SUPPLEMENTAL MATERIALS

MATERIALS & METHODS:

Mutagenesis, expression, and purification of PLN

The protocol used for mutagenesis, expression and purification of PLN has been previously described (1,2). Alanine mutations in full-length human PLN were carried out using two-step PCR. Mutated primers were designed and obtained from Integrated DNA Technologies (Coralville, Iowa, USA). Successful mutations were confirmed using Sanger DNA Sequencing (Molecular Biology Facility, University of Alberta. The N-terminus of recombinant human PLN was fused to the C-terminus of maltose-binding protein (MBP) with a Tobacco Etched Virus (TEV) protease cleavage site inserted between PLN and MBP. Fused recombinant MBP-PLN was grown as a 4 L batch in *E. Coli* BL21(DE3) competent cells obtained from New England BioLabs (Whitby, ON) at 37℃ until the optical density reached 0.6 at 600 nm. MBP-PLN fusion protein expression was induced with the addition of 75 µM Isopropyl-β-D-thiogalactopyranoside (IPTG) at 22℃ for 24 hours. The cells were then collected and sonicated (0.5 sec on, 0.5 sec off for 45 sec for 4 cycles with a 1-minute interval between each cycle) in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 M NaCl, 56 mM Glycerol, 0.5% Triton X-100 and 1 mM DTT). The sonicated cell extract was centrifuged, and the supernatant was incubated with the amylose affinity resin (New England BioLabs) for 1.5 hours. The affinity resin then washed with wash buffer (10 column volumes of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.2 M NaCl) and the MBP-PLN fusion protein was eluted with 50 ml of 80 mM maltose and 200 ml of 40 mM maltose in stepwise additions, followed by 200 ml of wash buffer. The eluted MBP-PLN fusion protein was concentrated and cleaved with TEV protease overnight at 22℃. PLN tended to precipitate upon TEV protease cleavage and the peptide was collected by centrifugation. MBP was separated from PLN by a series of 1M Guanidine-HCl washes, followed by solubilisation of PLN in 7M Guanidine-HCl. Solubilized PLN was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a ZORBAX column (300SB-C8, 9.4 x 250mm, 0.5 µM; Agilent Technologies Canada, Inc., Mississauga, ON) and a water-isopropanol-TFA gradient. The resulting purified PLN peptide was lyophilized and stored at -80℃ as 100 µg aliquots for future use.

Reconstitution of SERCA and PLN into proteoliposome.

SERCA was purified from the SR of rabbit hind leg muscle and reconstituted into membrane vesicles (proteoliposomes) as described (3). Purified recombinant human PLN (100 µg) was thawed and solubilized in 100 µl of 80% trifluoroethanol (TFE) followed by the addition of lipids, egg yolk phosphatidylcholine (EYPC; 310 µg) and egg yolk phosphatidic acid (EYPA; 40 µg). Lipids EYPA and EYPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). This mixture was dried under a stream of N_2 gas while vortexing in order to form a thin peptide-lipid film. The resulting thin film was dried further overnight under vacuum. The next day, water (94 µl) was added to the thin film and incubated for 15 minutes at 50℃. Following this rehydration step, 6 µl of 10% octaethylene glycol monododecyl ether ($C_{12}E_8$; Nikko Chemicals, Tokyo, Japan) was added and the reaction mixture was vigorously vortexed for 3 minutes and the solution was brought to appropriate buffer conditions (20 mM imidazole, pH 7.4, 100 mM NaCl, 0.02% sodium azide). From this solubilized peptide-lipid-detergent mixture, the reconstitution process was then initiated by adding 300 µg of previously purified SERCA in a buffer containing 1 mg/ml $C_{12}E_8$,

50 mM MOPS, pH 7.0, 1 mM DTT, 20% glycerol, 1 mM $CaCl_2$ and 0.17 mg/ml of EYPC. Detergent $C_{12}E_8$ was gradually removed over a period of 4 hours to induce vesicle formation using SM2 Bio-Beads (Bio-Rad Laboratories, Hercules, CA, USA) with constant stirring. For the first 2 hours, 1 mg of Bio-Beads was added every 30 minutes then 5 mg addition with 1-hour incubation and finally 10 mg addition with another 1 hour of incubation. The reconstituted vesicles were then collected by sucrose gradient density centrifugation at the interface between 20% sucrose layer and a 50% sucrose layer. The collected reconstitution vesicles were then flash-frozen and stored at -80°C for future experiments.

