SYSTEM DEVELOPMENT FOR TIME-RESOLVED FRET IN SERCA

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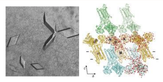
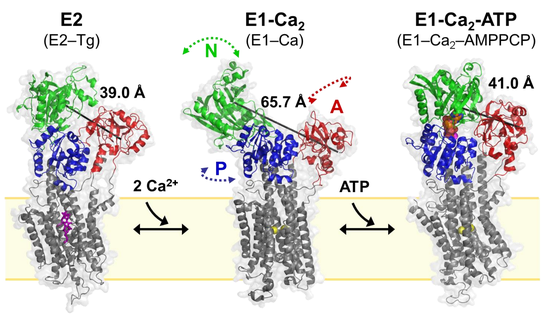
Abstract

We have compared mammalian (HEK) and insect (Sf21) cell expression systems for detecting structural dynamics of the sarcoplasmic reticulum Ca-ATPase (SERCA) by time-resolved fluorescence resonance energy transfer (FRET). X-ray crystallography suggests that calcium binding opens the cytoplasmic headpiece of SERCA, producing large increases in distance between nucleotide-binding, phosphorylation, and actuator domains (1.0–2.5 nm). To test this hypothesis using FRET, SERCA was labeled with cyan fluorescent protein (CFP) at the N-terminus in the actuator domain and fluorescein isothiocyanate (FITC) at Lys-515 in the nucleotide-binding domain. We expressed fluorescently-labeled SERCA in both HEK and Sf21 cells, purified endoplasmic reticulum membrane fragments from the cells, and then measured time-resolved FRET between CFP donor and FITC acceptor in the presence and absence of calcium. FRET determined that (1) the nucleotide-binding and actuator domain are closed in the calcium-free state and (2) calcium-binding induces a small opening (<0.2 nm) between these two domains. Similar results were detected by FRET in both mammalian and insect cell systems. We conclude that the cytoplasmic headpiece of SERCA is predominantly closed in biological membranes.

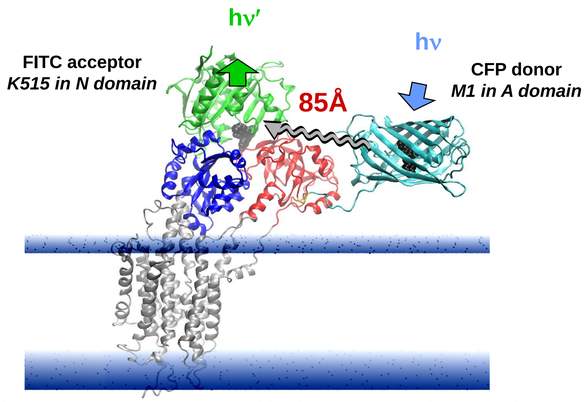
Proposed Structural Transitions from X-ray Crystallography





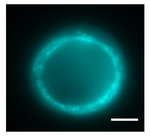
X-ray crystallographers (Toyoshima, Nissen, and Young Labs) have solved atomic-resolution structures for SERCA in ligand-stabilized states. In particular, three domains within the cytoplasmic 'headpiece' (nucleotide-binding [N], phosphorylation [P], and actuator [A] domains) are proposed to undergo dramatic rearrangements in response to substrate binding and phosphoenzyme formation. These data indicate that calcium binding causes a large reorientation of N and A domains with a 20Å increase in N-A distance (E2-Tg to E1-Ca) and that nucleotide binding in the presence of calcium causes a large closure of cytoplasmic headpiece with 20Å decrease in the N–A distance (E1-Ca to E1-AMPPCP).

FRET to Detect N-A Domain Distance in physiological conditions

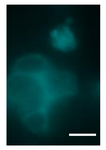


CFP cDNA was fused to the 5' end of SERCA cDNA, and the fluorescent fusion protein was heterologously expressed . CFP-SERCA was labeled with FITC at K515. Quantitative molecular modeling of dual-labeled SERCA (based on X-ray crystal structures) was used to calculate orientation factor and distance between probes.

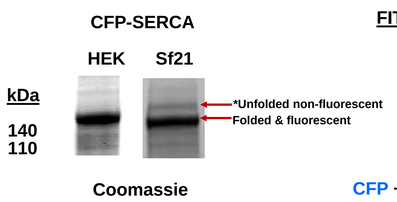
High-level Expression and Fluorescent Labeling of CFP-FITC-SERCA



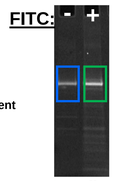
Sf21 cell



HEK cell



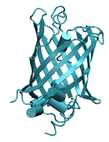
Coomassie



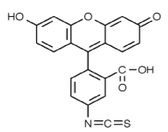
CFP + FITC fluorescence

- CFP-SERCA was efficiently expressed in active form and was specifically labeled with FITC in microsomes from Sf21 and HEK cells.
- A small amount (10-15%) of SERCA was unfolded and non-fluorescent in Sf21 cells.

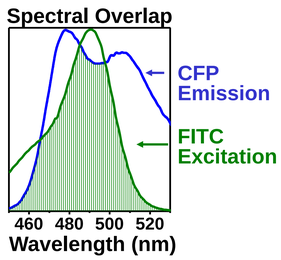
Donor-Acceptor Spectral Properties

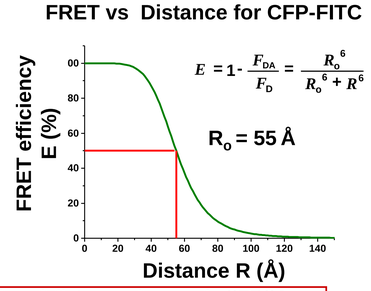


CFP Donor
ε 477 = 26,000
ϕ = 0.4



FITC Acceptor
ε 519 = 77,000
ϕ = 0.9

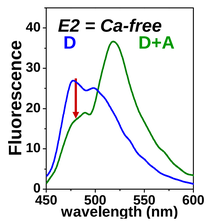




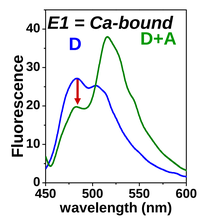
CFP and FITC are an optimal donor/acceptor probes for FRET detection of ligand-induced distance changes in SERCA.

