Nucleotide Activation of the Ca-ATPase

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We have used fluorescence spectroscopy, molecular modeling, and limited proteolysis to examine structural dynamics of the sarcoplasmic reticulum Ca-ATPase (SERCA). The Ca-ATPase in sarcoplasmic reticulum vesicles from fast twitch muscle (SERCA1a isoform) was selectively labeled with fluorescein isothiocyanate (FITC), a probe that specifically reacts with lys-515 (17–20) in the nucleotide-binding pocket of the N domain (21–23). Time-resolved fluorescence lifetime, anisotropy, and quenching was used to characterize FITC-SERCA (ATP-E2 state) versus FITC-SERCA in Ca2+-free, Ca2+-bound, and actively cycling phosphoenzyme states (E2, E1, and EP). Time-resolved spectroscopy revealed that FMP-SERCA exhibits increased probe dynamics but decreased probe accessibility compared with FITC-SERCA, indicating that ATP exhibits enhanced dynamics within a closed cytoplasmic headpiece. Molecular modeling was used to calculate the solvent-accessible surface area of FITC and FMP bound to SERCA crystal structures, revealing a positive correlation of solvent-accessible surface area with quenching but not anisotropy. Thus, headpiece closure is coupled to substrate binding but not active site dynamics. We propose that dynamics in the nucleotide-binding site of SERCA is important for Ca2+ binding (distal allosteroy) and phosphoenzyme formation (direct activation).

SERCA3 is a 110-kDa membrane protein that relaxes muscle by transporting calcium from the cytoplasm into SR (1, 2). SERCA comprises 10 transmembrane (TM) helices, plus a large cytoplasmic headpiece with three domains as follows: nucleotide binding (N), phosphorylation (P), and phosphatase activity (A) (Fig. 1) (3). SERCA binds two Ca2+ ions in the TM domain, which are pumped into the SR lumen using energy derived from ATP hydrolysis and proton exchange (4, 5). The kinetic cycle of SERCA is a series of structural and chemical transitions, including intermediates with high Ca2+ affinity (E1), low Ca2+ affinity (E2), and phosphoenzyme formation at Asp-351 (EP) (Scheme 1) (6). SERCA is a member of the “P-type” ion motive ATPase family, forming a transient aspartyl phosphate intermediate during the transport cycle (7). The three cytoplasmic domains are collectively responsible for phosphoryl transfer and phosphoenzyme turnover, resulting in energy transduction to the TM domain for Ca2+ transport (black arrow in Scheme 1) (2, 8).

Catalysis by model enzymes is dependent on protein dynamics (domain, backbone, and sidechain), as demonstrated for dihydrofolate reductase, adenylate kinase, and cAMP-dependent protein kinase (9–14). However, detailed connections between SERCA structure, dynamics, and mechanism remain largely unknown. Ligand-induced changes in SERCA have been successfully detected using spectroscopic probes attached to naturally reactive residues and genetically encoded sites (15, 16). Fluorescein isothiocyanate (FITC) selectively reacts with lys-515 (17–20) in the nucleotide-binding pocket of the N domain (Fig. 1). Fluorescence of FITC-SERCA decreases by 5% upon Ca2+ binding, indicating long range coupling between Ca2+ binding in the TM domain and FITC fluorescence in the N domain (21–23). Time-resolved phosphorescence anisotropy of erythrosin iodoacetamide at Cys-674 in the P domain detected increased microsecond dynamics upon ATP binding to a Ca2+-free enzyme (E2 to ATP-E2), revealing nucleotide-de-
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**FIGURE 1. Molecular models of FITC-SERCA and FMP-SERCA.** Atomic resolution models were constructed using x-ray crystal structures. Structural state, probe identity, and PDB code are indicated at bottom (see also Scheme 1). Green, nucleotide-binding domain. Blue, phosphorylation domain. Red, actuator domain. Gray, transmembrane domain. Orange, FITC. Purple, FMP.

**SCHEME 1.** SERCA kinetic cycle. Active Ca\(^{2+}\) transport into SR utilizes cytosolic ATP (black arrows). Reverse mode is driven by Ca\(^{2+}\) efflux and cytosolic phosphate (green arrow), using ADP or FITC as phosphate acceptor. FMP-SERCA is the fluorescent analog of SERCA in the ATP-E2 state (green box). E2 is the ground state (black box). ATP-E1-2Ca is the fully activated enzyme (red box), leading to phosphoryl transfer and Ca\(^{2+}\) transport.

Serca is competent to synthesize ATP using enzyme reverse mode (Ca\(^{2+}\) efflux) in SR vesicles preloaded with Ca\(^{2+}\) (Scheme 1) (27). A unique low fluorescence state (LFS) of FITC-SERCA, in which fluorescence is decreased by \(~50\%\) compared with other states, was observed long ago following Ca\(^{2+}\) chelation and efflux from preloaded vesicles (28). FITC-SERCA in LFS was initially assigned as a standard phosphoenzyme (phospho-Asp-351) in the “E1P-like” or E1Papo state (28–32). More recently, McIntosh et al. (33) have rigorously demonstrated that FITC is the phosphorylated species in LFS, becoming FITC monophosphate (FMP) with a stable phosphoester bond in the N domain, instead of a labile acylphosphate bond at Asp-351 in the P domain (34). Thus, for FITC-SERCA in preloaded vesicles, chelation of external Ca\(^{2+}\) induces enzyme reverse mode (Ca\(^{2+}\) efflux) using FITC as phosphoryl acceptor (green arrow in Scheme 1), thereby synthesizing FMP tethered in the active site of SERCA (33). For comparison, TNP-8N\(_3\)-ATP is a similar covalent pseudosubstrate that serves to drive Ca\(^{2+}\) transport into SR (black arrow in Scheme 1) when tethered in the active site of SERCA (35). Both FMP-SERCA and TNP-8N\(_3\)-ATP-SERCA have been characterized by steady-state fluorescence and absorbance measurements (33, 35, 36).

Time-resolved spectroscopy and molecular modeling bridge the gap between enzyme kinetics and static crystal structures. FITC remains a highly useful fluorescent probe of SERCA structural dynamics. Previously, we used steady-state emission of FITC-SERCA to monitor Ca\(^{2+}\) binding in the TM domain (37, 38). We also used FITC as a FRET acceptor to monitor distances from FRET donors in the P and A domains (16, 39). Here, we examined structural dynamics of FITC-SERCA and FMP-SERCA using time-correlated single photon counting (TCSPC), a technique in which a subnanosecond laser pulse excites the sample, followed by subnanosecond-resolved detection of fluorescence emission (40). This experiment detected directly excited-state lifetimes, thereby resolving structural states of SERCA. Time-resolved detection of fluorescence anisotropy and iodide quenching provided additional information on dynamics and accessibility in the active site. Results from time-resolved spectroscopy were correlated with molecular modeling and conformation-specific proteolysis, thereby identifying an ATP-induced order-to-disorder transition that precedes Ca\(^{2+}\) binding and phosphoryl transfer. We propose that internal ATP contributes to long range coupling and catalysis in SERCA.

**EXPERIMENTAL PROCEDURES**

**Materials**—FITC (fluorescein 5-isothiocyanate “isomer 1”) (Fig. 2A) was purchased from Invitrogen. Nucleotides (Fig. 2A), proteinase K, and other chemicals were purchased from Sigma. SDS-polycrylamide gels were purchased from Bio-Rad.

**FITC Labeling of SERCA**—SR vesicles were isolated from rabbit fast twitch muscle using differential centrifugation (41). SR vesicles were resuspended in 300 mM sucrose and 30 mM MOPS (pH 7.0), flash-frozen in liquid nitrogen, and stored at \(-80^\circ C\). Protein concentration of SR vesicles was determined by the Biuret method using bovine serum albumin (BSA) as standard. SERCA was labeled in SR vesicles (2 mg/ml) with 10 \(\mu M\) FITC for 20 min at 25 \(^\circ\)C in 100 mM KCl, 5 mM MgCl\(_2\), and 30 mM Tris (pH 8.9) (16, 39). The labeling reaction was terminated by 5-fold dilution in 300 mM sucrose and 50 mM MOPS (pH 7.0) with bovine serum albumin (1 mg/ml) as a scavenger of unreacted dye. Labeled SR vesicles were collected by centrifugation (62,000 \(\times\) g for 30 min at 4 \(^\circ\)C), washed once, and resuspended in 300 mM sucrose and 30 mM MOPS (pH 7.0). Stoichiometry of labeling was determined by absorbance of SR vesicles solubilized in 0.1% SDS and 1.0 \(\frac{n}{\text{NaOH}}\), using an extinction coefficient of 69,300 \(M^{-1}\) \(cm^{-1}\) at 494 nm for FITC conjugated to SERCA (16, 39). Specificity of labeling was verified by in-gel fluorescence.