Calcium-Dependent ATPase Activity Assays

Proteoliposomes containing SERCA and PLN were then measured for their calcium-dependent ATPase activity using a coupled-enzyme assay as previously described (4). Proteoliposomes were added to 150 µl of assay buffer (50 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 2.4 mM ATP at pH 7.0, 0.18 mM NADH, 0.5 mM PEP, 0.01 µg/µl calcium ionophore A23187, and Lactate Dehydrogenase (LDH) and Pyruvate Kinase (PK) enzymes at 9.6 units/ml). Reagents ATP, NADH, PEP, calcium ionophore A23187, LDH and PK were obtained from Sigma-Aldrich (Oakville, ON, CA). The assay was performed using a 96-well plate and to each of the wells in the plate a variable concentration of $CaCl_2$ was added to achieve free calcium concentrations in the range of 0.1 to 10 µM. The coupled-enzyme assay links ATP hydrolysis to NADH oxidation through the activity of LDH and PK, which serves to replenish ATP and provide a measurable absorbance at 340 nm as NADH is oxidized to NAD^+ in the reaction well. The absorbance at 340 nm was measured every 29 seconds for 30 minutes at different free calcium concentrations and the data were fit to the Hill equation (SigmaPlot, Systat Software Inc.) to determine the apparent calcium affinity (K_{Ca}). This was performed for each alanine substitution of PLN, where SERCA in the absence of PLN served as a negative control and SERCA in the presence of wild-type PLN served as a positive control. Standard errors were calculated for a minimum of three independent reconstitutions for each PLN variant. Comparison of K_{Ca} values was carried out using one-way analysis of variance (between subjects), followed by the Holm-Sidak test for pairwise comparisons.

Supplementary Table 1 (Table S1): The apparent calcium affinity of SERCA in the presence of PLN alanine substitutions and the calculated free-energies for PLN alanine substitutions in the SERCA-PLN complex, PLN monomer, and PLN pentamer in a membrane environment.

