Published in final edited form as:

Bioconj Chem. 2016 September 21; 27(9): 2176–2187. doi:10.1021/acs.bioconjchem.6b00387.

Engineering a Prototypic P-type ATPase *Listeria monocytogenes* Ca²⁺-ATPase 1 for Single-Molecule FRET Studies

Mateusz Dyla^{†,‡}, Jacob Lauwring Andersen[†], Magnus Kjaergaard^{†,‡,§}, Victoria Birkedal[‡], Daniel S. Terry^{||}, Roger B. Altman^{||}, Scott C. Blanchard^{||}, Poul Nissen^{*,†,‡}, and Charlotte R. Knudsen^{*,†}

[†]Centre for Membrane Pumps in Cells and Disease – PUMPKIN. Danish National Research Foundation & Danish Research Institute of Translational Neuroscience – DANDRITE, Nordic-EMBL Partnership for Molecular Medicine, and Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark

[‡]Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wieds Vej 14, DK-8000 Aarhus C, Denmark

[§]AIAS, Aarhus Institute of Advanced Studies, Aarhus University, DK-8000 Aarhus C, Denmark

^{||}Department of Physiology and Biophysics, Weill Cornell Medicine, New York, New York 10021, United States

Abstract

Approximately 30% of the ATP generated in the living cell is utilized by P-type ATPase primary active transporters to generate and maintain electrochemical gradients across biological membranes. P-type ATPases undergo large conformational changes during their functional cycle to couple ATP hydrolysis in the cytoplasmic domains to ion transport across the membrane. The Ca²⁺-ATPase from *Listeria monocytogenes*, LMCA1, was found to be a suitable model of P-type ATPases and was engineered to facilitate single-molecule FRET studies of transport-related structural changes. Mutational analyses of the endogenous cysteine residues in LMCA1 were performed to reduce background labeling without compromising activity. Pairs of cysteines were introduced into the optimized low-reactivity background, and labeled with maleimide derivatives of Cy3 and Cy5 resulting in site-specifically double-labeled protein with moderate activity. Ensemble and confocal single-molecule FRET studies revealed changes in FRET distribution related to structural changes during the transport cycle, consistent with those observed by X-ray crystallography for the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA). Notably, the cytosolic headpiece of LMCA1 was found to be distinctly more compact in the E1 state than in the E2 state. Thus, the established experimental system should allow future real-time FRET studies of the structural dynamics of LMCA1 as a representative P-type ATPase.

*Corresponding Authors: pn@mbg.au.dk, crk@mbg.au.dk.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.6b00387. Additional figures and Tcl script used to design the labeling sites (PDF)

Notes

The authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

Phosphorylation-type (P-type) ATPases undergo large-scale conformational changes when pumping typically cations across membranes to generate and maintain concentration gradients. The transport of cations against an electrochemical gradient is tightly coupled to ATP hydrolysis via formation and breakdown of a phosphoenzyme intermediate. All P-type ATPases contain a transmembrane (TM) domain linked to three cytosolic domains: the nucleotide-binding (N) domain, the actuator (A) domain, and the phosphorylation (P) domain.

The sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), belonging to subclass P_{IIA} , is one of the best characterized P-type ATPases, from both a biochemical and a structural point of view. In animals, Ca^{2+} is released from the sarco/endoplasmic reticulum (SR) to induce, e.g., muscle contraction. Subsequent termination of an SR-induced Ca^{2+} signal such as in muscle relaxation requires the removal of Ca^{2+} from the cytosol, which is primarily done by re-sequestration to the SR by the action of SERCA.¹

In most bacteria, Ca^{2+} -levels are maintained in the submicromolar range by a variety of secondary and primary transporters, including P-type ATPases.^{2,3} In the pathogenic bacteria *Listeria monocytogenes* and *Streptococcus pneumoniae*, as well as in fungal pathogens, P-type ATPases are associated with virulence and survival at high extracellular Ca^{2+} concentrations present in infected host cells.^{4–6} Recently, a *L. monocytogenes* P-type ATPase, LMCA1, was identified and characterized.^{7,8} LMCA1 shows 38% sequence identity with SERCA and differs from its eukaryotic counterpart by displaying a lower Ca^{2+} affinity and transporting only one Ca^{2+} ion and one H^{+} counterion per cycle. Furthermore, LMCA1 exhibits a higher pH optimum and is up-regulated at the transcriptional level upon exposure to alkaline pH.⁹

So far, only a preliminary structural analysis has been performed for LMCA1 in the Ca^{2+} -free state stabilized by AlF_4^- , representing an occluded E2-P* intermediate state of dephosphorylation with a fold similar to that observed for SERCA under identical conditions.¹⁰ In contrast, SERCA has been captured in several conformations along its

functional cycle and subjected to structural characterization by X-ray crystallography.^{1,11–13} Also, other P-type ATPases have been analyzed^{14–16} and show a similar structural architecture despite low overall sequence similarity.

The majority of P-type ATPases, including Ca^{2+} -ATPases, possesses ten TM helices. In SERCA, two Ca^{2+} binding sites (I and II) are located between helices M4, M5, M6, and M8,¹¹ while only LMCA1 site II is conserved and functional.⁷ The TM domain is connected to the cytoplasmic domains (A, N, and P) via extended helices and linkers, which allow the coupling of conformational changes in the cytoplasmic domains to the actual transport of the ions in the TM domain.

The structural conservation of P-type ATPases suggests a common reaction mechanism based on the alteration between two major conformational regimes, namely, the E1 and E2 states. In the E1 state, the TM domain of the pump exhibits high affinity for the primary substrate (i.e., Ca^{2+} for LMCA1 and SERCA). Following Ca^{2+} binding, a series of conformational changes lead to the occlusion of the cytosolic ion pathway as well as phosphorylation of a conserved Asp residue in the P domain via transfer of the γ -phosphate of ATP present at the interface with the N domain. This leads to a conformational change resulting in the phosphorylated E2 state (E2P) now with an outward-open pathway of the TM domain, where the bound Ca^{2+} ion(s) are exchanged for H^{+} counterion(s). Dephosphorylation is stimulated by the TGES motif of the A domain, which undergoes large movements during the phosphorylation/dephosphorylation cycle to actuate the ion transport through the TM domain.¹³

X-ray crystallography in combination with molecular dynamics simulations and biochemical data have provided a model of the conformational changes accompanying the functional cycle of SERCA.^{1,17} However, crystallography favors compact, well-ordered structures, while transient, functionally important conformations may be overlooked. In addition, some crystallized states may be influenced by crystallization conditions (e.g., detergents, lipids, additives, inhibitors). Thus, complementary high-resolution methods are needed to elucidate the dynamics of P-type ATPases during their functional cycle. Previous studies of SERCA tagged with GFP variants on the intracellular domains have shown that FRET can probe the structural changes accompanying the functional cycle.^{18–20} However, due to the large size of fluorescent proteins and their relatively poor photostability, it is desirable to use small, extrinsic organic fluorophores, which have recently undergone a great leap in stability and brightness.²¹ These enable the detection of dynamics at the single-molecule level with high resolution in time and space.²²

In this study, LMCA1 was biochemically validated to be a prototypic P-type ATPase that can be engineered to allow single-molecule studies of the dynamics during functional cycling. An LMCA1 variant optimized for maleimide labeling was developed, and pairs of cysteines were introduced to permit labeling, while maintaining functional activity and reducing background labeling. Ensemble and confocal single-molecule FRET studies enabled the observation of distinct FRET states related to structurally well-characterized conformations consistent with those observed for SERCA. Interestingly, the cytosolic headpiece of LMCA1 was found to become more compact upon Ca^{2+} binding.