**SDS-PAGE, In-gel Fluorescence, and Coomassie Densitometry**—Electrophoresis was performed using Laemmlie gels with 4–15%
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A. molecular structure of nucleotides and fluorophores: adenosine triphosphate (ATP), adenosine diphosphate (ADP), fluorescein monophosphate isothiocyanate (FMP), and fluorescein isothiocyanate (FITC). B, structural overlay of FMP and ATP in the active site of SERCA in the ATP-E2 state. FMP is shown in gray (molecular model), ATP in yellow (crystal structure 3AR4), and oxygen atoms in red. The phosphoryl group of FMP overlaps with the γ-phosphoryl group of ATP (circle) in the active site of SERCA.

Acrylamide. SR vesicles were solubilized for 10 min at 25 °C in 2.5% SDS, 5% glycerol, and 62.5 mM Tris (pH 6.8). In-gel fluorescence of labeled SERCA was quantified using the Storm 860 Imaging System (GE Healthcare) in blue fluorescence mode (excitation = 450 ± 30 nm; emission >520 nm). SERCA content in SR vesicles was quantified by Coomassie densitometry using the Odyssey Imaging System (LI-COR, Lincoln NE).

ATPase Assay—SERCA activity was assayed at 25 °C in 100 mM KCl, 5 mM MgCl2, 3 mM Na2ATP, and 50 mM MOPS (pH 7.0). ADP production by SERCA was coupled to NADH oxidation by an ATP-regenerating system (0.2 mM NADH, 0.6 mM phosphoenolpyruvate, 10 units/ml lactate dehydrogenase, and 10 units/ml pyruvate kinase) (16). The rate of ATP hydrolysis was calculated as the rate of NADH oxidation, measured by the decrease in NADH absorbance at 340 nm using an extinction coefficient of 6220 M⁻¹ cm⁻¹. The Ca²⁺ ionophore A23187 was added (3 μg/ml) to eliminate the buildup of a Ca²⁺ gradient inside SR vesicles (i.e. product inhibition) (42). Specific activity is expressed in international units (1 IU = 1 μmol·min⁻¹·mg⁻¹ at 25 °C), calculated using Ca²⁺-dependent ATPase activity and fractional SERCA content in SR vesicles.

Ligand Stabilization of SERCA Structural States—We carefully followed the protocol previously described for formation of LFS (29, 30, 32, 33). FITC-SERCA was run through the series of five states as follows: 1) Ca²⁺-free E2; 2) Ca²⁺-bound E1; 3) actively cycling phosphoenzyme E-P; 4) single-turnover enzyme reverse mode to produce the low fluorescence state FMP—free state (E2) was stabilized by adding 40 μM EGTA to chelate contaminating Ca²⁺ from water and solution chemicals. After 1 min in E2, the Ca²⁺-free state (E2) was stabilized by adding 40 μM EGTA. After 1 min in E1, 10 mM acetyl phosphate (AcP) was added to initiate Ca²⁺ transport and phosphoenzyme cycling (E-P). After 3 min of Ca²⁺ loading of SR vesicles, FMP was formed by chelating all extravesicular Ca²⁺ with 2 mM EGTA ([Ca²⁺], <7.0 mM), thus inducing Ca²⁺ efflux and single-turnover FMP synthesis, as described previously (29, 30, 32, 33). After 2 min as FMP-SERCA (ATP-E2 analog), actively cycling phosphoenzyme was re-formed (E-Pfinal) by adding 2 mM CaCl₂. Ligand-stabilized states of FITC-SERCA and FMP-SERCA were analyzed by proteolytic cleavage and fluorescence spectroscopy, as described below.

Proteolysis Assay—Limited proteolysis by proteinase K (ProtK) was used to identify conformational states of FITC-SERCA and FMP-SERCA (16, 43–46). SR vesicles were incubated at 0.5 mg/ml with 12.5 μg/ml ProtK (40:1 w/w) for 60 min at 30 °C (16). The standard solution contained 50 mM NaCl and 0.5 mM MgCl₂ with 0.1 mM CaCl₂ (E1 cleavage) or 20 mM EGTA (E2 cleavage). Proteolysis of FITC-SERCA and FMP-SERCA was compared with unlabeled SERCA with 0.1 mM nucleotide (ATP, ADP, AMPPCP) as a diagnostic tool of conformational state. The pH was set at 6.0 (25 mM MES), 7.0 (25 mM MOPS), or 8.0 (25 mM EPPS). Other additions included 1 μM thapsigargin (Tg), 20 mM MgCl₂, or 20 mM KH₂PO₄. Proteolysis was stopped by adding ice-cold trichloroacetic acid (2.5% w/v TCA). Proteolytic fragments were analyzed by SDS-PAGE (in-gel fluorescence imaging, Coomassie densitometry).

Steady-state Fluorescence Spectroscopy—Steady-state measurements were recorded on a Varian Cary Eclipse fluorometer using a xenon lamp as excitation source (47). Fluorescence was measured at 25 °C with 25 μg/ml SR protein (~0.16 μM SERCA). Samples were preincubated for 3 min at 25 °C and stirred continuously. The standard solution contained 100 mM KCl, 3 mM MgCl₂, and 50 mM MOPS (pH 7.0). Ionized Ca²⁺ concentrations were calculated using the FREE1 program. At the beginning of the series, the Ca²⁺-free state (E2) was stabilized by adding 40 μM EGTA to chelate contaminating Ca²⁺ from water and solution chemicals. After 1 min in E2, the Ca²⁺-free state (E2) was stabilized by adding 50 μM CaCl₂. After 1 min in E1, 10 mM acetyl phosphate (AcP) was added to initiate Ca²⁺ transport and phosphoenzyme cycling (E-P). After 3 min of Ca²⁺ loading of SR vesicles, FMP was formed by chelating all extravesicular Ca²⁺ with 2 mM EGTA ([Ca²⁺], <7.0 mM), thus inducing Ca²⁺ efflux and single-turnover FMP synthesis, as described previously (29, 30, 32, 33). After 2 min as FMP-SERCA (ATP-E2 analog), actively cycling phosphoenzyme was re-formed (E-Pfinal) by adding 2 mM CaCl₂. Ligand-stabilized states of FITC-SERCA and FMP-SERCA were analyzed by proteolytic cleavage and fluorescence spectroscopy, as described below.

Time-resolved Fluorescence Spectroscopy—Time-resolved measurements were recorded using TCSPC (40). Samples were excited with a subnanosecond pulsed diode laser at 485 ± 10 nm (LDH 485 from Picoquant, Berlin, DE). The laser power was 0.6 milliwatt with a repetition rate of 10 MHz. The laser pulses are highly uniform in shape and intensity (full width at half-maximum <100 ps; 6 nl/pulse) (40). Emission was selected.
using a bandpass filter (519 ± 5 nm) and detected using a single photon avalanche photomultiplier module (PMN-100 from Photons Solutions, Edinburgh UK) with a photon-counting board (SPC-130-EM from Becker and Hickl, Berlin DE). To avoid anisotropy effects, the emission polarizer was set to the magic angle (54.7°) during lifetime measurements. The instrument response function (IRF) was acquired using scattered excitation light detected with emission polarizer set to vertical (0°) but without an emission filter.

Time-resolved fluorescence waveforms were analyzed by multiexponential decay simulation and nonlinear least squares minimization (24, 40). The observed waveform, \( F_{\text{obs}}(t) \), was fit by the decay simulation, \( F_{\text{sim}}(t) \), which had been iteratively convolved with the measured instrument response function (IRF) shown in Equations 1 and 2,

\[
F_{\text{obs}}(t) = F(0) \sum_{i=1}^{n} x_i \exp(-t/\tau_i) \tag{Eq. 1}
\]

\[
F_{\text{sim}}(t) = \int IRF(t - t')F(t')dt' = \tag{Eq. 2}
\]

where \( F(0) \) is the initial fluorescence intensity; \( x_i \) is the mole fraction, and \( \tau_i \) is the decay lifetime. The number of exponentials, \( n \), was determined by minimizing the \( \chi^2 \) value between \( F_{\text{obs}}(t) \) and \( F_{\text{sim}}(t) \) waveforms. The time-resolved fluorescence waveform of each biochemical state was independently fitted. Total emission was determined by integrating \( F_{\text{obs}}(t) \).

**Time-resolved Fluorescence Anisotropy**—Time-resolved fluorescence anisotropy (TFA) experiments were performed with emission polarizer oriented vertically (0°, \( F_v(t) \)), horizontally (90°, \( F_h(t) \)), and at the magic angle (54.7°, \( F_m(t) \)). TFA data analysis (48) was used to calculate anisotropy as shown in Equation 3,

\[
r(t) = \frac{(F_v(t) - F_h(t))/F_v(t) + 2gF_h(t)}{F_v(t) + 2gF_h(t)} \tag{Eq. 3}
\]

where \( g \) is a correction factor based on polarizer calibration. TFA curves were analyzed using a model-independent sum of exponentials plus a constant shown in Equation 4,

\[
r(t) = \sum_{i=1}^{n} r_i \exp(-t/\tau_i) + r_0 \tag{Eq. 4}
\]

where \( r_i \) is the pre-exponential factor of each correlation time component; \( \tau_i \) is the correlation time, and \( r_0 \) is the amplitude of immobilized component (residual anisotropy). We tested up to three correlation time components and found that two correlation time components are necessary and sufficient to fit \( r(t) \). Because all TFA decay curves fit best to two correlation times and had similar values for observed initial anisotropy (\( r(t) \) at \( t = 0 \)) and residual anisotropy (\( r_0 \)), we globally linked correlation times for anisotropy decay fitting of FITC-SERCA and FMP-SERCA.