Steady-state FRET: Ca-free, Ca-bound, and Ca-free phosphoenzyme states are mostly closed

Sf21homogenates emission scan

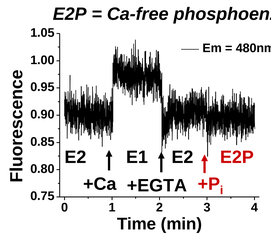


E = 0.34 ± 0.02
R = 62 ± 2.0 Å
X-ray/Model = 59 Å



E = 0.28 ± 0.02
R = 66 ± 1.4 Å
X-ray/Model = 86 Å

HEK homogenate kinetic scan



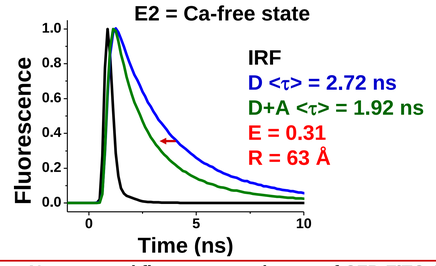
E = 0.36 ± 0.02
R = 60 ± 2.0 Å
X-ray/Model = 56Å

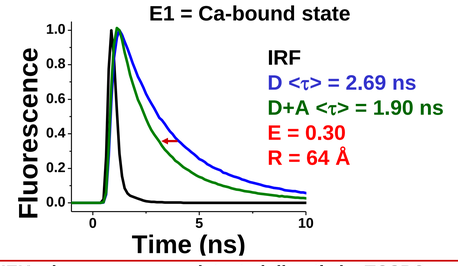
1) For the Ca-free E2 state (*left panel*), FRET measured in Sf21 system and crystallography indicate that the cytoplasmic headpiece is closed (N-A distance = 62 Å)

2) For the Ca-bound E1 state (*middle panel*), FRET measured in Sf21 system indicates that the cytoplasmic headpiece is mostly closed (N-A distance = 66 Å), in contrast to open by crystallography (N-A distance = 86 Å).

3) For the Ca-free phosphoenzyme state E2P (*right panel*), FRET measured in HEK system and crystallography indicate the cytoplasmic headpiece is most closed (N-A distance = 60 Å).

Time-resolved FRET in HEK microsomes: Time-correlated Single-photon Counting





Nanosecond fluorescence decays of CFP-FITC-SERCA in HEK microsomes were detected directly by TCSPC, confirming steady-state FRET results in both HEK and Sf21 homogenates:

1) The cytoplasmic headpiece of SERCA is closed in Ca-free and Ca-bound states

2) Ca slightly increases N-A domain distance by ~1-2 Å

Materials and Methods

- Cell homogenates were prepared by mechanical disruption using a Polytron.
- Cell microsomes were prepared by differential centrifugation, collected as the 100,000 × g pellet.
- Protein concentrations were determined using the bicinchoninic acid assay (BCA).
- SERCA was labeled with FITC using a 2:1 dye to protein ratio for 20 minutes at 25°C and pH 8.8.
- Fluorescence was measured using a Varian Eclipse spectrometer (steady-state), Fluorescence Innovations spectrometer (time-resolved) ,and Nikon 200TE microscope.

Cell Culture

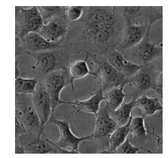
Growth curves, doubling times, and % viability were determined for insect and mammalian cell lines. Both cell lines showed optimal growth.

Sf21

Pass	[Start]	[Final]	Hours	Doubling Time	% Viable
13	0.25	1.20	71.0	32	97
14	0.50	1.70	47.5	30	97
15	0.50	1.63	48.0	30	98
16	0.25	1.34	71.5	31	97
17	0.50	1.82	47.5	26	96
18	0.50	1.79	48.0	27	98
19	0.25	1.30	71.0	30	98
21	0.50	1.90	41.0	26	97
22	0.25	1.59	72.5	28	98
24	0.50	1.82	50.5	28	99
			AVE	29	98
			STDEV	2	1

HEK QBI 293A

Pass	Seeded	Yield	Hours	Doubling Time	% Viable
13	4 E6	10.53 E6	48.0	37	96
14	1.5 E6	7.54 E6	47.0	31	94
15	2 E6	7.02 E6	48.0	27	95
16	2 E6	6.37 E6	48.0	29	94
17	1 E6	6.63 E6	48.0	22	94
18	2 E6	9.5 E6	50.5	22	95
19	1 E6	13.3 E6	101.0	27	95
20	1 E6	5.98	36.0	14	94
21	1 E6	6.63	73.0	27	96
22	2 E6	23.04	98.0	28	97
			AVE	26.30	95
			STDEV	6.11	1



40x objective of HEK cells

CONCLUSIONS

1) HEK and Sf21 are both sound systems for FRET measurements in SERCA. The HEK system resulted in more stable protein folding, and thus is a more accurate system for understanding mammalian SERCA.
2) In contrast to crystallography which found an open headpiece, the Ca-bound E1 headpiece of SERCA is closed in physiological conditions in both HEK and Sf21 cells.

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Further Information

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