RESULTS AND DISCUSSION

Validation of LMCA1 as a Prototypic P-type ATPase

LMCA1 has fewer cysteines than its mammalian P-type ATPase homologues and therefore presents a more accessible target for labeling with thiol-reactive fluorophores. Before embarking on the labeling of LMCA1, we validated LMCA1 as an appropriate model system to study general features of P-type ATPases. Traditionally, fluoride analogues of phosphate, such as BeF_x , AlF_x , and MgF_x , have been used as general inhibitors of P-type ATPases that trap the pumps in conformational states resembling enzyme phosphoforms (EP). These inhibitors have proven very useful in structural studies of SERCA and other P-type ATPases by stabilizing analogues of the E2·Pi product state ($\text{E2} \cdot \text{MgF}_4^{2-}$),²³ the E2-P* transition state ($\text{E2} \cdot \text{AlF}_4^-$),^{13,24} and the E2P ground state ($\text{E2} \cdot \text{BeF}_3^-$).^{13,24} Here, the capacities of these complexes as inhibitors of LMCA1 were characterized in ATPase assays.

NaF was found to inhibit LMCA1 with a high IC_{50} value of 2.3 mM (Figure 1A). This inhibitory effect is arguably caused by formation of a MgF_x complex from NaF and 1 mM MgCl_2 present in the buffer to sustain LMCA1 activity. The Hill coefficient was equal to -2.4 (Table 1), i.e., almost identical to the value reported for Na,K-ATPase under similar conditions.²⁵

BeF_x and AlF_x complexes were obtained by mixing 0.5 mM NaF (a concentration where no inhibition due to formation of MgF_x has yet been observed) with BeSO_4 or AlCl_3 , respectively. Both BeF_x and AlF_x complexes inhibited LMCA1 more efficiently than MgF_x . BeF_x was the most potent inhibitor, with an IC_{50} value of 1.7 μM (Figure 1B), compared to an IC_{50} value of 6.9 μM in the case of AlF_x (Figure 1C), in the presence of 0.5 mM NaF. In the absence of NaF, BeSO_4 and AlCl_3 were still capable of inhibiting LMCA1, albeit with higher IC_{50} values of 27 and 77 μM , respectively (Table 1). These data are in agreement with earlier studies of the Na,K-ATPase showing an inhibition of the ATPase by chloride complexes of beryllium or aluminum in the micromolar range.^{26,27} The two Hill coefficients obtained upon inhibition by BeSO_4 were approximately -1 , suggesting the binding of one metal ion at the phosphorylation site, while inhibition by AlCl_3 resulted in Hill coefficients of approximately -1.5 . In a structure of SERCA crystallized in the E2-P* transition state stabilized by AlF_4^- ,^{13,24} one Mg^{2+} ion binds at the phosphorylation site and coordinates the fluoride. It is likely that at high AlCl_3 concentrations, the second aluminum ion may bind and replace the Mg^{2+} ion, as has also been proposed for the Na,K-ATPase.²⁵

The effects of AlF_x and BeF_x on LMCA1 activity were found to be similar to their effects on SERCA, and thus, it is expected that the same specific structural states can be trapped with these metal fluorides for LMCA1. Therefore, we considered it sensible to apply LMCA1 as a model system in FRET studies of P-type ATPases.

Rational Design of a Cysteine-Deprived LMCA1 Mutant

LMCA1 contains five native cysteines, of which two are located in the N domain (C380 and C458), one in the P domain (C332), and two in the transmembrane region (C251 and C827) (Figure 2A). Estimates of the distance changes of these cysteines during the functional cycle based on equivalent positions of SERCA revealed only minor changes between the different

conformational states. Furthermore, the intrinsic cysteines did not seem to be completely solvent-exposed. Hence, none of these cysteines were suited for labeling for FRET studies. Instead, they were mutated to other amino acids to avoid their contribution to background labeling. Analysis of the conservation of the five intrinsic cysteines based on a multiple sequence alignment of over 1000 bacterial P_{IIA} ATPases (Figure 2B) indicated the possibility of introducing the mutations C251A, C332A, C380A, C458S, and C827S, corresponding to positions C268, C349, C420, C525, and S940 in rabbit SERCA1a, with only minor effects on activity.

The previously described (His)₆-tagged LMCA1 construct allowing expression in *E. coli* and subsequent purification by a two-step immobilized metal affinity chromatography (IMAC)⁷ was improved by the addition of four extra histidines. This enabled the use of higher imidazole concentrations during binding and washing steps of the purification, resulting in significantly improved purity after a single-step IMAC (Figure S1), as has been observed for other targets.²⁸

Single-point mutants of the cytoplasmic cysteines C332A, C380A, and C458S were purified (Figure 3A), and their ATPase activities were compared to that of wild-type LMCA1 (LMCA1_{WT}). All of the tested LMCA1 variants showed a linear, Ca²⁺-dependent production of inorganic phosphate (Pi) within the time course of the experiment (Figure 3B). Mutation of the cysteines located in the N domain (C380A and C458S) only had minor effects on the activity of LMCA1, while mutation of C332 resulted in a 50% decrease in specific activity, from 3.6 to 1.7 $\mu\text{mol}/(\text{min mg})$ (Figure 3C), corresponding to a decrease from approximately an average of 6 to 3 turnovers per second. This activity is lower than previously reported by Faxen et al.,⁷ primarily due to the lower concentration of Mg²⁺ and ATP applied in the present study. C332 is located in the P domain and is conserved in over 80% of all P_{IIA} ATPases. In canine SERCA2a, mutation of the corresponding cysteine (C349) to alanine leads to a 50% loss of activity²⁹ in agreement with the results obtained here for LMCA1. The importance of this cysteine is arguably caused by its proximity to the catalytic site D334 (D351 in rabbit SERCA1a), a part of the P-type ATPase hallmark DKTG phosphorylation motif. Since the C380A and C458S mutations did not considerably decrease the activity of LMCA1, these mutations were included in all subsequent constructs to reduce background labeling.

Next, four mutants harboring different combinations of intrinsic cysteines, henceforth referred to as “cysteine backgrounds”, were designed: a mutant completely devoid of intrinsic cysteines denoted LMCA1_{NC}, a mutant harboring only C332 denoted LMCA1_{C332}, a mutant carrying only the two transmembrane cysteines, C251 and C827, denoted LMCA1_{TM} and a mutant containing three cysteines, C332 and the two transmembrane cysteines, denoted LMCA1_{3C} (Figure 4A). For all four mutants, ATPase activities (Figure 4B) and background labeling (Figure 4C) were compared to those of LMCA1_{WT}. The removal of all intrinsic cysteines in LMCA1_{NC} caused a severe loss of activity, down to 15% of LMCA1_{WT}. As expected, LMCA1_{NC} showed a very low background labeling, ~4-fold lower than LMCA1_{WT}. The reintroduction of the most conserved cysteine in LMCA1_{C332} increased the activity to ~20% of LMCA1_{WT}, while the background labeling of LMCA1_{C332} was similar to LMCA1_{WT}, suggesting that C332 is by far the most reactive native cysteine.