**Fluorescence Quenching**—Solvent accessibility of FITC and FMP was assessed using iodide (I\(^-\)) quenching. Time-resolved detection demonstrated that iodide quenching was mostly independent of temperature (10, 25, and 37 °C), indicating little to no static quenching (~10%). Steady-state detection demonstrated that quenching was linear from 0 to 200 mM iodide, indicating that dynamic quenching is the predominant mechanism (collisional). Because iodide quenching was collisional, we used steady-state intensity to calculate \( K_{SV}\). The solvent accessibility of the probe was determined by plotting \( F_0/F \) against iodide concentration and fitting the data to Equation 5 for collisional quenching,

\[
(\tau_0)/\tau = F_0/F = 1 + K_{SV}[Q] \tag{Eq. 5}
\]

where \( \langle \tau \rangle \) is average fluorescence lifetime; \( F \) is steady-state emission intensity; \( [Q] \) is quencher concentration, and \( K_{SV} \) is the Stern-Volmer collisional quenching constant, an indicator of solvent accessibility (49, 50).

**Molecular Modeling**—FITC and FMP were modeled into atomic coordinates of SERCA using the DS Visualizer molecular modeling software (Accelrys, San Diego), as described previously for FITC linked to Lys-515 of CFP-SERCA (16). FITC models were built using x-ray crystal structures of E2-Tg (PDB code 1IWO) (51), ADP-E2-MgF\(^-\)-Tg (PDB code 1WPJ)(52), E1-2Ca (PDB code 1SU4) (3), and ADP-E1-2Ca-AlF\(_4\) (PDB code 2ZBD) (52). FMP and FITC were modeled into ATP-E2-Tg (PDB code 3AR4) (53). For models based on x-ray structures with bound nucleotide (ATP or ADP), the nucleotide was first removed, and then the fluorescent probe was linked to Lys-515 and manually docked in the nucleotide pocket of SERCA (Fig. 2B). The clean geometry function of DS Visualizer was used to energy-minimize the orientation of fluorescent probes and SERCA residues within 5 Å. The VMD program (54) was used to calculate solvent-accessible surface area (SASA) of FITC and FMP bound to SERCA.

**Statistical Analysis**—Experiments were performed in triplicate or greater (n ≥3). Data are presented as means ± S.E.

**RESULTS**

**FITC Labeling**—SERCA in SR vesicles from fast twitch skeletal muscle was labeled with FITC (Fig. 2A) (16, 38, 39). Coomassie densitometry demonstrated that SERCA comprises 67 ± 7% of the total protein in SR vesicles (Fig. 3C, left panel). In-gel fluorescence imaging demonstrated that virtually all of FITC is covalently bound to SERCA (Fig. 3C, right panel). The labeling stoichiometry was 1.2 ± 0.1 FITC molecules bound per SERCA molecule. This slight excess labeling is not enough to affect significantly the interpretation of the results below. ATPase activity of FITC-SERCA was inhibited >95%, indicating that FITC labeling blocks catalytic binding of ATP.

**Steady-state Fluorescence Spectroscopy**—To verify formation of FMP-SERCA (33), steady-state fluorescence of FITC-SERCA was monitored in ligand-stabilized structural states using excitation at 480 nm (Fig. 3). We followed the time course of Champel et al. (29), which included five consecutive ligand additions to stabilize FITC-SERCA in the following: 1) the Ca\(^{2+}\)-free state E2 (+40 μM EGTA); 2) the Ca\(^{2+}\)-bound state E1 (+50 μM Ca\(^{2+}\)).

**Active Site Dynamics and Domain Closure of SERCA**

Fluorescence anisotropy (TFA) experiments were performed with FITC-SERCA and FMP-SERCA. The observed waveform, \( F_{\text{obs}}(t) \), was fit by the decay simulation, \( F_{\text{sim}}(t) \), which had been iteratively convolved with the measured instrument response function (IRF) shown in Equations 1 and 2.

\[
(\tau_0)/\tau = F_0/F = 1 + K_{SV}[Q] \tag{Eq. 5}
\]
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Ca\(^{2+}\); 3) actively cycling phosphoenzyme \(EP (\pm 10 \text{ mM AcP})\); 4) the LFS, comprising FMP-SERCA as a structural analog of the ATP-E2 state \((\pm 2 \text{ mM EGTA})\), and 5) a return to actively cycling phosphoenzyme \(EP (\pm 2 \text{ mM Ca}^{2+})\) (Fig. 3). FITC-SERCA in E2 exhibited the highest steady-state fluorescence, nominally 100.0 ± 0.3% intensity (Fig. 3, A and B). E1 showed a small but significant decrease in fluorescence (4.2 ± 0.9%) (Fig. 3, A and B), similar to the well characterized Ca\(^{2+}\)-induced drop in FITC-SERCA fluorescence as reported previously (21–23, 29, 33, 55). Actively cycling phosphoenzyme \(EP\), which includes a mixture of all states in Scheme 1, produced a gradual decrease in fluorescence over 3 min to 85.3 ± 1.1% as SR vesicles filled with Ca\(^{2+}\). EGTA chelation of Ca\(^{2+}\) outside of preloaded vesicles induced enzyme reverse mode of the Ca-ATPase (green arrow in Scheme 1), thereby synthesizing FMP-SERCA using Ca\(^{2+}\) efflux (enzyme reverse mode). FMP-SERCA showed an immediate decrease in fluorescence to 51.2 ± 2.8% (Fig. 3, A and B), similar to previously reports (45–55%) (28–33). The absorption spectrum also changed substantially, increasing at 450 nm and decreasing at 500 nm (supplemental Fig. S1), in agreement with previous reports (29, 33, 56), thereby confirming formation of FMP-SERCA. Addition of Ca\(^{2+}\) hydrolyzed FMP-SERCA and returned FITC-SERCA to actively cycling phosphoenzyme \(EP\), increasing fluorescence to 82.9 ± 1.2%, similar to initial \(EP\) (Fig. 3, A and B, and see Table 1 for summary). We conclude that FITC-SERCA is competent for Ca\(^{2+}\) transport, phosphoenzyme formation, and reverse-mode synthesis of FMP.

To determine the effect of fluorescent labeling on SERCA, we measured steady-state Trp fluorescence of SERCA, FITC-SERCA, and FMP-SERCA (supplemental Fig. S2). Steady-state Trp fluorescence indicated that SERCA, FITC-SERCA, and FMP-SERCA show similar fluorescence changes in response to Ca\(^{2+}\) binding and phosphoenzyme formation (Table 1 and supplemental Fig. S2). We conclude that fluorescently labeled SERCA shows the same conformational coupling as unlabeled SERCA.

In-gel Fluorescence—SDS-PAGE was used to verify FMP-SERCA formation (Fig. 3C). Here, we observed that \(E2\) and \(E1\) states of FITC-SERCA show the same fluorescence on SDS-PAGE (Fig. 3D), unlike the small but significant difference observed in the absence of SDS (compare Fig. 3, B with D). Thus, SDS denaturation abolishes Ca\(^{2+}\)-induced fluorescence changes of FITC-SERCA. However, FMP-SERCA in LFS showed the same decrease in fluorescence both in the absence and presence of SDS (compare Fig. 3, B with D). Thus, our quantitative gel results for LFS/FMP fluorescence match the qualitative in-gel observations by McIntosh et al. (33), where LFS was shown to be FMP-SERCA. The fact that low fluorescence is preserved in the presence of SDS indicates that the fluorescence phenomenon for LFS is due to a chemical modification of FITC (i.e. FMP formation), instead of phosphoenzyme-induced structural changes in SERCA. Our in-gel fluorescence results further suggest that FMP-SERCA forms in low amounts in the \(EP\) state, under conditions where the forward and reverse cycles are in steady-state equilibrium but heavily favored toward the forward cycle. Thus, we provide the first quantitative correlation between solution and in-gel fluorescence, concluding that LFS is caused predominantly by FMP formation.

