solution (140 mM ascorbic acid, 5 mM ammonium heptamolybdate, 0.1% (w/v) SDS and 0.4 M HCl). Samples were incubated for 15 minutes and the color was stabilized by the addition of 75 μl arsenate solution (150 mM sodium arsenate, 70 mM sodium citrate and 350 mM acetic acid) and incubation for 30 minutes. The absorbance was measured at 860 nm in a VICTOR X plate reader (Perkin Elmer).

Phosphorylation of LMCA1 by [γ-³²P]ATP

The phosphorylation of LMCA1 by [γ-³²P]ATP was carried out at 25°C either by manual mixing or using a Bio-Logic quench-flow module QFM-5, as earlier described¹⁰. To monitor the phosphorylation of enzyme preincubated with Ca²⁺, LMCA1 (1 or 2 μg/ml) suspended in 50 mM Tris-HCl pH 7.6, 200 mM KCl, 10% v/v glycerol, 10 mM MgCl₂, 1 mM BME, 0.25 mg/ml C₁₂E₈, 1 mM CaCl₂ was mixed with an equal volume of the same buffer containing 10 μM [γ-³²P]ATP followed by acid quenching at the indicated time interval by further mixing with an equal volume of 25% (w/v) trichloroacetic acid containing 100 mM H₃PO₄. To monitor the phosphorylation of enzyme initially present in the Ca²⁺-deprived form, LMCA1 (1 or 2 μg/ml) suspended in 50 mM Tris-HCl pH 7.6, 200 mM KCl, 10% v/v glycerol, 10 mM MgCl₂, 1 mM BME, 0.25 mg/ml C₁₂E₈, 0.2 mM EGTA was mixed with an equal volume of 50 mM Tris-HCl pH 7.6, 200 mM KCl, 10% v/v glycerol, 10 mM MgCl₂, 1 mM BME, 0.25 mg/ml C₁₂E₈, 2.2 mM CaCl₂ (resulting in a final ~1 mM CaCl₂) and 10 μM [γ-³²P]ATP, followed by acid quenching at the indicated time interval as described above. The acid-precipitated protein was washed by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis in a 7% polyacrylamide gel at pH 6.0⁴⁵, and the radioactivity associated with the separated Ca²⁺-ATPase band was quantified by imaging with a Cyclone Storage Phosphor System (Perkin Elmer, Waltham, MA). Background correction and quantification of the bands was performed in ImageJ⁴⁶.

TIRF single-molecule fluorescence imaging

Microfluidic chambers passivated with polyethylene glycol (PEG) and a small percentage of biotin-PEG⁴⁷ were incubated for 5 min with 0.8 μM streptavidin (Invitrogen), followed by 4 nM biotin-*tris*-NTA-Ni²⁺⁴⁸ in T50 buffer (50 mM KCl, 10 mM Tris-acetate, pH 7.5).

LD550/LD650-labeled, His-tagged LMCA1 molecules were immobilized via the His-tag: Ni^{2+} interaction by incubating for 2 min at ~4 nM concentration. This immobilization strategy acted to further purify the protein. Subsequent imaging experiments were conducted in imaging buffer consisting of 200 mM KCl, 50 mM Tris/Mes (pH 7.5), 10 mM MgCl_2 , 10% glycerol, 1 mM β -mercaptanol, and 0.25 mg/ml C_{12}E_8 detergent, unless otherwise specified. An oxygen scavenging system consisting of 0.1% w/v glucose, 0.2 units/mL glucose oxidase (Sigma), and 1.8 units/ μL catalase (Sigma) was added to minimize photobleaching. Both enzymes were purified by gel filtration prior to use. Microfluidic chambers were reused up to ten times by eluting the protein from the surface with 0.3 M imidazole in imaging buffer (Extended Data Fig. 3B–C).

Single-molecule FRET imaging of LMCA1 dynamics was performed at 25 °C using a custom-built prism-based total internal reflection (TIR) microscope, as previously described^{16, 19, 49}. Surface-bound LD550 fluorophores were excited by the evanescent wave generated by TIR of an Opus 532 nm solid state laser (Laser Quantum). Illumination intensities were chosen so that the observed mean total intensity (donor+acceptor) was ~400 detected photons per frame. Fluorescence emission was collected by a 1.27 N.A. 60 $\times$ PlanApo water immersion objective (Nikon), spectrally separated using a MultiCam-LS device (Cairn) with a T635lpxr-UF2 dichroic (Chroma), and imaged onto two Hamamatsu ORCA-Flash 4.0 v2 sCMOS cameras¹⁷ at a rate of 66.7 or 200 s^{-1} (15 or 5 ms time resolution, respectively). Scattered excitation light was removed by a ET555lp filter (Chroma) between the objective and the MultiCam. Synchronization was ensured with a pulse generator and verified with an oscilloscope. Data were acquired with 2 $\times$ 2 hardware binning using custom software implemented in LabView (National Instruments).