The background labeling of LMCA1_{TM} was similar to that of LMCA1_{NC}, while the ATPase activity corresponded to ~50% of LMCA1_{WT}. In LMCA1_{3C}, C322 was reintroduced into LMCA1_{TM} leading to an expected increase in activity (~70% of LMCA1_{WT}). However, the background labeling of LMCA1_{3C} was as high as LMCA1_{WT}.

In an attempt to improve the activity of LMCA1_{TM}, alternative substitutions at position 332 were endeavored: C332L, C332S, and C332D. Leucine was chosen as the third most common amino acid at position 332 (Figure 2B), serine was a structurally conservative mutation, while aspartate was chosen to mimic the negatively charged thiolate ion of cysteine, which reacts with maleimide. The high labeling efficiency of C332 indicated that the latter strategy might be fruitful. Disappointingly, all mutants showed a very low ATPase activity (Figure S2).

Thus, the LMCA1_{TM} mutant containing an alanine at position 332 displayed the best compromise between low background labeling (20% of LMCA1_{WT}) and high activity (~50% of LMCA1_{WT}) and was chosen for the introduction of pairs of cysteines for labeling.

Design of Cysteine Labeling Sites in LMCA1 and Optimization of Labeling

An automated script was written and used to identify residues in LMCA1_{TM} homology models (made in MODELLER³⁰ based on structures of SERCA) appropriate for the introduction of cysteine pairs for labeling (Supporting Information). Pairs of residues in the cytoplasmic A, P, and N domains showing the greatest distance changes during the conformational cycle were identified (Figure 5A). In addition, ideal pairs should not be conserved (Figure 5B) and should be solvent exposed. Reactivity is highly variable even between fully surface exposed cysteines,³¹ and ideally this should be taken into account. However, due to the large uncertainty in cysteine pK_a predictions,³² we chose to approach reactivity empirically. Based on these rationales, two combinations of labeling sites were identified (Figure 5). One cysteine was introduced into the A domain (at residue 18 or 24), which undergoes the largest relative movements during the functional cycle. The second cysteine was introduced into either the N domain at position 413 or into the P domain at position 530. The resulting mutants were denoted LMCA1_{TM}-A/N (labeled at positions 18 and 413) and LMCA1_{TM}-A/P (labeled at positions 24 and 530). Since both the A and N domains show significant dynamics, the first mutant was expected to be a very potent FRET reporter, but might at the same time be more difficult to interpret, as the observed distance changes result from the movements of two mobile domains. In the second mutant, the measured distance change mainly reflects the rotation of the A domain during the functional cycle.

Dual labeling with maleimide-derivatives of Cy3 and Cy5 was optimized with respect to reaction time and molar excess of dye over protein (Figure S3). Initially, 12 and 15 times molar excess of Cy3 and Cy5, respectively, was applied for the mutant LMCA1_{WT}-A/P during 15, 30, or 60 min. Unreacted dye was removed on a desalting column and the first fraction containing pure LMCA1 was used for determination of labeling efficiency and ATPase activity. The labeling efficiency remained constant at approximately 50% during the chosen time span (Figure S3A). Under these conditions, the mean background labeling of LMCA1_{WT} was ~8%, while the background labeling of LMCA1_{NC} was only ~2%. The

effect of introducing labeling sites and labeling on the ATPase activity of LMCA1_{WT}-A/P was found to be minimal (Figure S3B).

Subsequently, the dye-to-protein ratio was optimized to increase the labeling efficiency. Labeling of LMCA1_{NC}-A/P was evaluated at three different dye-to-protein ratios: 4/5, 8/10, and 16/20 (molecular excess of Cy3/Cy5, respectively, over protein). Cy3 and Cy5 were always used in a 4:5 ratio to decrease the fraction of LMCA1 molecules double-labeled with Cy3, causing zero-FRET background peaks. The labeling was performed during 60 min to ensure that time was not a limiting factor at lower dye-to-protein ratios. The labeling efficiency increased steadily to approximately 50% at 16/20-fold excess without reaching a plateau (Figure S3C). Previous studies have reached much higher degrees of double labeling for soluble proteins.^{33,34} The modest yield and the need for a large excess of labeling reagent stems from the relatively low protein concentration (10 μ M), where bimolecular maleimide–thiol reactions compete with the unimolecular inactivation of the reactive maleimide. Since higher dye and protein concentrations will favor bimolecular labeling reactions over unimolecular reactions, it is thus likely that even higher concentrations would lead to a higher labeling efficiency. However, a labeling efficiency of 50% was judged to be sufficient for single-molecule studies, and therefore higher dye or protein concentrations were not pursued to avoid undesirable effects from high concentrations of DMSO used to solvate the dyes or protein aggregation. The level of background labeling of both LMCA1_{WT} and LMCA1_{NC} was low, and did not increase at higher dye-to-protein ratios for LMCA1_{NC}. Therefore, all subsequent labeling reactions were performed for 60 min at a 16/20 molecular excess of Cy3/Cy5 dyes over LMCA1.

When size exclusion chromatography (SEC) was employed to remove unbound dyes after labeling, labeled LMCA1 was separated into a monomeric fraction and a fraction containing oligomers and aggregates. LMCA1 is present in both peaks, but only the monomeric peak demonstrates Ca²⁺-dependent activity (Figure S4). In addition, differential background labeling of the monomeric and oligomeric peak was observed, especially in LMCA1_{WT} and LMCA1_{TM}, in which the monomeric fraction was essentially nonlabeled, as opposed to the oligomeric one. Thus, SEC was introduced as an additional purification step after labeling to isolate the monomeric fraction of labeled LMCA1 and to remove unreacted dyes.

Applying the optimized labeling strategy, LMCA1_{TM}-A/N and LMCA1_{TM}-A/P were labeled to approximately 50% (Figure 6A). The labeling efficiencies of the negative controls, LMCA1_{WT} and LMCA1_{TM}, were ~2% and ~0.5%, respectively, indicating a lower background labeling of the monomeric fraction as compared to the background labeling without the SEC purification step. No significant effects on ATPase activity of introducing cysteines for labeling were detected (Figure 6B). The activities after the labeling were also measured and found to be unaffected for LMCA1_{WT}, LMCA1_{TM} and LMCA1_{TM}-A/N, whereas the activity of LMCA1_{TM}-A/P was reduced by 50% after labeling (Figure 6B). This was surprising given that no decrease in activity was observed after the labeling of LMCA1_{WT}-A/P (Figure S3B), suggesting that it was not necessarily the labeling of the cysteines at positions 24 and 530 (A/P) that had a marked effect on the activity.