Proteolysis Identifies the Predominant Structural State of FMP-SERCA—Limited ProtK cleavage is an effective assay to identify the predominant structural state of SERCA in a variety of ligand-stabilized conditions (16, 43, 44, 57). Unlabeled SERCA shows specific ProtK cleavage patterns for the Ca\(^{2+}\)-free \((E2)\) and Ca\(^{2+}\)-bound \((E1)\) states, producing 96- and 83-kDa fragments, respectively (1st and 4th lanes for SERCA in Fig. 4A). ATP slightly but significantly protects SERCA from ProtK digestion, preserving the 110-kDa band (1st and 2nd lane).

![Figure 3](image-url)

**FIGURE 3.** Steady-state fluorescence of FITC-SERCA and FMP-SERCA.

A, time course of LFS formation (FMP-SERCA) using sequential ligand addition and cuvette spectroscopy. B, average fluorescence intensity from A, normalized to FITC-SERCA in E2 (mean ± S.E., n = 9). C, SDS-PAGE quantitation of LFS formation (FMP-SERCA) using Coomassie densitometry (left panel) and in-gel fluorescence imaging (right panel). D, average fluorescence intensity from C, normalized to FITC-SERCA in E2 (mean ± S.E., n = 6).

**TABLE 1**

Summary of fluorescence results

Steady-state fluorescence, in-gel fluorescence, and integrated time-resolved fluorescence are reported for FITC-SERCA (\(E2\), \(E1\), and \(EP\)) and FMP-SERCA (ATP-E2), normalized to FITC-SERCA in \(E2\). Average lifetime, ratio of fast to slow rotational component, and Stern-Volmer quenching constant are also reported. Data are presented as mean ± S.E.

| Biochemical state                      | \(E2\)         | \(E1\)          | \(EP\)          | \(E2\)/ATP | \(EP\)/ATP |
|---------------------------------------|---------------|----------------|----------------|-----------|-----------|
| Steady-state fluorescence             | 100.0 ± 0.3   | 95.8 ± 0.9     | 85.3 ± 3.3     | 51.2 ± 8.4| 82.9 ± 3.5|
| In-gel fluorescence                    | 100.0 ± 2.5   | 101.2 ± 2.5    | 75.1 ± 3.7     | 38.8 ± 1.8| 80.6 ± 3.8|
| Time-resolved fluorescence            | 100.0 ± 0.5   | 94.2 ± 0.4     | 81.0 ± 0.2     | 51.7 ± 0.3| 80.5 ± 1.2|
| Lifetime (\(\tau\)) (ns)              | 2.57 ± 0.02   | 2.58 ± 0.02    | 2.66 ± 0.02    | 2.65 ± 0.02| 2.68 ± 0.03|
| Anisotropy \(r_{F/P}PTS\) (\%max)     | 0.45 ± 0.09   | 0.53 ± 0.06    | 0.63 ± 0.11    | 1.05 ± 0.19| 0.53 ± 0.03|
| Iodide quenching \(K_{IV}\) (M\(^{-1}\)) | 1.78 ± 0.07   | 2.79 ± 0.05    | 2.32 ± 0.04    | 1.23 ± 0.08| 1.58 ± 0.08|
lanes for SERCA in Fig. 4). ATP also slightly but significantly protects the primary proteolytic fragment of E2 (96 kDa) from secondary cleavage by ProtK (1st and 2nd lanes for SERCA in Fig. 4). Unlike ATP, ADP does not provide protection of SERCA in E2 (1st and 3rd lanes for SERCA in Fig. 4). Thus, proteolysis distinguishes the predominant headpiece structure of ATP in E2 from those of ADP-E2, E1, and E1.

FITC-SERCA was assayed for ligand-induced changes in ProtK digestion. The same cut patterns are found for FITC-SERCA and SERCA in E2 and E1 states (1st and 4th lanes in Fig. 4, A and B), indicating the following: (i) FITC-SERCA undergoes similar Ca\(^{2+}\)-dependent conformational changes as unlabeled SERCA and (ii) FITC labeling does not mimic ATP-induced protection from ProtK cleavage (16). We previously demonstrated that addition of AMP-PCP to FITC-SERCA in the E1 state does not provide additional ProtK protection, indicating that FITC labeling blocks the catalytic nucleotide-binding site (16). Here, we further demonstrated that neither ATP nor ADP provides protection for FITC-SERCA in the E2 state (1st and 3rd lanes for FITC-SERCA in Fig. 4). Thus, proteolysis indicates that the structure of FITC-SERCA is similar to that of nucleotide-free SERCA.

ProtK digestion of FMP-SERCA in LFS, however, demonstrated that FMP formation gives similar protection as ATP binding to SERCA (1st to 3rd lanes for FITC-SERCA versus 1st and 2nd lanes for SERCA in Fig. 4, A and B). In particular, FMP-SERCA in the absence of Ca\(^{2+}\) shows high amounts of 110-kDa (uncut) and 96-kDa (E2 cut) bands, similar to unlabeled SERCA in the ATP-E2 state (compare 1st lane of FMP-SERCA versus 2nd lane of SERCA in Fig. 4). Unlike unlabeled SERCA, FMP-SERCA shows no additional protection with ATP, indicating that FMP blocks the catalytic nucleotide-binding site, similar to FITC (compare 1st and 2nd lanes for SERCA, FITC-SERCA, and FMP-SERCA in Fig. 4). We propose that FMP mimics ATP bound to SERCA, presumably due to similar positioning of each respective high energy phosphoryl group utilized in enzyme catalysis.

**Time-resolved Fluorescence Spectroscopy**—The free dyes fluorescein monophosphate and fluorescein are reported to have a “very high” quantum yield (56). However, the absolute value for the quantum yield of fluorescein monophosphate has not been reported, and fluorescein monophosphate is not commercially available. To compare quantum yields, FMP was synthesized from FITC-SERCA by Ca-ATPase reverse mode, and fluorescence lifetimes were determined by TCSPC using excitation at 485 nm. Time-resolved fluorescence was analyzed by waveform integration (Fig. 5, A and B) and lifetime fitting (Fig. 5, C and D) to determine whether changes in emission or nonradiative relaxation (58) contribute to the change in absorbance (FITC-SERCA versus FMP-SERCA) to produce LFS.

The integrated intensities of fluorescence waveforms, which are proportional to initial fluorescence intensity F(0) (Equation 1) reveal the same conformation-dependent changes observed by steady-state fluorescence, including ~50% decrease in FMP-SERCA (compare Figs. 3, A and B, and 5, A and B, and also see Table 1). Normalized waveforms exhibit similar decay rates, revealing that the fluorescence lifetime of FMP-SERCA (green trace in Fig. 5C) is nearly identical to FITC-SERCA in E2, E1, and EP states. Quantitative lifetime fitting of fluorescence waveforms (Equations 1 and 2) determined that two exponential components were necessary and sufficient to generate an optimal fit for FMP-SERCA and FITC-SERCA (supplemental Fig. S3). Both FMP-SERCA and FITC-SERCA have a long lifetime component (\(\tau_1 \approx 3.30\) ns) with a predominant mole fraction (\(x_1 \approx 0.65\)), plus a short lifetime component (\(\tau_2 \approx 1.33\) ns) with a minor mole fraction (\(x_2 \approx 0.35\)) (supplemental Table S1), indicating that the decrease in steady-state fluorescence of LFS is not due to a change in radiative relaxation processes of FMP-SERCA.
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Analysis of average lifetime ($\tau$) determined from two-lifetime components and their mole fractions (supplemental Eq. S1) was used to compare quantum yield between states. All measured states exhibit an similar average lifetime ($\tau$) ≈2.6 ns (Fig. 5D and Table 1), demonstrating that both FITC-SERCA and FMP-SERCA have the same quantum yield. Thus, the decrease in fluorescence for FMP-SERCA was due to optical ground-state phenomena (for decrease in absorbance, see supplemental Fig. S1) and possibly due to the formation of an unobserved population of FMP-SERCA molecules (lifetime-independent static quenching), which is beyond the detection capability of our instrument. There is a correlation between decreased absorbance at 480 nm (supplemental Fig. S1) and decreased fluorescence at 520 nm (Figs. 3 and 5) for FMP-SERCA, indicating that the primary cause of the apparent low steady-state fluorescence is decreased absorbance (33). Here, for the first time we have measured the lifetime of FMP-SERCA in LFS, thereby eliminating changes in radiative and nonradiative relaxation rates as secondary causes for low fluorescence (supplemental Table S1), as compared with FITC-SERCA.

Fluorescence lifetimes of FITC-SERCA determined here by TCSPC are similar to lifetimes previously reported using TCSPC and phase domain spectroscopy (50, 59–62). Most of these studies measured the average lifetime ($\tau$) of FITC-SERCA in a single ligand-stabilized structural state (59–62). Two of these previous studies measured time-resolved fluorescence of FITC-SERCA in two structural states ($E_1$ versus $E_2$), finding that Ca$^{2+}$ binding has no effect on ($\tau$) (50, 60), similar to current results (Fig. 5, C and D). Here, we have extended previous work by comparing FMP-SERCA to FITC-SERCA in $E_2$, $E_1$, and $E_P$ states and by analyzing the distribution of lifetimes and amplitudes. We conclude that FMP-SERCA has the same two fluorescence lifetime components and associated mole fractions as FITC-SERCA in $E_2$, $E_1$, and $E_P$.