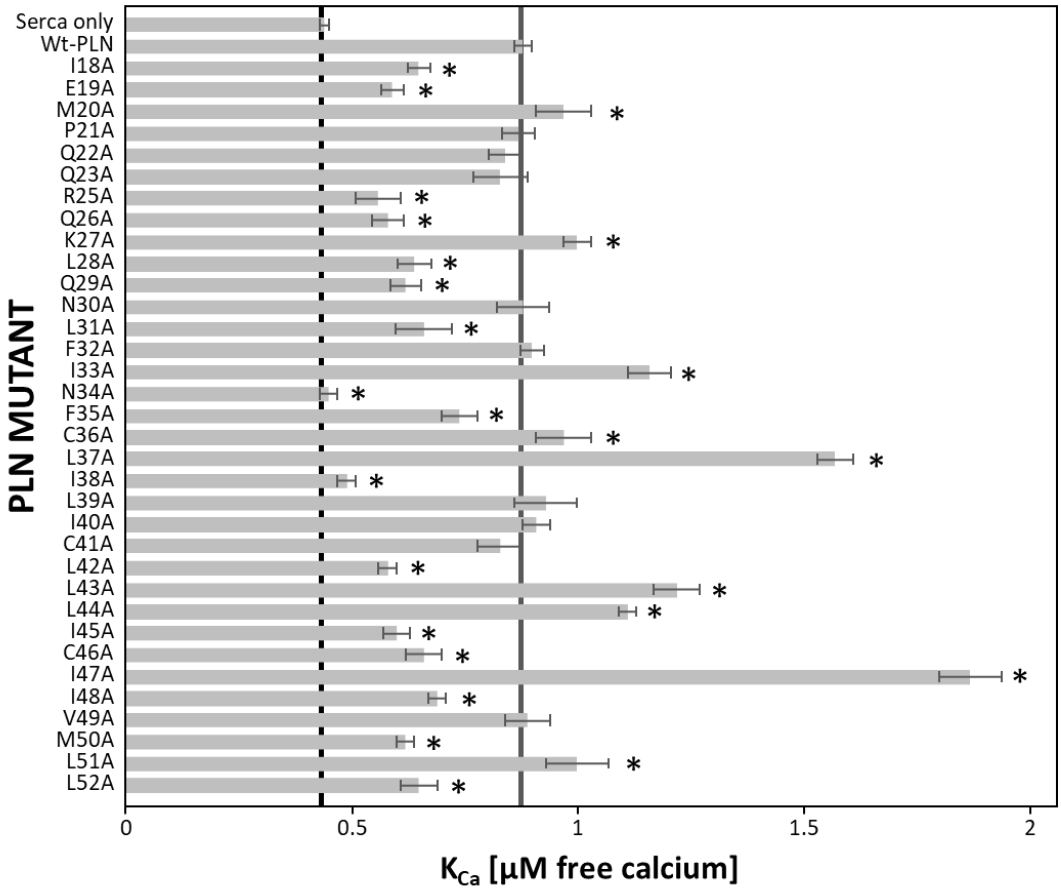
PLN variant	K_{Ca} (μM)	SERCA-PLN $\Delta G_{bound\text{-}alchemical}$ (kcal/mol)	PLN monomer $\Delta G_{unbound\text{-}alchemical}$ (kcal/mol)	PLN pentamer $\Delta G_{unbound\text{-}alchemical}$ (kcal/mol)
SERCA only	0.44 ± 0.01			
wt-PLN	0.88 ± 0.02			
I18A	0.65 ± 0.03	-6.36 ± 0.04	-6.51 ± 0.06	-6.09 ± 0.02
E19A	0.59 ± 0.02	104.74 ± 0.13	109.64 ± 0.10	108.85 ± 0.13
M20A	0.97 ± 0.06	3.24 ± 0.08	3.98 ± 0.13	2.56 ± 0.06
P21A	0.87 ± 0.04			
Q22A	0.84 ± 0.03	55.23 ± 0.10	55.46 ± 0.13	56.85 ± 0.05
Q23A	0.83 ± 0.06	54.14 ± 0.11	56.52 ± 0.10	56.33 ± 0.04
R25A	0.56 ± 0.05	255.53 ± 0.58	262.87 ± 0.39	264.43 ± 0.24
Q26A	0.58 ± 0.04	55.79 ± 0.11	55.65 ± 0.10	56.54 ± 0.05
K27A	1.00 ± 0.03	40.24 ± 0.27	39.20 ± 0.29	42.90 ± 0.19
L28A	0.64 ± 0.04	12.49 ± 0.08	13.26 ± 0.08	11.68 ± 0.04
Q29A	0.62 ± 0.04	52.46 ± 0.17	54.97 ± 0.23	55.70 ± 0.07
N30A	0.88 ± 0.06	77.11 ± 0.07	78.08 ± 0.08	76.96 ± 0.04
L31A	0.66 ± 0.06	14.17 ± 0.08	12.90 ± 0.11	13.26 ± 0.04
F32A	0.90 ± 0.05	-6.41 ± 0.12	-5.94 ± 0.11	-6.13 ± 0.07
I33A	1.16 ± 0.08	-7.76 ± 0.10	-4.94 ± 0.09	-5.93 ± 0.04
N34A	0.45 ± 0.02	76.38 ± 0.07	74.03 ± 0.11	74.77 ± 0.04
F35A	0.74 ± 0.04	-4.35 ± 0.09	-5.87 ± 0.07	-7.20 ± 0.04
C36A	0.97 ± 0.06	0.31 ± 0.06	1.42 ± 0.05	1.07 ± 0.04
L37A	1.57 ± 0.04	13.08 ± 0.06	13.73 ± 0.10	15.36 ± 0.04
I38A	0.49 ± 0.02	-2.91 ± 0.08	-6.57 ± 0.08	-5.75 ± 0.04
L39A	0.93 ± 0.07	14.24 ± 0.08	12.92 ± 0.07	14.54 ± 0.04
I40A	0.91 ± 0.03	-4.79 ± 0.08	-6.18 ± 0.07	-3.85 ± 0.04
C41A	0.83 ± 0.05	0.83 ± 0.04	-0.27 ± 0.05	0.63 ± 0.02
L42A	0.58 ± 0.02	14.68 ± 0.05	14.24 ± 0.08	14.12 ± 0.03
L43A	1.22 ± 0.05	13.28 ± 0.08	14.61 ± 0.10	16.13 ± 0.04
L44A	1.11 ± 0.02	12.21 ± 0.09	13.62 ± 0.10	16.85 ± 0.02
I45A	0.60 ± 0.03	-4.52 ± 0.09	-5.80 ± 0.08	-5.16 ± 0.03
C46A	0.66 ± 0.04	1.03 ± 0.05	0.68 ± 0.05	0.86 ± 0.02
I47A	1.87 ± 0.07	-5.57 ± 0.08	-5.58 ± 0.06	-2.99 ± 0.04
I48A	0.69 ± 0.02	-3.39 ± 0.09	-5.29 ± 0.08	-3.54 ± 0.03
V49A	0.89 ± 0.05	0.25 ± 0.04	-0.08 ± 0.05	-0.58 ± 0.03
M50A	0.62 ± 0.02	0.94 ± 0.14	2.81 ± 0.14	3.19 ± 0.32
L51A	1.00 ± 0.07	12.20 ± 0.10	12.95 ± 0.10	15.39 ± 0.04
L52A	0.65 ± 0.04	12.61 ± 0.08	13.36 ± 0.11	12.36 ± 0.04