Fluorescence Characteristics of Labeled LMCA1_{TM} Mutants

In order to determine intramolecular distances from FRET efficiencies, fluorophores must be freely rotating on the time scale of imaging ($\kappa^2 = 2/3$) and their quantum yield must remain relatively constant over the imaging period.³⁵ Thus, fluorescence anisotropy (r) measurements were conducted to evaluate the extent of rotational freedom of the donor fluorophore attached to LMCA1. The anisotropy was assessed individually at both sites of labeling in the Cy3-labeled LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants under four buffer conditions including saturating concentrations of different ligands intended for use in subsequent FRET experiments (Figure 7A). The measured anisotropy values ($r \sim 0.30$) were only slightly higher than anisotropy values of the free Cy3 ($r \sim 0.25$). The high anisotropy of free Cy3 is in good agreement with previously reported anisotropy values for carbocyanines³⁶ and consistent with the short Cy3 fluorescence lifetime.³⁷ Importantly, no changes in anisotropy were observed in the presence of different ligands. In both pairs of labeling sites, anisotropy values were independent of the labeling position, suggesting that similar FRET signals will result irrespective of the specific position to which each dye is randomly attached in the dual-labeled LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants.

To exclude the possibility that potential changes in FRET efficiency could (partially) reflect changes in fluorescence quantum yield, relative quantum yield measurements of Cy3-labeled LMCA1_{TM}-18, -413, -24, or -530 were performed under two extreme ligand conditions including either 10 mM CaCl₂ or 1 mM EGTA with 0.1 mM BeF_x (Figure 7B). None of the mutants exhibited any changes in quantum yields under these two conditions. The relative quantum yield values of free Cy3 were lower than for the Cy3-labeled mutants, consistent with the model in which the activation energy for photoisomerization of Cy3, which is responsible for the low fluorescence quantum yield and short lifetime of free Cy3, increases upon binding to a macromolecule.³⁷

Thus, the results of fluorescence anisotropy and quantum yield analyses validate the suitability of the LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants for FRET measurements of relative distance changes induced by different ligand conditions. The differences in quantum yields and anisotropies between the labeling sites, however, suggest that direct comparisons of the FRET efficiencies between the two labeling schemes are not feasible in ensemble and confocal single-molecule FRET experiments.

Ensemble and Confocal Single-Molecule FRET Studies

The presence of a FRET signal from the LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants labeled with a mixture of photostabilized Cy3 and Cy5 dyes (LD550 and LD650)³⁸ was confirmed by excitation of Cy3 at 530 nm and concomitant recording of emission spectra (Figure 8). Expectedly, an emission peak at ~ 570 nm caused by Cy3 emission was observed. The other emission peak emerging at ~ 670 nm was indicative of sensitized Cy5 emission, and confirmed the fluorescence resonance energy transfer from Cy3 to Cy5 for both LMCA1_{TM}-A/N and LMCA1_{TM}-A/P.

The labeled LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants were further investigated with the aid of confocal-based smFRET to test the eligibility of these mutants for single-molecule

FRET investigations. The results are presented as the proximity ratio, also known as “relative FRET”, E_{rel} . Proximity ratio is equivalent to FRET efficiency, but without the correction for detection efficiency and quantum yield. When there are site-specific differences in quantum yield, the correction factor would be different between different stochastically labeled species, and single-event determination of the correction factor is thus necessary for determination of true FRET efficiencies. Single-event correction is not possible due to the short observation times in the confocal setup, but would be possible in future TIRF based studies. Thus, only relative distance changes can be inferred from these FRET measurements.

The results clearly demonstrated the presence of FRET signals in both mutants (Figure 9), which changed in response to distinct buffer compositions. Under all of the applied conditions, a zero FRET peak was present, which stems from molecules missing an active acceptor, primarily due to the stochastic labeling. In principle the presence of the zero peak could obscure very low FRET states, but the distances predicted from crystal structures should correspond to FRET values that are distinguishable from the zero peak.

The remaining distribution accounts for sensitized FRET signal and was best modeled by two Gaussians in all conditions except for apo LMCA1_{TM}-A/N, where a single Gaussian peak fitted the data best (Figure 9). This suggests the presence of at least two populations of conformations, which in the LMCA1_{TM}-A/P mutant correspond well with the high-FRET E1 and low-FRET E2 states predicted by the structural model (Figure 4A). In the presence of 10 mM or 0.5 mM CaCl₂, the high-FRET peak predominates ($E_{\text{rel}} \sim 0.74$ or 0.72 , respectively), in agreement with Ca²⁺ stabilizing the E1 conformations. In the apo condition, the LMCA1_{TM}-A/P mutant displayed a lower high-FRET peak ($E_{\text{rel}} \sim 0.64$) with a broad distribution, suggesting either a novel conformational state or a dynamic equilibrium between E1 and E2 states. Also the LMCA1_{TM}-A/N mutant exhibited a single, broad FRET distribution ($E_{\text{rel}} \sim 0.44$) in the apo condition, consistent with a dynamic equilibrium between E1 and E2 states as proposed for SERCA.¹⁸ The phosphate analogues BeF_x and AlF_x were used to stabilize the E2P and E2-P* states, respectively, in the absence of Ca²⁺. Both mutants responded to these inhibitors by shifting the distribution toward lower FRET, in accordance with the structural models.

There are two different classes of crystal structures of the Ca²⁺-bound state of SERCA. The first structure was determined in the absence of nucleotides and showed a wide open cytoplasmic headpiece.¹¹ Subsequent structures of SERCA in complex with Ca²⁺ and ATP-analogues have been much more compact.¹² Thus, the functional relevance of the open structure has been controversial.³⁹ The two types of Ca²⁺ states can be directly probed in smFRET studies by monitoring the interdyer distance in LMCA1_{TM}-A/N. We observed that in the presence of both 10 mM CaCl₂ (high concentration used to crystallize SERCA with an open cytoplasmic headpiece) and 0.5 mM CaCl₂ (where maximum activity of LMCA1 was observed), a high-FRET peak ($E_{\text{rel}} \sim 0.57$ and 0.58 , respectively) predominated. These FRET values are higher than in the apo condition or in the presence of metal fluorides, which suggests a more compact conformation of the cytoplasmic headpiece of LMCA1 to prevail upon Ca²⁺ binding in solution. Thus, the compact headpiece structure seems to be physiologically relevant, while the open structure may represent a transient structure that can

be captured by crystallization at high concentrations of Ca^{2+} . This conclusion is in concordance with a previous proposal based on FRET studies of a SERCA-CFP fusion protein.¹⁸

In conclusion, an experimental system for single-molecule FRET measurements was established, which should allow high-resolution, real-time FRET studies of the conformational dynamics of a functional cycle of LMCA1 in the future. The high degree of structural conservation among P-type ATPases suggests that mechanistic conclusions drawn from LMCA1 can be generalized to large parts of the family, which justifies the use of a convenient bacterial homologue as a model system for studying general features of the pumping cycle. Furthermore, inhibition studies confirmed LMCA1 to be a representative P-type ATPase, which responds to metal fluorides in the same manner as SERCA. The states trapped by BeF_x and AlF_x were demonstrated to be structurally distinct via confocal smFRET measurements. Additionally, our smFRET data suggest that the cytoplasmic headpiece of LMCA1 becomes more compact after Ca^{2+} binding. As opposed to previous FRET studies of SERCA engineered with fluorescent proteins,^{18–20} our approach is based on the site-specific labeling with small organic fluorophores characterized by remarkable stability and brightness.²¹ This strategy potentially allows the detection of dynamics at a single-molecule level by total internal reflection fluorescence (TIRF) microscopy.