**Time-resolved Fluorescence Anisotropy**—TFA, a technique sensitive to nanosecond dynamics (58), was used to examine the active site of SERCA. TFA is sensitive to probe motion and backbone dynamics but not uniaxial rotation of SERCA in the membrane (rotational correlation time >1000 ns) or tumbling of SR vesicles (63). TFA decay of FITC-SERCA has not been previously reported. Here, we used TCSPC with excitation at 485 nm to detect TFA of FMP-SERCA and FITC-SERCA. FMP-SERCA exhibits the fastest anisotropy decay (green trace in Fig. 6A), as compared with FITC-SERCA in $E_2$, $E_1$, and $E_P$ states, which show slower anisotropy decays that are nearly identical (Fig. 6A). FMP-SERCA and all measured states of FITC-SERCA have similar initial anisotropy (~0.37) and similar residual anisotropy (~0.14) (Fig. 6A), indicating restricted motion on the nanosecond time scale.

TFA data were analyzed by fitting to multieponential decays. Two exponential components were necessary and sufficient to generate an optimal fit for FMP-SERCA and FITC-SERCA (Fig. 6 and supplemental Fig. S4). Fitting of TFA data determined that both FMP-SERCA and FITC-SERCA have a fast correlation time ($\phi_{fast} = 0.285 \pm 0.071$ ns) and a slow correlation time ($\phi_{slow} = 2.42 \pm 0.076$ ns) (supplemental Fig. S4) but exhibit distinct distributions of fast and slow components ($r_{fast}$ and $r_{slow}$) (Fig. 6, B and C). The ratio $r_{fast}/r_{slow}$ qualitatively indicates the dynamic disorder of the active site in each structural state (Fig. 6C). FMP-SERCA exhibits a higher ratio of $r_{fast}/r_{slow}$ indicating higher dynamic disorder for FMP-SERCA than all biochemical states of FITC-SERCA, which have the same $r_{fast}/r_{slow}$ (Fig. 6C). Here, we observe changes in rotational motion on the nanosecond time scale, consistent with protein backbone motion, and changes on the subnanosecond time scale, consistent with probe motion (58). We propose that the active site of SERCA is dynamically disordered and that the ATP-$E_2$ state has greater disorder than $E_2$, $E_1$, and $E_P$ states.

**FIGURE 5.** Time-resolved fluorescence of FITC-SERCA and FMP-SERCA. A, emission of FITC-SERCA and FMP-SERCA following excitation pulse (instrument response function). B, average integrated intensity of fluorescence waveforms from A, normalized to FITC-SERCA in $E_2$ (mean ± S.E., n = 3). C, emission normalized to individual peak amplitude. D, average lifetime ($\tau$) of fluorescence waveforms from C using two-component fits (mean ± S.E., n = 3).

**FIGURE 6.** Time-resolved fluorescence anisotropy of FITC-SERCA and FMP-SERCA. A, fluorescence anisotropy decays following excitation pulse. B, anisotropy decays were globally fitted to two rotational correlation times ($\phi_{fast} = 0.285 \pm 0.071$ ns, $\phi_{slow} = 2.42 \pm 0.076$ ns). The pre-exponential factor of each rotational component ($r$) is reported (mean ± S.E., n = 3). C, anisotropy ratio of fast to slow rotational component is enhanced in FMP-SERCA (mean ± S.E., n = 3).
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Fluorescence Quenching—To further examine structural changes in the cytoplasmic headpiece of SERCA, iodide quenching was used as an indicator of FITC and FMP accessibility in the active site (Fig. 7A and Table 1). Ligand-stabilized structural states were assayed by adding 0–279 mM KI, and a standard Stern-Volmer plot was used to quantitate fluorescence quenching. For FITC-SERCA, the $K_{SV}$ of the Ca$^{2+}$-free ground state (E2) is 1.78 ± 0.07, whereas the $K_{SV}$ of the Ca$^{2+}$-activated state (E1) is 2.79 ± 0.05 (Fig. 7A, Table 1), indicating that Ca$^{2+}$ increases active site accessibility (16, 51). Iodide quenching results for E2 and E1 here (Fig. 7A) are similar to those of Highsmith (50), who first identified a Ca$^{2+}$-induced increase in $K_{SV}$ (greater accessibility) for FITC-SERCA. Thus, FITC-SERCA in E2 and E1 states show a large difference in active site accessibility (quenching) yet have similar fluorescence emission (lifetime) and dynamics (anisotropy) (summarized in Table 1). FMP-SERCA exhibits the lowest quenching ($K_{SV} = 1.23 ± 0.08$) of all states tested, indicating that the active site of the ATP-E2 state has the most restricted solvent accessibility (Fig. 7A). Actively cycling phosphoenzyme (EP) has a $K_{SV}$ of 2.32 ± 0.04 (Fig. 7A), an intermediate value consistent with EP comprising all structural states in the SERCA kinetic cycle (Scheme 1). We conclude that the cytoplasmic headpiece of SERCA is predominantly closed in the ATP-E2 state.

Molecular Modeling of the Active Site—Molecular modeling was used to examine FMP and FITC binding to SERCA (Fig. 1). FITC was docked in four crystal structures of SERCA (E2-Tg, E1-2Ca, ADP-E1P-2Ca, and ADP-E2P-Tg), with conjugation of isothiocyanate to Lys-515 (thiourea linkage). FMP and FITC were also docked into the ATP-E2-Tg crystal structure. Because of overlapping binding sites, ATP and ADP were removed from the ATP-E2-Tg and ADP-E1P-2Ca structures prior to FMP and FITC docking. Fluorescent probes and surrounding SERCA side chains were energy-minimized to attain more accurate positioning of the probe in the nucleotide-binding pocket. Models of FMP-SERCA and FITC-SERCA revealed major differences in the interactions of FMP and FITC with residues in the nucleotide-binding pocket, presumably due to large differences in the SERCA headpiece structure in different crystal structures (Fig. 1).

Our energy-minimized model of FMP-SERCA in the ATP-E2-Tg crystal structure predicts that FMP and ATP exhibit multiple, overlapping structural motifs within the nucleotide-binding site (Fig. 2B). For example, the thiourea linkage of FMP overlaps with the amide group of adenine; the benzoate ring of FMP shows similar stacking interactions with Phe-487 as the adenine ring, the benzoate oxygens of FMP match location with ribose oxygens, and the phosphate group of FMP is in the same location as the γ-phosphate of ATP. Thus, our molecular modeling results predict that FMP utilizes the same nucleotide-binding motif as ATP and suggests mechanisms for synthesis and hydrolysis of FMP, where the 3-O oxygen of FITC is in optimal position to accept or donate the phosphoryl group from Asp-351 (Fig. 2B).

The first molecular model of FITC-SERCA in ADP-E1P-2Ca (with ADP removed and FITC hand-docked) placed the phenolic 3-O of the xanthenone ring system adjacent to AlF$_4^-$ at the normally occupied β-phosphate position of ADP (32); this model is based on a hybrid structure of PDB 1T5T (64) and EM 1KJU (65). When we tried to align AlF$_4^-$ with the same 3-O of FITC-SERCA in ADP-E1P-2Ca based on PDB 2ZBD (66) (with ADP removed and FITC hand-docked and energy-minimized), it resulted in severe ring distortion of FITC due to torsional strain. However, these seemingly contradictory results are not necessarily inconsistent. Because of the high dynamic disorder of the nucleotide-binding site preceding ATP hydrolysis, it is not surprising that molecular models of SERCA show variability in probe orientation following ATP hydrolysis. We suggest that the ring structure of our model of FITC-SERCA in ADP-E1P-2Ca represents a pre-ADP release state, where the xanthenone ring system has rotated away from Asp-351 (supplemental Fig. S5), whereas the first molecular model of FITC-SERCA in ADP-E1P-2Ca represents the transition state between phosphoryl hydrolysis and transfer, prior to rotation of the ring system (32).