Analysis of smFRET data

Analysis of single-molecule fluorescence data was performed using the SPARTAN analysis software package MATLAB¹⁷. Single-molecule fluorescence traces were extracted from wide-field movies and corrected for background, spectral crosstalk, and unequal apparent brightness¹⁸. FRET trajectories were calculated as $E_{\text{FRET}} = I_A/(I_A + I_D)$, where I_A and I_D are the acceptor and donor fluorescence intensities at each frame, respectively. Traces were selected for further analysis according to the following criteria: (1) single-step photobleaching, (2) signal to background noise ratio > 8, (3) less than four donor blinking events, and (4) FRET efficiency above baseline levels for at least 100 frames (1.5 s). FRET histograms were calculated from the first 100 frames with bin sizes of 0.03 and shown as spline interpolations to enhance readability. Mean FRET values and Pearson correlation coefficients of fluorescence traces were determined from FRET values above a baseline threshold of 0.2 within the first 100 frames, to exclude fluorophore dark states. Fitting to dose-response functions and preparation of figures were performed with Origin 8 (OriginLab), with a Hill coefficient fixed at 1.0. Where noted, data were idealized using the segmental K-means algorithm⁵⁰ and a model with FRET values of 0.0 ± 0.1 (photobleached), 0.4 ± 0.1 (E2), and 0.68 ± 0.1 (E1), with model parameters optimized for each trace individually. Throughout, analysis was performed separately for each movie containing typically 1,000 selected molecules. Mean transition rates, dwell times, and transition density

plots exclude transitions to and from fluorophore dark states. Transition rates and state occupancies were calculated using only the first 300 frames of selected traces.

Estimation of rate constants for the kinetic model

Rate constants listed in Fig. 4 are estimates of the kinetic behavior of the wild-type protein in solution conditions that maximize transport rates (10 mM Mg^{2+} , 1 mM Ca^{2+} , 1 mM ATP, pH 7.5). $\text{E1}'$ [H]E2 exchange rates were estimated to be at or beyond the imaging time resolution (5–15 ms) based on the lack of dwells between distinct states in the absence of ligands at pH 7.5 (Extended Data Fig. 4B) and the short low-FRET state lifetime of wild type LMCA1 in the presence of Ca^{2+} and ATP (Fig. 1I; Extended Data Fig. 4J). Ligand binding rates in E1 states were derived from the response in the D334N mutant (Extended Data Fig. 5B–C). The 6 s^{-1} rate of (Ca)E1'-ATP state formation was estimated from the rate of low-FRET formation in E167Q-LMCA1 after Ca^{2+} and ATP delivery (Fig. 2H). The rate of transiting through the [Ca]E1P-ADP state after (Ca)E1'-ATP formation was estimated to be at or beyond our imaging frame rate (66 s^{-1}) based the observation that dwells in high-FRET states are only rarely detected on pathway to E2P state formation in the E167Q mutant (Extended Data Fig. 6A–C). The rates of transiting from [Ca]E1P to [Ca]E2P and finally [H]E2 states all appear to be at or beyond our imaging frame rate (5–15 ms) based on the characteristic single-frame dwells in lower-FRET states in wild-type LMCA1 in the presence of Ca^{2+} and ATP (Fig. 1H,I; Extended Data Fig. 4J) and the instantaneous rapid transitions from high to low FRET states in E167Q-LMCA1 (Fig. 2D). The rate of the [Ca]E2P-ADP $\rightarrow$ [Ca]E1P-ADP reverse transition was estimated from the lifetime of the [Ca]E2P-ADP state (Extended Data Fig. 9) and the observed rate of return to equilibrium after removal of ATP or Ca^{2+} (or the addition of ATP γ S) in G₄-LMCA1 (Extended Data Fig. 8G–I).