MATERIALS AND METHODS

Sequence Alignment, Homology Models, and Calculation of Intramolecular Distances

1121 bacterial P_{IIA} - and P_{O} - (unclassified) ATPases from the UNIPROT database were found using the PUMPKIN P-type ATPase database: <http://octo3.bioxray.au.dk/pump-classifier/p-type-atpase-database/>. A multiple-sequence alignment of those sequences was constructed using MUSCLE.⁴⁰ When plotting conservation scores, a column in the alignment corresponding to a given residue in LMCA1 was plotted as amino acid frequencies in MATLAB (MathWorks). PDB entries 4H1W, 1SU4, 1T5T, 3B9B, 3B9R, and 2C88 were used as template structures in MODELLER³⁰ to model LMCA1 in the inward-open E1 state, the nucleotide-free E1 state, the calcium-bound E1~P state, the phosphorylated E2P ground state, the dephosphorylated E2-P* transition-like state, and the calcium-free E2 state, respectively. Homology models were made using an online version of the program provided by the Bioinformatics Toolkit from Max-Planck Institute for Developmental Biology (<http://toolkit.tuebingen.mpg.de/modeller>). A sequence alignment of LMCA1 and SERCA1a performed in MUSCLE⁴⁰ was used as an input file together with the aforementioned PDB structures. Structural figures were prepared in PyMol (v 1.7, Schrödinger LLC, <http://www.pymol.org>).

Intramolecular distances in LMCA1 were evaluated using a script written in Tool Command Language (Tcl) and designed to work under Tk console in VMD.⁴¹ It enabled loading of multiple structures of the LMCA1 homology model, choosing two selections in the protein and calculating the distances between all residues within these selections and within all the structures loaded. In this way, pairs of residues suitable for reporting distance changes using FRET were identified. The code is provided in Supporting Information.

Site-Directed Mutagenesis, Expression, and Purification

A pET-22b plasmid (Novagen) containing the LMCA1 gene followed by a nucleotide sequence encoding a C-terminal linker (DYDIPTT sequence), a Tobacco Etch Virus (TEV) protease site (ENLYFG sequence), an XhoI restriction site (CTCGAG sequence), and a six histidine tag (6x CAC sequence), previously described in Faxen et al.,⁷ was used as a template for introducing four additional histidines into the histidine tag using the QuikChange mutagenesis kit (Agilent Technologies). The resulting construct, pET-22b:LMCA1–10xHis, was used as the template for the introduction of mutations removing or introducing cysteines for labeling purposes. All mutations were verified by Sanger sequencing (Eurofins, MWG).

The applied protocol for LMCA1 expression and purification was adapted from Faxen et al.⁷ 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 BME, 0.25 mg/mL C₁₂E₈) with 50 mM imidazole. After washing of the column in the same buffer, bound protein was eluted with buffer C containing 150 mM imidazole.

Determination of ATPase Activity

The ATPase activity of LMCA1 was measured by determining the liberation of inorganic phosphate by the Baginski method.⁴² 1 μ L of purified LMCA1 at a concentration of 0.1–5 μ g/ μ L was added to a final volume of 50 μ L in reaction buffer (20 mM Tris-HCl pH 7.6, 200 mM KCl, 20% v/v glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 0.4 mM NaMoO₄, 10 mM NaN₃, 40 mM KNO₃, 5 mM BME, 0.25 mg/mL C₁₂E₈) in a 96-well microtiter plate at room temperature. Reactions were initiated by the addition of ATP to a final concentration of 3 mM. The reactions were stopped after 1–30 min by addition of 50 μ L ascorbic acid solution (140 mM ascorbic acid, 5 mM ammonium heptamolybdate, 0.1% (w/v) SDS and 0.4 M HCl) and incubated for 15 min. The ascorbic acid solution was freshly prepared and stored on ice. The color of the reduced heteropolymolybdate-phosphate complex (molybdenum blue) was stabilized by the addition of 75 μ L arsenate solution (150 mM sodium arsenate, 70 mM sodium citrate, and 350 mM acetic acid). The absorbance of molybdenum blue was measured at 860 nm in a VICTOR X plate reader (PerkinElmer).

Maleimide-Based Labeling of Cysteines

LMCA1 was dialyzed against the labeling buffer (50 mM MOPS-KOH pH 6.8, 200 mM KCl, 20% glycerol, 1 mM MgCl₂, 0.2 mM *tris*(2-carboxyethyl)phosphine (TCEP), 0.25 mg/mL C₁₂E₈). Cy3 and Cy5 (~0.25 mg) (GE Healthcare) or LD550 and LD 650 dyes (Lumidyne Technologies) were dissolved in 31 μ L DMSO, degassed, and the concentrations of the dyes were determined by measuring the absorbance of 4000 $\times$ diluted dyes in methanol. 4–16 molar excess of Cy3 and 5–20 molar excess of Cy5 were added to the protein. Typically, the LMCA1 concentration was 10–20 μ M and the labeling reaction volume was 500 μ L. Labeling was performed for 15–60 min at room temperature and stopped by the addition of 140 mM BME. LMCA1 was separated from the unbound dyes either by dialysis followed by passage over a PD-10 desalting column (GE Healthcare) or by size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE

Healthcare). SDS-PAGE of fractions collected from the PD-10 column was run and in-gel fluorescence was visualized using a Typhoon gel imager (Amersham Biosciences). Molar concentrations of the dyes and of the protein were determined by absorption measurements on a NanoDrop (Thermo Scientific) or directly during size exclusion chromatography, if it was used to separate unreacted dyes. Absorption was measured at 550, 650, and 280 nm with extinction coefficients of 150 000, 250 000, and 48 100 M⁻¹ cm⁻¹ for Cy3, Cy5, and LMCA1, respectively. Labeling efficiency was calculated as a dye to LMCA1 molar ratio, corrected for absorption of Cy3 at 280 nm (0.08 × Cy3 absorption at 550 nm), absorption of Cy5 at 280 nm (0.05 × Cy5 absorption at 650 nm), and by absorption of Cy5 at 550 nm (0.06 × Cy5 absorption at 650 nm).

Ensemble Anisotropy Measurements

Anisotropy measurements were performed using a Fluoromax 3 spectrofluorometer (HORIBA Jobin Yvon) with polarization filters. 50 nM solutions of Cy3-labeled LMCA1_{TM} variants in the imaging buffer (50 mM Tris-HCl, pH 7.6, 200 mM KCl, 20% glycerol, 1 mM MgCl₂, 0.2 mM TCEP, 0.25 mg/mL C₁₂E₈) were used for measurements in a 60 μL quartz cuvette. Data were acquired using three excitation wavelengths of 530, 540, and 550 nm and a 10 nm emission range with 1 nm increments around the emission peak (~570 nm), and presented as an average and standard deviation from the emission range upon three excitations. Fluorescence anisotropy (*r*) was calculated as

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}}$$

where *I*_{VV} and *I*_{VH} are the fluorescence intensities with polarizations vertical and horizontal to the vertically polarized excitation beam, and *G* is a grating factor defined as *G* = *I*_{HV}/*I*_{HH}, where *I*_{HV} and *I*_{HH} are the fluorescence intensities with polarizations vertical and horizontal to the horizontally polarized excitation beam.