Solvent Accessibility in the Active Site—As a quantitative measure of probe-protein interactions and headpiece closure, we calculated SASA of FMP and FITC in five models (Fig. 7B and Table 1). The model of FITC-SERCA in E1-2Ca had the highest SASA (282 Å$^2$) of all models, illustrating the openness of the cytoplasmic headpiece in the Ca$^{2+}$-bound crystal structure (Fig. 7B). The ADP-E1P-2Ca model had the lowest SASA (29 Å$^2$), indicating tight headpiece closure in the nucleotide-bound structure. The ADP-E2P and E2-Tg models of FITC-SERCA showed moderate headpiece opening, with SASA of 160 and 147 Å$^2$, respectively (Fig. 7B). For the model of FMP-SERCA, we calculated a SASA of 171 Å$^2$, indicating that the headpiece is partially closed in ATP-E2-Tg but that further headpiece closure is required following Ca$^{2+}$ binding to initiate ATP hydrolysis (ATP-E1-2Ca in Scheme 1). For comparison, SASA of ATP in the E2-Tg crystal structure is 172 Å$^2$, very similar to FMP-SERCA (171 Å$^2$). It is apparent that FMP-SERCA shows low accessibility (iodide quenching and predicted SASA) but high mobility (time-resolved anisotropy), indicating that headpiece closure is not coupled to active site dynamics. We propose that ATP increases active site dynamics within a closed cytoplasmic headpiece of SERCA.
DISCUSSION

**ATP in the Active Site of SERCA**—Nearly 20 residues have been identified that participate in nucleotide binding by SERCA in various structural states, including residues from N, P, and A domains, as determined by mutagenesis (1, 57, 67–70) and crystallography (53, 64, 71–74). The nucleotide-binding site of SERCA is malleable, with alternate modes of binding, including catalytic and regulatory (2, 53, 72, 74). The commonality of binding is stabilized by an intricate network of H-bonds, hydrophobic interactions, and charge-charge interactions between nucleotides and SERCA. For catalytic binding of ATP, Phe-487 (N domain) and Lys-515 (N domain) interact with adenine, whereas Arg-489 (N domain) and Arg-560 (N domain) interact with the polyphosphate tail (64, 71). In one proposed mode for regulatory binding of ADP, adenine is bound between “pinchers” formed by Arg-489 (N) and Arg-678 (P domain) (74); this mode of adenine binding is also observed in crystal structures of SERCA with bound TNP-ATP and TNP-ADP (53). In another proposed mode of regulatory binding of ATP, adenine interacts with Phe-487 (N) and Lys-515 (N), but the polyphosphate tail interacts with Arg-678 (P) and Lys-202 (A domain) (2, 72). Thus, ATP binding by SERCA is adaptable, with a range of nucleotide orientations and interactions in the active site.

Crystal structures show stable nucleotide binding but provide no information on differences in active site dynamics for substrate and product complexes (ATP→E2, ATP→E1-Ca, ADP→E1P-2Ca, and ADP→E2P). Prior to the availability of crystal structures of SERCA with bound nucleotide, NMR spectroscopy identified a handful of residues in an N-domain fragment of SERCA that interacts with ATP (75). NMR further identified coupling of nucleotide binding to internal dynamics of the N domain, with six residues showing increased backbone mobility and four residues showing decreased backbone mobility (76). Thus, ATP binding induces changes in SERCA internal dynamics.

**FITC in the Active Site**—FITC and ATP bind competitively in the active site; FITC labeling precludes ATP hydrolysis (55). There is no crystal structure for FITC-SERCA, so the precise location and orientation of FITC are not known. Proteolysis studies indicate that FITC labeling does not induce cytoplasmic localization, indicating that FITC emission is insensitive to active site environmental changes and that changes in steady-state intensity are due to conformation-specific changes in absorbance. Anisotropy and quenching data demonstrate that FITC-SERCA in E2 and E1-2Ca (nucleotide-free analogs) have the same active site dynamics but different solvent accessibility (Figs. 6 and 7A), emphasizing the need for complementary high resolution fluorescence assays to detect structural changes between states.

**FMP in the Active Site**—Strong biochemical evidence, including absorbance, fluorescence, tryptophanolysis, and 32P localization, indicate that FITC on SERCA in LFS is phosphorylated, thereby forming FMP-SERCA (33). FMP-SERCA has approximately the same quantum yield as FITC-SERCA, but a much lower absorption at 500 nm (supplemental Fig. S1), which provides an explanation for the phenomenon of low fluorescence detected at 520 nm (Figs. 3 and 5) (33, 56). Here, we performed in-gel fluorescence and confirmed that LFS is preserved in the presence of SDS (Fig. 3), strongly suggesting that a chemical modification occurs to FITC to account for low fluorescence. We performed time-resolved fluorescence spectroscopy to measure the lifetime of FITC-SERCA in different biochemical states. Here, we observed that the average lifetime of FITC-SERCA, which is proportional to quantum yield, was the same for all biochemical states tested, including FMP-SERCA in LFS (Fig. 5). Our in-gel fluorescence of EP (Fig. 3) demonstrates reversibility of the Ca-ATPase kinetic cycle (i.e. low amount of FMP-SERCA synthesis) even under conditions that heavily favor the forward reaction mechanism (i.e. Ca2+ transport by FITC-SERCA). Results obtained here are consistent with previous biochemical evidence that formation of FMP-SERCA is responsible for LFS (33).

Both SERCA and the plasma membrane Ca-ATPase are able to utilize the commercially available 3-methoxy-FMP (free dye) as a substrate for Ca2+ transport (33, 78–80). Both SERCA and plasma membrane Ca-ATPase are also able to synthesize 3-methoxy-FMP using enzyme reverse mode (green arrow in Scheme 1) (33, 78–80). Thus, it is likely that FMP (which is not commercially available) can also substitute for ATP as when tethered to SERCA as a pseudosubstrate in the active site (Fig. 2). To test this hypothesis, we performed proteolysis assays to assess the protective effects of nucleotides and fluorophores on ligand-stabilized structural states. We found that for unlabeled SERCA, the nonhydrolyzable ATP analog AMPPCP protects SERCA in the E2 and E1 states from proteolysis (Fig. 4) (16). Likewise, we found that FMP-SERCA is protected from proteolysis, indicating that FMP formation induces the same structural change as ATP binding (Fig. 4). However, FITC labeling does not provide protection from proteolysis for the E2 or E1 states, verifying the assignment of the active site of FITC-SERCA as nucleotide-free (Fig. 4). Therefore, we propose that FMP acts as an ATP analog for SERCA, thereby serving as a useful fluorescent reporter of ATP dynamics in the nucleotide-binding site.
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Anisotropy measurements revealed that FMP-SERCA (ATP-E2) has a faster anisotropy decay than FITC-SERCA in the E2 state, suggesting that ATP induces an order-to-disorder transition in the active site of the Ca$^{2+}$-free enzyme (Fig. 6). Iodide quenching revealed that FMP-SERCA has the lowest solvent accessibility, whereas FITC-SERCA in the E1 state has the highest solvent accessibility (Fig. 7). These quenching results were supported by molecular modeling, which was used to calculate SASA (Figs. 1 and 7). We propose that the active site of SERCA in the ATP-E2 state has low accessibility but high disorder.

Active Site Dynamics Mediates ATP Activation of Ca$^{2+}$—Fluorescence spectroscopy and molecular modeling have provided new insights into the mechanism of nucleotide activation of SERCA. Classic kinetics studies have measured most transition rates in the catalytic cycle. However, these same kinetics studies provide no structural information about SERCA. Conversely, new crystal structures have provided atomic details of many key intermediates but provide no information on the role of thermodynamics in catalysis. Here, we have measured time-resolved fluorescence anisotropy of FITC and FMP in the nucleotide-binding site and interpret our results in light of recent articles that propose active site conformational dynamics mediates substrate binding, enzyme catalysis, and product release (9–14). An inherent advantage of our study is that FITC and FMP are tethered to the nucleotide-binding site of SERCA, which prevents probe release. Thus, our high resolution fluorescence assays bridge outstanding questions between SERCA kinetics and structure.

At first glance, it seems paradoxical that the ATP-E2 state has both a closed headpiece and disordered nucleotide-binding site. The simplest explanation for the apparent paradox is that the key factor for catalysis of ATP is the dynamic disorder of the active site. We propose that active-site disorder enhances ATP hydrolysis by increasing the entropy of the transient intermediate, thereby decreasing the activation energy and increasing the forward reaction rate for ATP hydrolysis and phosphoryl transfer (Fig. 8) (9, 10, 13, 81). Other studies support our interpretation of active site dynamic disorder. For comparison, SERCA shows conformation-specific site-directed labeling by ATP-1,2-pyridoxal, which reacts with Lys-684 in the ATP-E2 state, but Lys-492 in the ATP-E1 state, indicating movement of the γ-phosphate group of ATP upon subsequent enzyme activation by Ca$^{2+}$ (82). Time-resolved phosphorescence anisotropy shows that microsecond dynamics in the SERCA headpiece is increased by ATP binding to the Ca$^{2+}$-free enzyme (ATP-E2) (24). It has been proposed that the γ-phosphate of ATP and Asp-351 of the P domain exhibit electrostatic repulsion during phosphoryl transfer and that mutation of Asp-351 to neutral or electropositive residues reduces the effects of these repulsive forces (45, 67, 71). Thus, it is likely that electrostatic repulsion increases dynamic disorder for ATP in the active site, helping to catalyze ATP hydrolysis and phosphoenzyme formation within a closed cytoplasmic headpiece.