Statistical analysis of smFRET data

Comparisons of ensemble average FRET values, state occupancies, dwell times, and transition rates were made in MATLAB using a two-sample, two-tailed, unpaired Student's *t*-test. A threshold of $p < 0.01$ was chosen for determining statistical significance. For simplicity, only significant *p* values are shown. Replicates (*n*) are defined as data acquired from independent immobilizations of LMCA1, generally performed from different frozen aliquots on separate days with newly prepared buffer solutions. Throughout the paper, error bars (e.g., Fig. 1C) represent the error in estimating true population means across multiple repeats, each consisting of ~1,000 single molecule observations. Single molecules within the ensemble display a distribution of behaviors that may differ substantially from the population mean, relative to the error bars.

Code availability

The full source code of SPARTAN¹⁷, which was used for all analysis of smFRET data, is publicly available: <http://www.scottcblanchardlab.com/software>

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Extended Data

Extended Data Fig. 1. Functional cycle of P-type ATPases illustrated with SERCA crystal structures

SERCA crystal structures (PDB accession numbers in clockwise order starting from E1: 4H1W⁶, 1T5S², 1T5T², 3B9B⁵, 3B9R⁵, 2C88⁴). LMCA1 labeling sites (T24C and A530C, corresponding to SERCA P26 and E606) are depicted as green spheres connected with a black line and the distance between them is shown. The N, P and A domains are shown as red, blue and yellow cartoons, respectively. Transmembrane helices M1-M2, M3-M4 and M5-M10 are shown as pink, wheat, and grey cartoons, respectively. Ca^{2+} ions are shown as orange spheres. Sarcolipin associated with the 4H1W crystal structure is shown in a cyan cartoon representation. Mutations used to inhibit or slow down partial reactions are depicted in red. Square brackets denote ion occlusion. Ion stoichiometry in the scheme refers to LMCA1.

Extended Data Fig. 2. Bulk ATPase activity of LMCA1

(A) Activity measurements were performed under conditions resembling those used for smFRET experiments (1 mM Ca^{2+} , 1 mM ATP, 10 mM Mg^{2+} and pH 7.6). Time course measurements performed in duplicates were subjected to linear regression to provide a measure of activity (slope of the line). Error bars correspond to 95% confidence intervals of the fit. (B) 4.5 nmol of LMCA1_{TM}A/P was labeled with various amounts of Cy3 and Cy5 dyes (0, 22.5, 45, 90 nmol) in a final volume of 286 μ l for 30 min. Labeling efficiency was defined as a corrected ratio of Cy3 or Cy5 absorbance to LMCA1 absorbance. Horizontal error bars show standard deviation of labeling with Cy3 and Cy5 dyes. Activity measurements were performed and analyzed as described above.

Extended Data Fig. 3. Single molecule TIRF imaging of LMCA1

(A) Schematic of the surface immobilization and imaging strategy. Fluorophores, shown as green sticks, are attached to the engineered cysteine labeling sites in LMCA1, which is depicted as a cartoon with A, N, P, and TM domains in yellow, red, blue, and wheat, respectively. (B) Wide-field fluorescence images of surface immobilized LMCA1 particles. N is the total number of particles detected. (C) Image after treatment of the surface with 0.3 M imidazole.

Extended Data Fig. 4. Effects of ligands on rapid FRET dynamics in LMCA1

(A) FRET values from all traces were summed into population histograms to reveal subtle shifts in mean FRET efficiency across conditions: 0.1 mM EGTA (black), 1 mM Ca²⁺ (orange), 1 mM Ca²⁺ with 1 mM ATP (purple), 1 mM Ca²⁺ with 1 mM ATPγS (green), and 0.1 mM EGTA with 0.2 mM BeF_x (blue). (B–C) Representative single molecule fluorescence (donor in green and acceptor in red) traces of LMCA1 in (B) 0.1 mM EGTA and (C) 1 mM Ca²⁺. (D) Mean FRET efficiency from experiments in the presence of the indicated concentration of Ca²⁺ and buffer at pH 7.5 (○) or 6.0 (□), fit to Hill equations with EC₅₀ values of 250 μM and 2.5 mM, respectively (vertical lines). Error bars are mean ± SEM, *n* = 3. (E–H) Representative traces in (E) EGTA and BeF_x; (F) 1 mM Ca²⁺ and 1 mM ATP; (G) 1 mM Ca²⁺ and 1 mM ATPγS; and (H) 1 mM each of Ca²⁺, ADP, and AlF_x. (I) Distribution of dwell times within the high- and (J) low-FRET states in the presence of 1 mM Ca²⁺ and 1 mM ATP imaged at 5 ms time resolution. Error bars are mean ± SEM, *n* = 5.