Supplemental Table 2 (Table S2): The apparent calcium affinity of SERCA in the presence of PLN alanine substitutions and the calculated change in free-energy for PLN alanine substitutions in the formation of the SERCA-PLN complex from the PLN monomer or the PLN pentamer in a membrane environment. The calculated change in free-energy for PLN alanine substitutions in the formation of the PLN monomer from the PLN pentamer in a membrane environment.

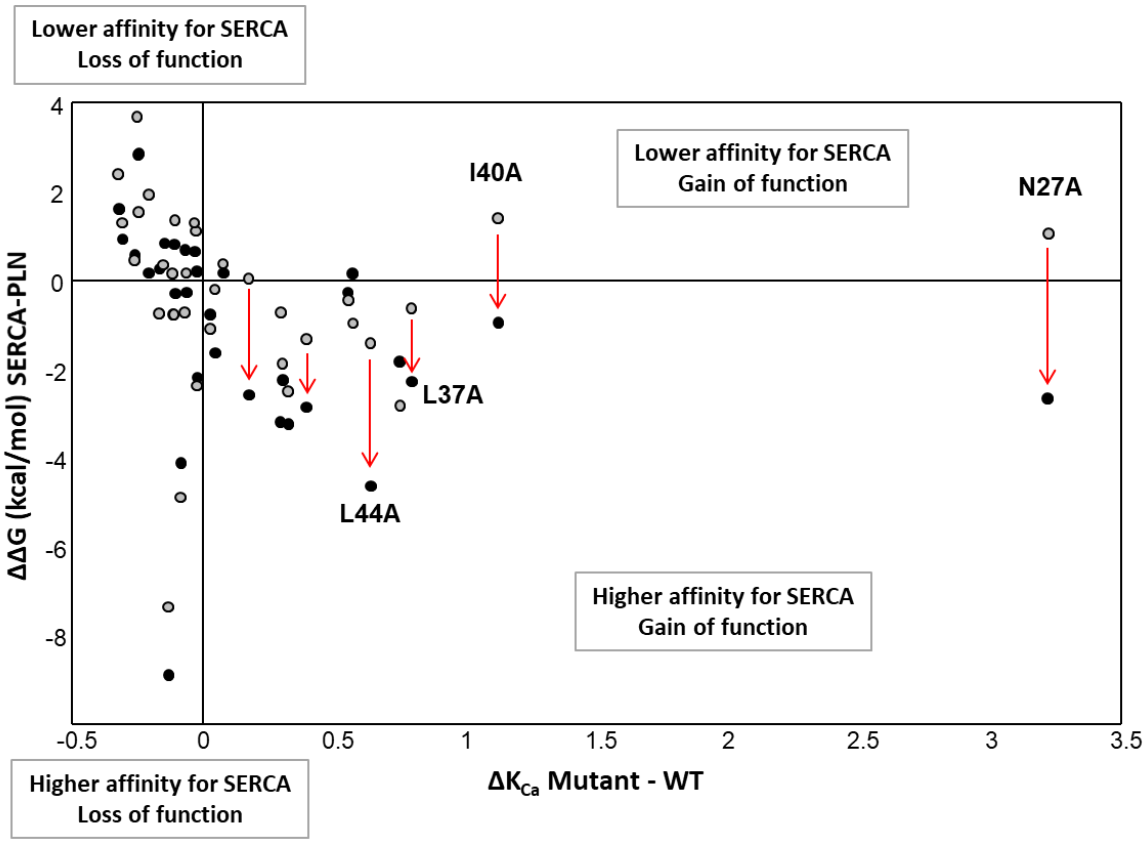
PLN variant	K_{Ca} (μM)	SERCA-PLN from monomer ΔΔG (kcal/mol)	SERCA-PLN from pentamer ΔΔG (kcal/mol)	PLN monomer from pentamer ΔΔG (kcal/mol)
SERCA only	0.44 ± 0.01			
wt-PLN	0.88 ± 0.02			
I18A	0.65 ± 0.03	0.14 ± 0.10	-0.28 ± 0.07	-0.42 ± 0.08
E19A	0.59 ± 0.02	-4.89 ± 0.28	-4.11 ± 0.31	0.78 ± 0.22
M20A	0.97 ± 0.06	-0.74 ± 0.21	0.68 ± 0.14	1.42 ± 0.19
P21A	0.87 ± 0.04			
Q22A	0.84 ± 0.03	-0.23 ± 0.23	-1.63 ± 0.16	-1.40 ± 0.18
Q23A	0.83 ± 0.06	-2.38 ± 0.21	-2.19 ± 0.15	0.20 ± 0.14
R25A	0.56 ± 0.05	-7.34 ± 0.97	-8.90 ± 0.82	-1.56 ± 0.63
Q26A	0.58 ± 0.04	0.13 ± 0.25	-0.76 ± 0.16	-0.89 ± 0.19
K27A	1.00 ± 0.03	1.03 ± 0.56	-2.67 ± 0.46	-3.70 ± 0.48
L28A	0.64 ± 0.04	-0.77 ± 0.16	0.81 ± 0.12	1.58 ± 0.12
Q29A	0.62 ± 0.04	-2.52 ± 0.40	-3.24 ± 0.24	-0.73 ± 0.30
N30A	0.88 ± 0.06	-0.98 ± 0.15	0.15 ± 0.11	1.12 ± 0.12
L31A	0.66 ± 0.06	1.27 ± 0.18	0.92 ± 0.12	-0.35 ± 0.15
F32A	0.90 ± 0.05	-0.46 ± 0.23	-0.28 ± 0.18	0.18 ± 0.18
I33A	1.16 ± 0.08	-2.81 ± 0.20	-1.83 ± 0.15	0.98 ± 0.14