Relative Quantum Yield Measurements

The relative fluorescence quantum yield of Cy3-labeled LMCA1_{TM} variants in the imaging buffer (50 mM Tris-HCl, pH 7.6, 200 mM KCl, 20% glycerol, 1 mM MgCl₂, 0.2 mM TCEP, 0.25 mg/mL C₁₂E₈ supplemented either with 10 mM CaCl₂ or 1 mM EGTA, 0.1 mM BeSO₄, and 1 mM NaF) was determined using absorption and fluorescence measurements in a quartz cuvette with 1 cm path length. The measurements were performed at two sample concentrations and the absorbance values were kept below 0.1. The relative fluorescence quantum yield (*Φ*_f) was calculated as

$$\Phi_f = \frac{I}{\text{Abs}}$$

where Abs is the corrected absorbance at the fluorescence excitation wavelength and *I* is the integrated area under the corrected fluorescence spectrum. The data were presented as an

average and standard deviation of measurements at four excitation wavelengths: 520, 530, 540, and 550 nm.

Ensemble FRET Measurements

FRET measurements in solution were performed using a Fluoromax 3 spectrofluorometer (HORIBA Jobin Yvon). 50 nM solutions of dual-labeled LMCA1_{TM}-A/N or LMCA1_{TM}-A/P in the imaging buffer (50 mM Tris-HCl, pH 7.6, 200 mM KCl, 20% glycerol, 1 mM MgCl₂, 0.2 mM TCEP, 0.25 mg/mL (~0.46 mM) C₁₂E₈) were used for measurements in a 60 μ L quartz cuvette. Emission spectra were recorded in the range of 540–720 nm after excitation at 530 nm.

Confocal Single-Molecule FRET Measurements

Dual-labeled LMCA1_{TM}-A/N or LMCA1_{TM}-A/P was diluted to a final concentration of 40 pM in 50 mM Tris-HCl, pH 7.6, 200 mM KCl, 20% glycerol, 1 mM MgCl₂, 0.2 mM TCEP, 0.25 mg/mL C₁₂E₈ additionally containing either 10 mM CaCl₂, 0.5 mM CaCl₂, 0.5 mM EGTA, or 0.5 mM EGTA with 1 mM NaF and with 0.1 mM BeSO₄ or 0.1 mM AlCl₃. Single-molecule measurements were recorded at 20 °C on a custom built instrument: A collimated Gaussian laser beam (532 nm, final power 100 μ W) was directed to the back port of a Nikon TiE inverted microscope, and focused 10 μ m into a sealed solution containing the protein solution. Fluorescence emission was collected through an oil-immersion objective (Apochromat 60 NA 1.40, Nikon), directed to the detection pathway by a bandpass dichroic (Chroma: zt532dcrb-UF1), and focused onto a 50 μ m pinhole. Donor and acceptor fluorescence was separated with a dichroic mirror (Chroma: ZT633rdc-UF1) and filtered by wavelength (Donor channel: Chroma: ET600/75m, Acceptor channel: Chroma ET720/150m, Semrock: LP02–647) before being focused onto avalanche photodiodes (SPCM-AQR-14, PerkinElmer). Photon counts were binned into 1 ms bins and recorded for 30 min for each sample. Event-selection based on a simple SUM threshold of 50 photons and correction for autofluorescence and cross-talk were done using pyFRET.⁴³ Proximity ratio, also known as “relative FRET” (E_{rel}) was calculated as $E_{\text{rel}} = I_A/(I_A + I_D)$ without correction for differences in relative quantum yields of the two dyes or for the relative detection efficiency of the two channels (γ -factor of 1). The proximity ratio histograms were fitted with a sum of two or three Gaussians in GraphPad Prism 7.0. The appropriate model was chosen based on the extra sum-of-squares F test with the P value set to 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a center of excellence grant from the Danish National Research Foundation and a fellowship from the Lundbeck Foundation to M.K. We appreciate the excellent technical assistance from Anna Marie Nielsen, Tetyana Klymchuk, and Lotte Thue Pedersen. We are grateful to Jesper Vuust Møller, Department of Biophysics, Aarhus University for fruitful discussions.

References

1. Møller JV, Olesen C, Winther AM, Nissen P. The sarcoplasmic Ca^{2+} -ATPase: design of a perfect chemi-osmotic pump. *Q Rev Biophys.* 2010; 43:501–66. [PubMed: 20809990]
2. Rosen BP. Bacterial calcium transport. *Biochim Biophys Acta, Rev Biomembr.* 1987; 906:101–10.
3. Dominguez DC. Calcium signalling in bacteria. *Mol Microbiol.* 2004; 54:291–7. [PubMed: 15469503]
4. Rosch JW, Sublett J, Gao G, Wang YD, Tuomanen EI. Calcium efflux is essential for bacterial survival in the eukaryotic host. *Mol Microbiol.* 2008; 70:435–44. [PubMed: 18761687]
5. Francis MS, Thomas CJ. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. *Microb Pathog.* 1997; 22:67–78. [PubMed: 9049996]
6. Kmetzsch L, Staats CC, Cupertino JB, Fonseca FL, Rodrigues ML, Schrank A, Vainstein MH. The calcium transporter Pmc1 provides Ca^{2+} tolerance and influences the progression of murine cryptococcal infection. *FEBS J.* 2013; 280:4853–64. [PubMed: 23895559]
7. Faxen K, Andersen JL, Gourdon P, Fedosova N, Morth JP, Nissen P, Møller JV. Characterization of a *Listeria monocytogenes* Ca^{2+} pump: a SERCA-type ATPase with only one Ca^{2+} -binding site. *J Biol Chem.* 2011; 286:1609–17. [PubMed: 21047776]
8. Kotsubei A, Gorgel M, Morth JP, Nissen P, Andersen JL. Probing determinants of cyclopiazonic acid sensitivity of bacterial Ca^{2+} -ATPases. *FEBS J.* 2013; 280:5441–9. [PubMed: 23621633]
9. Giotis E, Muthaiyan A, Blair I, Wilkinson B, McDowell D. Genomic and proteomic analysis of the Alkali-Tolerance Response (AlTR) in *Listeria monocytogenes* 10403S. *BMC Microbiol.* 2008; 8:102. [PubMed: 18577215]
10. Andersen JL, Gourdon P, Møller JV, Morth JP, Nissen P. Crystallization and preliminary structural analysis of the *Listeria monocytogenes* Ca^{2+} -ATPase LMCA1. *Acta Crystallogr, Sect F: Struct Biol Cryst Commun.* 2011; 67:718–22.
11. Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature.* 2000; 405:647–55. [PubMed: 10864315]
12. Sorensen TL, Møller JV, Nissen P. Phosphoryl transfer and calcium ion occlusion in the calcium pump. *Science.* 2004; 304:1672–5. [PubMed: 15192230]
13. Olesen C, Picard M, Winther AM, Gyryp C, Morth JP, Oxvig C, Møller JV, Nissen P. The structural basis of calcium transport by the calcium pump. *Nature.* 2007; 450:1036–42. [PubMed: 18075584]
14. Morth JP, Pedersen BP, Toustrup-Jensen MS, Sorensen TLM, Petersen J, Andersen JP, Vilsen B, Nissen P. Crystal structure of the sodium-potassium pump. *Nature.* 2007; 450:1043–1049. [PubMed: 18075585]
15. Wang K, Sitsel O, Meloni G, Autzen HE, Andersson M, Klymchuk T, Nielsen AM, Rees DC, Nissen P, Gourdon P. Structure and mechanism of Zn^{2+} -transporting P-type ATPases. *Nature.* 2014; 514:518–22. [PubMed: 25132545]
16. Andersson M, Mattle D, Sitsel O, Klymchuk T, Nielsen AM, Møller LB, White SH, Nissen P, Gourdon P. Copper-transporting P-type ATPases use a unique ion-release pathway. *Nat Struct Mol Biol.* 2013; 21:43–8. [PubMed: 24317491]
17. Espinoza-Fonseca LM, Autry JM, Thomas DD. Microsecond molecular dynamics simulations of Mg^{2+} - and K^{+} -bound E1 intermediate states of the calcium pump. *PLoS One.* 2014; 9:e95979. [PubMed: 24760008]
18. Winters DL, Autry JM, Svensson B, Thomas DD. Interdomain fluorescence resonance energy transfer in SERCA probed by cyan-fluorescent protein fused to the actuator domain. *Biochemistry.* 2008; 47:4246–56. [PubMed: 18338856]
19. Hou Z, Hu Z, Blackwell DJ, Miller TD, Thomas DD, Robia SL. 2-Color calcium pump reveals closure of the cytoplasmic headpiece with calcium binding. *PLoS One.* 2012; 7:e40369. [PubMed: 22808146]
20. Pallikkuth S, Blackwell DJ, Hu Z, Hou Z, Zieman DT, Svensson B, Thomas DD, Robia SL. Phosphorylated phospholamban stabilizes a compact conformation of the cardiac calcium-ATPase. *Biophys J.* 2013; 105:1812–21. [PubMed: 24138857]