Active site dynamics probably plays an important role on pre-catalysis arrangement of ATP for phosphoryl transfer. SERCA utilizes bimolecular nucleophilic substitution (S$_2$) for phosphoryl transfer (67, 83, 84), a reaction mechanism that relies heavily on both the collisional frequency of the two reacting molecules and their orientation (83, 85). SERCA hydrolyzes ATP at an extremely slow rate in the absence of Ca$^{2+}$, suggesting that SERCA employs a mechanism to prevent nonproductive phosphoryl transfer in the Ca$^{2+}$-free ATPase (6, 45, 86). We propose that the ATP-E2 state in the kinetic cycle has high dynamics but that the orientation and location of the phosphate tail is nonoptimal for phosphoryl transfer. Ca$^{2+}$ binding further closes the cytoplasmic headpiece (25, 26) while maintaining high active site disorder, aiding in the orientation of the two chemical groups (γ-phosphate and Asp-351) needed for proper transition-state geometry, thus enabling the ATP hydrolysis step to proceed rapidly in ATP-E1-2Ca (Fig. 8) (6, 45, 85, 86). It is possible that dynamic disorder in the active site is even further increased following Ca$^{2+}$ binding, because the two electro-negative groups of Asp-351 and γ-phosphate of ATP are brought closer together. Thus, we propose that active site disorder in the Ca-ATPase increases the probability of productive collision and phosphoryl transfer between ATP and Asp-351. Further spectroscopic and kinetics studies, preferably using new technology that resolves structural and kinetics states simultaneously (87), will be needed to test this hypothesis more rigorously.

A Branched Kinetic Pathway for Initial Activation of SERCA

by ATP or Ca$^{2+}$—Traditional kinetic schemes often show SERCA binding Ca$^{2+}$ before ATP (1, 15, 66), but in muscle cells SERCA probably binds ATP before Ca$^{2+}$ (2, 6, 88), indicating that there is a branched pathway of sequential ligand activation of SERCA (supplemental Scheme S1). We previously examined the effects of Ca$^{2+}$ binding to nucleotide-free ATPase (bottom pathway in supplemental Scheme S1) using all-atom molecular dynamics simulations, which demonstrated that Ca$^{2+}$ induces an activated, but empty, nucleotide-binding site in a closed cytoplasmic headpiece (25). Here, we used FMP as a fluorescent ATP-like pseudosubstrate to examine nucleotide activation of SERCA (top pathway in supplemental Scheme S1). For a two-ligand enzyme, binding of the first ligand is able to allosterically regulate binding of the second ligand binding, where an increase in entropy at the primary active site decreases the activation energy for ligand binding at the secondary active site (89). Thus, we propose that increased disorder (entropy) upon ATP binding in the nucleotide site facilitates Ca$^{2+}$ binding in the TM domain of SERCA (Fig. 8).

It is likely that these two branched pathways are not mutually exclusive, rather, both pathways are utilized in muscle...
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(supplemental Scheme S1). Kinetic studies demonstrate that ATP accelerates Ca\(^{2+}\) binding to the ATPase (6, 86, 90), indicating that the rate of Ca\(^{2+}\) binding to ATP-bound SERCA (ATP-E2) (step 2 in top pathway of supplemental Scheme S1) is greater than the rate of Ca\(^{2+}\) binding to ATP-free SERCA (E2) (step 1 in bottom pathway). Because of distinct kinetic and thermodynamic properties of SERCA, there are probably physiological differences in muscle that result from changes in ligand concentration and therefore the relative flux through one pathway or the other. Thus, the key determinant for pathway selection by SERCA is the concentration of Ca\(^{2+}\) and ATP in muscle (91).

“Saturating ATP” Hypothesis Suggests ATP Activation of Ca\(^{2+}\) Release and Phosphoenzyme Decay—The ATP binding affinity of SERCA at the catalytic site is 5–10 \(\mu\text{M}\) (45), whereas the concentration of ATP in muscle cells is 5–8 \(\mu\text{M}\) (2). The importance of saturating ATP in muscle has recently been emphasized, proposing that ATP is bound to SERCA through most of the kinetic cycle (2, 72, 92). Not only does ATP increase Ca\(^{2+}\) binding and phosphoryl transfer (Fig. 8), but ATP also accelerates Ca\(^{2+}\) release from E2P2Ca and E2P decomposition (Scheme 1, bottom row) (2, 6, 93). Thus, we propose that active site dynamics is also increased by rebinding of ATP immediately following ADP release, thereby accelerating subsequent Ca\(^{2+}\) release and phosphoenzyme decay.

Conclusions—We used high resolution fluorescence assays to characterize FMP-SERCA synthesized using FITC-SERCA and enzyme reverse mode. Using conformation-specific proteolysis, we provide evidence that FITC-SERCA is a structural analog of SERCA in the ATP-E2 state. Quenching and anisotropy measurements of FMP-SERCA suggest that the ATP-E2 state of SERCA has a closed headpiece but disordered active site. These data reveal new insights into structural transitions required for coupling ATP activation to Ca\(^{2+}\) transport by SERCA. These results, together with our recently published molecular dynamics simulations of Ca\(^{2+}\) activation (25), provide a compelling mechanistic model for ligand activation of the Ca-ATPase.

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REFERENCES

1. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) The mechanism of Ca\(^{2+}\) transport by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases. J. Biol. Chem. 272, 28815–28818
2. Möller, J. V., Olesen, C., Winther, A. M., and Nissen, P. (2010) The sarco- plasmic Ca\(^{2+}\)-ATPase. Design of a perfect chemi-osmotic pump. Q. Rev. Biophys. 43, 501–566
3. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 405, 647–655
4. Levy, D., Seigneuret, M., Bluzat, A., and Rigaud, J. L. (1990) Evidence for proton countertransport by the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase during calcium transport in reconstituted proteoliposomes with low ionic permeability. J. Biol. Chem. 265, 19524–19534
5. Yu, X., Carroll, S., Rigaud, J. L., and Inesi, G. (1993) \(\text{H}^+\) counterrtransport and electrogenicity of the sarcoplasmic reticulum Ca\(^{2+}\) pump in reconstituted proteoliposomes. Biophys. J. 64, 1322–1324
6. Inesi, G. (1985) Mechanism of calcium transport. Annu. Rev. Physiol. 47, 573–601
7. Palmgren, M. G., and Nissen, P. (2011) P-type ATPases. Annu. Rev. Biophys. 40, 243–266
8. Toyoshima, C. (2008) Structural aspects of ion pumping by Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum. Arch. Biochem. Biophys. 476, 3–11
9. Bohr, D. D., McElheny, D., Dyson, H. J., and Wright, P. E. (2010) Milli- second timescale fluctuations in dihydrofolate reductase are exquisitely sensitive to the bound ligands. Proc. Natl. Acad. Sci. U.S.A. 107, 1373–1378
10. Bhabha, G., Lee, J., Ekert, D. C., Gam, J., Wilson, I. A., Dyson, H. J., Benkovic, S. J., and Wright, P. E. (2011) A dynamic knockout reveals that conformational fluctuations influence the chemical step of enzyme catalysis. Science 332, 234–238
11. Henzler-Wildman, K. A., Lei, M., Thai, V., Kerns, S. J., Karplus, M., and Kern, D. (2007) A hierarchy of timescales in protein dynamics is linked to enzyme catalysis. Nature 450, 913–916
12. Henzler-Wildman, K. A., Thai, V., Lei, M., Ott, M., Wolf-Watz, M., Fenn, T., Pozharski, E., Wilson, M. A., Petsko, G. A., Karplus, M., Hübner, C. G., and Kern, D. (2007) Intrinsic motions along an enzymatic reaction trajec- tory. Nature 450, 838–844
13. Li, F., Gangal, M., Juliano, C., Gorfain, E., Taylor, S. S., and Johnson, D. A. (2002) Evidence for an internal entropy contribution to phosphoryl transfer. A study of domain closure, backbone flexibility, and the catalytic cycle of cAMP-dependent protein kinase. J. Mol. Biol. 315, 459–469
14. Masterson, L. R., Cheng, C., Yu, T., Tonelli, M., Kornev, A., Taylor, S. S., and Veglia, G. (2010) Dynamics connect substrate recognition to catalysis in protein kinase A. Nat. Chem. Biol. 6, 821–828
15. Bigelow, D. J., and Inesi, G. (1992) Contributions of chemical derivatiza- tion and spectroscopic studies to the characterization of the Ca\(^{2+}\) trans- port ATPase of sarcoplasmic reticulum. Biochim. Biophys. Acta 1113, 323–338
16. Winters, D. L., Autry, J. M., Svensson, B., and Thomas, D. D. (2008) Inter- domain fluorescence resonance energy transfer in SERCA probed by cyan fluorescent protein fused to the actuator domain. Biochemistry 47, 4246–4256
17. Mitchinson, C., Wilderspin, A. F., Trinnaman, B. J., and Green, N. M. (1982) Identification of a labeled peptide after stoichiometric reaction of fluorescein isothiocyanate with the Ca\(^{2+}\)-dependent adenosine triphosphatase of sarcoplasmic reticulum. FEBS Lett. 146, 87–92
18. Kirley, T. L., Wang, T., Wallick, E. T., and Lane, L. K. (1985) Homology of ATP binding sites from Ca\(^{2+}\) and (Na,K)-ATPases. Comparison of the amino acid sequences of fluorescein isothiocyanate-labeled peptides. Biochem. Biophys. Res. Commun. 130, 732–738
19. Briggs, F. N., Cable, M. B., Geisow, M. G., and Green, N. M. (1986) Primary structure of the nucleotide binding domain of the Ca,Mg-ATPase from cardiac sarcoplasmic reticulum. Biochem. Biophys. Res. Commun. 135, 864–869
20. Wawrzynów, A., and Collins, J. H. (1993) Chemical modification of the Ca\(^{2+}\)-ATPase of rabbit skeletal muscle sarcoplasmic reticulum. Identifi- cation of sites labeled with aryl isothiocyanates and thiol-directed confor- mational probes. Biochim. Biophys. Acta 1203, 60–70
21. Pick, U., and Karlsh, S. J. (1980) Indications for an oligomeric structure and for conformational changes in sarcoplasmic reticulum Ca\(^{2+}\)-ATPase labeled selectively with fluorescein. Biochim. Biophys. Acta 626, 255–261
22. Pick, U., and Bassilian, S. (1981) Modification of the ATP-binding site of the Ca\(^{2+}\)-ATPase from sarcoplasmic reticulum by fluorescein isothiocyanate. FEBS Lett. 123, 127–130
23. Pick, U., and Karlsh, S. J. (1982) Regulation of the conformation transition in the Ca-ATPase from sarcoplasmic reticulum by pH, temperature, and calcium ions. J. Biol. Chem. 257, 6120–6126
24. Mueller, B., Zhao, M., Negrashov, I. V., Bennett, R., and Thomas, D. D. (2004) SERCA structural dynamics induced by ATP and calcium. Bio-
Active Site Dynamics and Domain Closure of SERCA