Extended Data Fig. 5. Rapid response to Ca^{2+} and ATP binding to D334N-LMCA1

(A) Representative trace of D334N-LMCA1 in the presence of 10 mM Ca^{2+} and 1 mM ATP. Compare to Fig. 1H and Extended Data Fig. 4G. (B) Population average FRET efficiency from representative experiments starting in 0.1 mM EGTA, followed by rapid stopped-flow delivery (vertical dotted line) of 10 mM Ca^{2+} and 1 mM ATP (◄), 10 mM Ca^{2+} (◻), 1 mM ATP with Mg^{2+} (◄), or 1 mM ATP without Mg^{2+} (◄). Experiments were also performed starting in 1 mM ATP with the addition of 10 mM Ca^{2+} (◄) and starting in 10 mM Ca^{2+} with the addition of 1 mM ATP (◄). Values were normalized to the initial FRET value before injection. Lines are fits to exponential functions and represent a lower bound for the rate of ligand binding and the subsequent conformational change, limited both by finite mixing time and the time resolution of imaging. (C) Time constants from the curves in panel B. Slashes separate the condition before and after buffer exchange. Error bars are mean $\pm$ SEM, $n = 3$. The ATP/Ca+ATP and Ca/Ca+ATP experiments have higher uncertainty due to the smaller magnitude change. A response time on the order of a single 15 ms frame (horizontal dotted line) is consistent with the data in all cases.

Extended Data Fig. 6. [Ca]E1P-ADP intermediate dwells prior to E2P formation in E167Q-LMCA1 are rare and transient

(A) Example trace of E167Q-LMCA1 imaged in EGTA, followed by rapid exchange with 1 mM ATP and 10 μM Ca^{2+} (vertical dotted line). The expected FRET value of Ca^{2+} -bound states is indicated by a horizontal dotted green line. (B) Traces were summed into a population contour plot, showing accumulation in low FRET over time after injection of substrates (vertical dotted line). (C) Traces from panel B were individually post-synchronized to the transition into the low-FRET, E2P state (vertical dotted line). No accumulation in higher-FRET states is apparent prior to the transition. Such accumulation would appear as increased density at 0.75 FRET and decreased density at lower FRET values just prior to the vertical dotted line. (D) LMCA1 was pre-incubated with EGTA and 1 mM ATP. Shown is the occupancy in the low-FRET, E2P state following addition of Ca^{2+} and ATP (vertical dotted line) in the absence (◻) and presence (◄) of 1 mM ADP. Lines are fits to exponential functions with time constants of 0.16 ± 0.02 and 0.18 ± 0.01 , respectively. Error bars are mean $\pm$ SD, $n = 3$.

Extended Data Fig. 7. Rapid phosphorylation of LMCA1 by [γ - ^{32}P]ATP

(A) Phosphorylation of E167Q-LMCA1_{WT} and LMCA1_{WT} preincubated with Ca^{2+} was carried out for 10 s at 25°C in a reaction buffer containing 1 mM CaCl_2 and indicated concentrations of ATP, followed by acid quenching. The solid lines are the best fits to the Hill equation with the Hill coefficient constrained to one. The data come from a single experiment with half of the points performed in duplicates. (B) Quench-flow experiment was carried out at 25 °C by mixing E167Q-LMCA1_{WT} pre-incubated in a reaction buffer supplemented with 0.2 mM EGTA with an equal volume of the same buffer supplemented with 2.2 mM CaCl_2 and 10 μM [γ - ^{32}P]ATP (final ~1 mM CaCl_2 , 5 μM ATP), followed by acid quenching at the indicated time intervals. Phosphorylation is shown relative to the maximum level reached. Error bars are mean $\pm$ SD, $n = 2$. Solid line is the best fit of a mono-exponential function. (C) Quenched-flow experiments were performed at 25 °C by mixing labeled E167Q-LMCA1_{TM}-A/P pre-incubated in a reaction buffer containing 1 mM CaCl_2 with an equal volume of the same buffer containing different concentrations of [γ - ^{32}P]ATP. Phosphorylation rates were calculated and plotted as a function of [γ - ^{32}P]ATP concentration. Error bars are 95% confidence intervals of the fit. The solid line shows the Michaelis-Menten equation resulting in the listed values of V_{max} and K_m .