21. Juette MF, Terry DS, Wasserman MR, Zhou Z, Altman RB, Zheng Q, Blanchard SC. The bright future of single-molecule fluorescence imaging. *Curr Opin Chem Biol.* 2014; 20:103–11. [PubMed: 24956235]
22. Roy R, Hohng S, Ha T. A practical guide to single-molecule FRET. *Nat Methods.* 2008; 5:507–16. [PubMed: 18511918]
23. Toyoshima C, Nomura H, Tsuda T. Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. *Nature.* 2004; 432:361–368. [PubMed: 15448704]
24. Toyoshima C, Norimatsu Y, Iwasawa S, Tsuda T, Ogawa H. How processing of aspartylphosphate is coupled to lumenal gating of the ion pathway in the calcium pump. *Proc Natl Acad Sci U S A.* 2007; 104:19831–19836. [PubMed: 18077416]
25. Cornelius F, Mahmmoud YA, Toyoshima C. Metal Fluoride Complexes of Na,K-ATPase: characterization of fluoride-stabilized phosphoenzyme analogues and their interaction with cardiotonic steroids. *J Biol Chem.* 2011; 286:29882–29892. [PubMed: 21708939]
26. Robinson JD. Variable affinity of the (Na⁺ + K⁺)-dependent adenosine triphosphatase for potassium: Studies using beryllium inactivation. *Arch Biochem Biophys.* 1973; 156:232–243. [PubMed: 4269801]
27. Caspers ML, Dow MJ, Fu MJ, Jacques PS, Kwaiser TM. Aluminum-induced alterations in [3H]ouabain binding and ATP hydrolysis catalyzed by the rat brain synaptosomal (Na⁺)+K⁺-ATPase. *Mol Chem Neuropathol.* 1994; 22:43–55. [PubMed: 7916767]
28. Grisshammer R, Tucker J. Quantitative evaluation of neurotensin receptor purification by immobilized metal affinity chromatography. *Protein Expression Purif.* 1997; 11:53–60.
29. Jones LR, Cornea RL, Chen Z. Close Proximity between Residue 30 of Phospholamban and Cysteine 318 of the Cardiac Ca²⁺ Pump Revealed by Intermolecular Thiol Cross-linking. *J Biol Chem.* 2002; 277:28319–28329. [PubMed: 12015326]
30. Sali A, Potterton L, Yuan F, van Vlijmen H, Karplus M. Evaluation of comparative protein modeling by MODELLER. *Proteins: Struct, Funct, Genet.* 1995; 23:318–26. [PubMed: 8710825]
31. Jacob MH, Amir D, Ratner V, Gussakowsky E, Haas E. Predicting reactivities of protein surface cysteines as part of a strategy for selective multiple labeling. *Biochemistry.* 2005; 44:13664–72. [PubMed: 16229456]
32. Ugur I, Marion A, Parant S, Jensen JH, Monard G. Rationalization of the pKa values of alcohols and thiols using atomic charge descriptors and its application to the prediction of amino acid pKa's. *J Chem Inf Model.* 2014; 54:2200–13. [PubMed: 25089727]
33. Ratner V, Kahana E, Eichler M, Haas E. A general strategy for site-specific double labeling of globular proteins for kinetic FRET studies. *Bioconjugate Chem.* 2002; 13:1163–70.
34. Jager M, Nir E, Weiss S. Site-specific labeling of proteins for single-molecule FRET by combining chemical and enzymatic modification. *Protein Sci.* 2006; 15:640–6. [PubMed: 16452617]
35. Zhao Y, Terry D, Shi L, Weinstein H, Blanchard SC, Javitch JA. Single-molecule dynamics of gating in a neurotransmitter transporter homologue. *Nature.* 2010; 465:188–93. [PubMed: 20463731]
36. Levitus M, Negri RM, Aramendia PF. Rotational Relaxation of Carbocyanines. Comparative Study with the Isomerization Dynamics. *J Phys Chem.* 1995; 99:14231–14239.
37. Sanborn ME, Connolly BK, Gurunathan K, Levitus M. Fluorescence properties and photophysics of the sulfoindocyanine Cy3 linked covalently to DNA. *J Phys Chem B.* 2007; 111:11064–74. [PubMed: 17718469]
38. Zheng Q, Juette MF, Jockusch S, Wasserman MR, Zhou Z, Altman RB, Blanchard SC. Ultra-stable organic fluorophores for single-molecule research. *Chem Soc Rev.* 2014; 43:1044–56. [PubMed: 24177677]
39. Jensen AM, Sorensen TL, Olesen C, Møller JV, Nissen P. Modulatory and catalytic modes of ATP binding by the calcium pump. *EMBO J.* 2006; 25:2305–14. [PubMed: 16710301]
40. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004; 32:1792–7. [PubMed: 15034147]
41. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graphics.* 1996; 14:33–8. 27–8.