J. Biol. Chem. 274, 25227–25236
68. McIntosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1996) Mutagenesis of segment 467-Phe-Ser-Arg-Asp-Arg-Lys-492 of sarcoplasmic reticulum Ca2+-ATPase produces pumps defective in ATP binding. J. Biol. Chem. 271, 25778–25789
69. McIntosh, D. B., Clausen, J. D., Woolley, D. G., MacLennan, D. H., Vilsen, B., and Andersen, J. P. (2004) Roles of conserved P domain residues and Mg2+ in ATP binding in the ground and Ca2+-activated states of sarcoplasmic reticulum Ca2+-ATPase. J. Biol. Chem. 279, 32515–32523
70. Hua, S., Ma, H., Lewis, D., Inesi, G., and Toyoshima, C. (2002) Functional role of "N" (nucleotide) and "P" (phosphorylation) domain interactions in the sarcoplasmic reticulum (SERCA) ATPase. Biochemistry 41, 2264–2272
71. Toyoshima, C., and Mizutani, T. (2004) Crystal structure of the calcium pump with a bound ATP analog. Nature 430, 529–535
72. Jensen, A. M., Sørensen, T. L., Olesen, C., Møller, J. V., and Nissen, P. (2006) Modulatory and catalytic modes of ATP binding by the calcium pump. EMBO J. 25, 2305–2314
73. Laursen, M., Bublitz, M., Moncoq, K., Olesen, C., Møller, J. V., Young, H. S., Nissen, P., and Morth, J. P. (2009) Cyclopiazonic acid is complexed to a divalent metal ion when bound to the sarcoplasmic reticulum Ca2+-ATPase. J. Biol. Chem. 284, 13513–13518
74. Moncoq, K., Trieber, C. A., and Young, H. S. (2007) The molecular basis for cyclopiazonic acid inhibition of the sarcoplasmic reticulum calcium pump. J. Biol. Chem. 282, 9748–9757
75. Abu-Abed, M., Mal, T. K., Kainocho, M., MacLennan, D. H., and Ikura, M. (2002) Characterization of the ATP-binding domain of the sarco(endoplasmic reticulum Ca2+-ATPase. Probing nucleotide binding by multidimensional NMR. Biochemistry 41, 1156–1164
76. Abu-Abed, M., Millet, O., MacLennan, D. H., and Ikura, M. (2004) Probing nucleotide-binding effects on backbone dynamics and folding of the nucleotide-binding domain of the sarcoplasmic/endoplasmic-reticulum Ca2+-ATPase. J. Biol. Chem. 282, 235–242
77. Bodley, A. L., and Jencks, W. P. (1987) Acetyl phosphate as a substrate for the calcium ATPase of sarcoplasmic reticulum. J. Biol. Chem. 262, 13997–14004
78. Benders, A. G., van Kuppevelt, T. H., Oosterhof, A., Wevers, R. A., and Veerkamp, J. H. (1992) Adenosine triphosphatases during maturation of cultured human skeletal muscle cells and in adult human muscle. Biochim. Biophys. Acta 1112, 89–98
79. Brandt, N. R., Caswell, A. H., and Brunschwig, J. P. (1980) ATP-energized Ca2+ pump in isolated transverse tubules of skeletal muscle. J. Biol. Chem. 255, 6290–6298
80. Freire, M. M., Mignaco, J. A., de Carvalho-Alves, P. C., Barabini, H., and Scofano, H. M. (2002) 3-O-Methylfluorescein phosphate as a fluorescent substrate for plasma membrane Ca2+-ATPase. Biochim. Biophys. Acta 1553, 238–248
81. Huntley, J. J., Scrofani, S. D., Osborne, M. J., Wright, P. E., and Dyson, H. J. (2000) Dynamics of the metallo-β-lactamase from Bacteroides fragilis in the presence and absence of a tight-binding inhibitor. Biochemistry 39, 13356–13364
82. Yamamoto, H., Imamura, Y., Tagaya, M., Fukui, T., and Kawakita, M. (1989) Ca2+-dependent conformational change of the ATP-binding site of Ca2+-transporting ATPase of sarcoplasmic reticulum as revealed by an alteration of the target site specificity of adenosine triphosphorydixol. J. Biochem. 106, 1121–1125
83. Pei, Q., Del Carpio, C., Tsuboi, H., Koyama, M., Endou, A., Kubo, M., Broclawik, E., Nishijima, K., Terasaki, T., and Miyamoto, A. (2007) Theoretical study on the ATP hydrolysis mechanism of HisP protein, the ATP-binding subunit of ABC transporter. Materials Transactions 48, 735–739
84. Møller, J. V., Nissen, P., Sørensen, T. L., and le Maire, M. (2005) Transport mechanism of the sarcoplasmic reticulum Ca2+-ATPase pump. Curr. Opin. Struct. Biol. 15, 387–393
85. Lassila, J. K., Zalatan, J. G., and Herschlag, D. (2011) Biological phosphoryl-transfer reactions. Understanding mechanism and catalysis. Annu. Rev. Biochem. 80, 669–702
86. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. E. (1980) Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. J. Biol. Chem. 255, 3025–3031
87. Nesmeyov, Y. E., Agafonov, R. V., Negrashov, I. V., Blakely, S. E., Titus, M. A., and Thomas, D. D. (2011) Structural kinetics of myosin by transient time-resolved FRET. Proc. Natl. Acad. Sci. U.S.A. 108, 1891–1896
88. Tran, K., Smith, N. P., Loiselle, D. S., and Crampin, E. J. (2009) A thermodynamic model of the cardiac sarcoplasmic/endoplasmic reticulum Ca2+-SERCA) pump. Biophys. J. 96, 2029–2042
89. Kern, D., and Zuiderweg, E. R. (2003) The role of dynamics in allosteric regulation. Curr. Opin. Struct. Biol. 13, 748–757
90. Fernandez-Belda, F., Garcia-Carmona, F., and Inesi, G. (1988) Accelerating effect of ATP on calcium binding to sarcoplasmic reticulum ATPase. Arch. Biochem. Biophys. 260, 118–124
91. Hammes, G. G., Chang, Y. C., and Oas, T. G. (2009) Conformational selection or induced fit. A flux description of reaction mechanism. Proc. Natl. Acad. Sci. U.S.A. 106, 13737–13741
92. Olesen, C., Sørensen, T. L., Nielsen, R. C., Møller, J. V., and Nissen, P. (2004) Dephosphorylation of the calcium pump coupled to counterion occlusion. Science 306, 2251–2255
93. Champaile, P., Riollet, S., Orlowski, S., Guillaume, F., Seebregts, C. J., and McIntosh, D. B. (1988) ATP regulation of sarcoplasmic reticulum Ca2+-ATPase. Metal-free ATP and 8-bromo-ATP bind with high affinity to the catalytic site of phosphorylated ATPase and accelerate dephosphorylation. J. Biol. Chem. 263, 12288–12294