Extended Data Fig. 8. Modulation of G₄-LMCA1 dynamics by ligands

(A) Population FRET histograms from experiments imaging G₄-LMCA1 in the presence of 0.1 mM EGTA (black); 1 mM Ca²⁺ (orange); 1 mM Ca²⁺ with 1 mM ATP (purple); 1 mM Ca²⁺ with 1 mM ATPγS (green); and 0.1 mM EGTA with 0.2 mM BeF_x (cyan). (B–D) Representative smFRET traces from experiments in the presence of (B) EGTA, followed by exchange (vertical line) to 1 mM Ca²⁺; (C) 1 mM Ca²⁺ and 1 mM ATPγS; and (D) Ca²⁺ and ATP at low illumination intensity and 200 ms time resolution to show the behavior of a single transporter molecule on the minutes timescale. (E) Low FRET state occupancy of G₄-LMCA1 imaged in the presence of varying concentrations of ATP (■), Ca²⁺ (○), or Mg²⁺ (△) at pH 7.5; or (F) varying pH. Lines are fits to dose response functions. Error bars are mean ± SEM; *n* = 3. Unless specified, the concentrations of ATP, Ca²⁺, and Mg²⁺ are 1 mM, 1 mM, and 10 mM, respectively. (G) Population FRET-time contour plot and (H) representative FRET trace from experiments starting in 1 mM Ca²⁺ and 1 mM ATP, followed by buffer exchange to replace ATP with 1 mM ATPγS. (I) Population average FRET over time from experiments replacing ATP with ATPγS (▲), removing ATP (▼), or removing Ca²⁺ (◆). Lines are fits to exponential functions with time constants of 380 ms on average. (J) Average transition rate in the presence of 1 mM Ca²⁺, 1 mM ATP, and the indicated concentrations of ADP. Error bars are mean ± SD, *n* = 2.

Extended Data Fig. 9. Dwell-time distributions of G₄-LMCA1 in the presence of various ligand concentrations and pH

G₄-LMCA1 was imaged, unless otherwise specified, in the presence of 1 mM ATP, 1 mM Ca²⁺, and 10 mM Mg²⁺ at pH 7.5 with varying ligand concentrations as specified below. The sampling of low- and high-FRET states was idealized as shown in Fig. 4B. Shown are the distributions of time spent in dwells in the high- (A–D) and low- (E–H) FRET states, with dwell counts normalized to the imaging time prior to photobleaching. Experiments were performed varying the concentration of (A,E) ATP; (B,F) Ca²⁺; (C,G) or Mg²⁺. In each case, the concentration of the ligand varied as: zero (0.1 mM EGTA; black), 1 μM (blue), 10 μM (green), 100 μM (orange), 1 mM (red), and 10 mM (dark red). (D,H) Varying pH: 6.0 (black), 6.5 (blue), 7.0 (green), 7.5 (orange), 8.0 (red), and 8.5 (dark red). Error bars are mean ± SEM, *n* = 3.

Extended Data Fig. 10. G₄-E167Q-LMCA1 transits a [Ca]E2P-ADP intermediate on path to Ca²⁺ site de-occlusion and stable E2P formation

G₄-E167Q-LMCA1 was pre-incubated in 1 mM Ca²⁺, followed by addition of 1 mM ATP (time zero). (A) Representative single molecule fluorescence and FRET traces from this experiment, with [Ca]E1-ATP, [Ca]E2P-ADP, and E2P phases of are highlighted with yellow, red, and green shadings, respectively. (B–C) Ensemble FRET histograms of (B) raw traces and (C) traces synchronized to the drop in FRET associated with [Ca]E2P-ADP state formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Single-molecule imaging of LMCA1 dynamics