42. Baginski ES, Foa PP, Zak B. Micro-determination of inorganic phosphate, phospholipids, and total phosphate in biologic materials. Clin Chem. 1967; 13:326–32. [PubMed: 6036717]
43. Murphy, RR., Jackson, SE., Klenerman, D. pyFRET: A Python Library for Single Molecule Fluorescence Data Analysis. 2014. [arXiv.org](https://arxiv.org/abs/1412.6402v1), e-Print Arch. 1412.6402v1

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Inhibition of LMCA1 activity by metal fluoride complexes was assessed in ATPase assays.

A. Inhibition of LMCA1 by NaF in the presence of 1 mM MgCl_2 . B. Inhibition of LMCA1 by BeSO_4 at a constant NaF concentration of 0.5 mM (solid symbols), or in the absence of NaF (open symbols). C. Inhibition of LMCA1 by AlCl_3 at a constant NaF concentration of 0.5 mM (solid symbols), or in the absence of NaF (open symbols). Error bars represent standard deviation of three measurements. The data were fitted by a Hill equation (solid and dashed lines). The resulting Hill coefficients and IC_{50} values are listed in Table 1.

Figure 2.

Intrinsic cysteines of LMCA1. A. Homology model of LMCA1 highlighting the five intrinsic cysteines represented as black spheres. 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 cyan cartoons, respectively. B. Conservation of intrinsic cysteines calculated as the percentage of amino acids occurring in a multiple sequence alignment at the position corresponding to the cysteines in the sequence of LMCA1. A dash (–) corresponds to an amino acid deletion.

Figure 3.

Effects of cytoplasmic cysteine mutations on the activity of LMCA1. A. SDS-PAGE analysis of purified LMCA1 variants mutated at the indicated positions. The band corresponding to LMCA1 is marked with a red arrow. B. Time-course ATPase activity measurements of LMCA1 mutants. Open symbols at 15 min represent a control measurement without Ca^{2+} . Duplicates were made for each condition tested. The points represent mean values and the error bars represent standard deviation. The linear part of every curve was subjected to linear regression (black lines) to provide a measure of activity. C. Specific ATPase activity of the LMCA1 mutants as obtained by linear regression of the time course in B. Error bars correspond to 95% confidence intervals of the fit.

Figure 4.

Characterization of LMCA1 cysteine backgrounds. A. Homology models illustrating the different LMCA1 cysteine backgrounds. Cysteines are represented as black spheres. LMCA1 coloring and representation is analogous to Figure 2A. B. Comparison of ATPase activity of LMCA1 cysteine backgrounds. The relative activity is presented as the percentage of the wild-type (WT) activity. Error bars correspond to standard deviations of two (LMCA1_{C332} and LMCA1_{3C}) or three (LMCA1_{NC} and LMCA1_{TM}) biological replicates. C. Comparison of maleimide-based background labeling of LMCA1 cysteine variants relative to LMCA1_{WT}. LMCA1_{NC} and LMCA1_{TM} are average values of two, and LMCA1_{WT} of three biological replicates. Absolute labeling efficiency values varied between replicas (approximately 2–8% for LMCA1_{WT}, depending on the conditions), but the ratios of mutant-to-LMCA1_{WT} were constant. Error bars show standard deviation of labeling with Cy3 and Cy5 dyes.

Figure 5.

Labeling sites of LMCA1. A. Homology models of two LMCA1 variants designed for labeling, denoted LMCA1_{TM}-A/N and LMCA1_{TM}-A/P, are shown as two rows, each displaying six different functional states (based on the following SERCA PDB entries from left to right: 1SU4, 4H1W, 1T5T, 3B9B, 3B9R, 2C88). Each of them is labeled with the corresponding Post-Albers state and additives used for crystallization (SLN – sarcolipin, TG – thapsigargin) on top of the picture. Three E1 states are shown on a blue background, whereas three E2 states are shown on a gray one. Amino acids constituting the labeling sites are shown as colored spheres, and are additionally labeled with amino acid name, number, and the domain it belongs to in every leftmost homology model. The distances between labeling sites are shown as black lines with distances in Å shown above. All labeling sites are shown in the TM cysteine background, hence cysteines C251 and C827 are shown as

black spheres. Coloring is analogous to Figure 2A. B. Conservation of labeling sites calculated analogously to Figure 2B.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Maleimide-based labeling and activity of LMCA1_{TM}-A/N and LMCA1_{TM}-A/P. A. Labeling efficiency after 60 min of labeling with 16× molar excess of Cy3 and 20× of Cy5, calculated as a corrected ratio of Cy3 or Cy5 absorbance to LMCA1 absorbance. Error bars correspond to standard deviation of two independent labeling reactions. B. ATPase activity of the indicated LMCA1 variants before and after a 60 min labeling reaction. Time course measurements performed in duplicate 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.

Figure 7.

Fluorescence characteristics of labeled LMCA1TM mutants. A. Fluorescence anisotropy (r) was measured for Cy3 free in solution or attached to individual cysteines in LMCA1TM-18, -413, -24, and -530. Error bars represent standard deviation of measurements from 10 nm emission range at three excitation wavelengths. B. Quantum yield (Φ_f) was measured relative to Cy3 free in solution for Cy3 attached to individual cysteines in LMCA1TM-18, -413, -24, and -530. Error bars represent standard deviation of measurements performed at four excitation wavelengths.

Figure 8.

Ensemble FRET imaging of LMCA1. Emission spectra of dual-labeled LMCA1_{TM}-A/N and LMCA1_{TM}-A/P mutants in the absence of ATP and Ca²⁺ (apo state). Cy3 was excited at 530 nm and the resulting emission spectra were recorded and normalized to Cy3 maximum emission (at ~570 nm).

Figure 9.

Confocal smFRET imaging of LMCA1. Proximity ratio (E_{Tel}) histograms of LMCA1_{TM}-A/N (left) and LMCA1_{TM}-A/P (right) in the presence of (top to bottom): 10 mM CaCl₂, 0.5 mM CaCl₂, 0.5 mM EGTA (apo), 0.5 mM EGTA + 0.1 mM BeSO₄ + 1 mM NaF (BeF_x), or 0.5 mM EGTA + 0.1 mM AlCl₃ + 1 mM NaF (AIF_x). The histograms were fitted to a sum of three (or two in case of apo LMCA1_{TM}-A/N) Gaussians with the mean E_{Tel} shown above

each peak. Proximity ratio (E_{rel}) is defined analogously to FRET, with the exception that the detection efficiencies are not corrected (gamma-factor correction).

Table 1

Inhibition Constants for Metal Fluorides

	IC ₅₀ [μ M]	Hill coefficient
NaF (1 mM MgCl ₂)	2300 $\pm$ 200	-2.4 $\pm$ 0.4
BeSO ₄ (0.5 mM NaF)	1.7 $\pm$ 0.2	-1.1 $\pm$ 0.1
BeSO ₄ (H ₂ O)	27 $\pm$ 5	-0.72 $\pm$ 0.08
AlCl ₃ (0.5 mM NaF)	6.9 $\pm$ 0.8	-1.4 $\pm$ 0.2
AlCl ₃ (H ₂ O)	77 $\pm$ 4	-1.7 $\pm$ 0.2