(A) SERCA crystal structures representing [Ca]E1P-ADP (left) and E2P (right) conformation classes (PDB: 1T5T² and 3B9B⁵), highlighting labeling sites (stars) and changes in inter-dye distance (black line). N, P, A, and TM domains are shown in red, blue, yellow, and green, respectively. (B) Schematic of structurally defined (upright) and hypothesized (italic) steps in transport with arrows representing the net progression. (C) Average FRET in the presence of the 1 mM of indicated ligands (except 0.1 mM EGTA and 0.2 mM BeF_x). (D) Representative smFRET trace in EGTA exchanged to Ca²⁺ (dotted line). (E) Average FRET (squares) in EGTA and various pH, fit to a dose-response function (red line). (F) Pearson's correlation coefficient of donor and acceptor fluorescence in the presence of the indicated ligands. Error bars: mean $\pm$ SEM, $n=3$. (G) Representative traces in the presence of EGTA and BeF_x or (H) Ca²⁺ followed by rapid addition of ATP (dotted line). Imaging was also performed with 5 ms time resolution in the presence of 1 mM Ca²⁺ and ATP with 10 mM Mg²⁺. (I) Representative smFRET trace (blue) with idealization (red), (J) transition density plot (transitions per second scale at right), and (K) average transition rate where the concentration of ATP (□), Ca²⁺ (○), or Mg²⁺ (△) was varied. Error bars: mean $\pm$ SD, $n=2$.

Fig. 2. Quantifying steps in the first half cycle of transport with E167Q-LMCA1

(A) Illustration (PDB 1XP5) of phosphorylation site D334 (blue sticks), catalytic residue E167 (yellow sticks), and phosphate mimic AlF_4^- (orange spheres). FRET histograms of (B) D334N-LMCA1 or (C) E167Q-LMCA1 imaged in EGTA (black), Ca^{2+} (orange), and Ca^{2+} with ATP (purple). (D) Representative smFRET trace of E167Q-LMCA1 exchanged (dotted line) from EGTA to Ca^{2+} with 10 μM ATP. Shadings indicate approximate apo E1/E2 (white), (Ca)E1 (red), and E2P (green) phases. (E–G) Low-FRET state occupancy of E167Q-LMCA1 imaged in EGTA, adding Ca^{2+} and ATP (vertical line), where ATP (E) or Ca^{2+} (F) concentrations were zero ($\square$), 1 ($\circ$), 10 ($\triangle$), 100 (∇), 1,000 μM ($\blacktriangleleft$). Lines are fits to single exponential functions. (G) Rates of transitioning to low FRET from panels E–G, where ATP ($\square$), Ca^{2+} ($\circ$), or Mg^{2+} ($\triangle$) was varied. Lines are fits to dose-response functions. Unless specified, ATP, Ca^{2+} , and Mg^{2+} concentrations were 1 mM, 1 mM, and 10 mM, respectively. (H) Quench-flow experiments (see Methods) were carried out by mixing the indicated LMCA1 variants pre-incubated in CaCl_2 with $[\gamma^{32}\text{P}]\text{ATP}$ (final 1 mM CaCl_2 , 5 μM ATP), followed by acid quenching at the indicated times. Phosphorylation is shown relative to the maximum level reached. Lines are fits to monoexponential functions. Error bars: mean $\pm$ SD, $n=2$.

Fig. 3. Glycine insertion into the A/M1 loop selectively slows the E2P to E1 return step
 (A) Position where glycines were inserted (arrow) to obtain G₄-LMCA1. (B) Representative smFRET trace (blue) and idealization (red) of G₄-LMCA1 pre-incubated in Ca²⁺ adding ATP (vertical line). (C) Average transition rate, varying the ATP (■), Ca²⁺ (●), or Mg²⁺ (▲) concentration or (D) pH. Lines are fits to dose-response functions. Error bars: mean ± SEM, *n*=3. (E) Representative smFRET trace of G₄-E167Q-LMCA1 pre-incubated in Ca²⁺, adding ATP (vertical line). Shadings indicate [Ca]E1-ATP (yellow), [Ca]E2P-ADP (red), and E2P (green) phases. (F) Average FRET of traces synchronized to the appearance of lower-FRET states in G₄-E167Q-LMCA1 (black) and E167Q-LMCA1 (red). (G) Zoom of the slow decay in mean FRET value of G₄-E167Q-LMCA1 from panel D (squares) fit to an exponential function (red line). Unless specified, experiments were conducted in the presence of 1 mM ATP, 1 mM Ca²⁺, and 10 mM Mg²⁺ at pH 7.5.

Fig. 4. Model of the conformational dynamics driving Ca^{2+} pumping by LMCA1

Schematic of the transport process, with high-FRET, E1 states shaded in red (top row) and low-FRET, E2 states shaded in green (bottom row), with approximate rate constants of each stage of the wild-type LMCA1 transport cycle in the presence of optimal ligand concentrations. See Methods for details.