N34A	0.45 ± 0.02	2.36 ± 0.18	1.61 ± 0.10	-0.75 ± 0.14
F35A	0.74 ± 0.04	1.52 ± 0.16	2.85 ± 0.13	1.33 ± 0.12
C36A	0.97 ± 0.06	-1.11 ± 0.10	-0.76 ± 0.09	0.36 ± 0.08
L37A	1.57 ± 0.04	-0.65 ± 0.16	-2.28 ± 0.10	-1.63 ± 0.13
I38A	0.49 ± 0.02	3.66 ± 0.16	2.84 ± 0.11	-0.81 ± 0.11
L39A	0.93 ± 0.07	1.32 ± 0.16	-0.29 ± 0.12	-1.62 ± 0.11
I40A	0.91 ± 0.03	1.39 ± 0.16	-0.94 ± 0.13	-2.33 ± 0.12
C41A	0.83 ± 0.05	1.09 ± 0.09	0.20 ± 0.06	-0.89 ± 0.07
L42A	0.58 ± 0.02	0.44 ± 0.13	0.56 ± 0.09	0.12 ± 0.11
L43A	1.22 ± 0.05	-1.32 ± 0.18	-2.85 ± 0.12	-1.53 ± 0.14
L44A	1.11 ± 0.02	-1.41 ± 0.18	-4.63 ± 0.11	-3.22 ± 0.12
I45A	0.60 ± 0.03	1.28 ± 0.16	0.64 ± 0.12	-0.64 ± 0.11
C46A	0.66 ± 0.04	0.35 ± 0.10	0.17 ± 0.07	-0.17 ± 0.07
I47A	1.87 ± 0.07	0.01 ± 0.15	-2.57 ± 0.12	-2.59 ± 0.10
I48A	0.69 ± 0.02	1.91 ± 0.17	0.16 ± 0.12	-1.75 ± 0.11
V49A	0.89 ± 0.05	0.33 ± 0.09	0.83 ± 0.07	0.50 ± 0.07
M50A	0.62 ± 0.02	-1.87 ± 0.28	-2.25 ± 0.46	-0.38 ± 0.46
L51A	1.00 ± 0.07	-0.74 ± 0.20	-3.19 ± 0.13	-2.44 ± 0.14
L52A	0.65 ± 0.04	-0.75 ± 0.19	0.26 ± 0.12	1.01 ± 0.15
Average ΔΔG (kcal/mol)		-0.39	-1.00	-0.61
Total ΔΔG (kcal/mol)		-12.76	-32.98	-20.22

Supplemental Table 3 (Table S3): The calculated change in free-energy for PLN alanine substitutions in the formation of the SERCA-PLN complex from the PLN monomer or the PLN pentamer. For a subset of residues, the alchemical FEP calculations were run for replicates at different simulation equilibration times (10, 25, 40, and 50 ns).

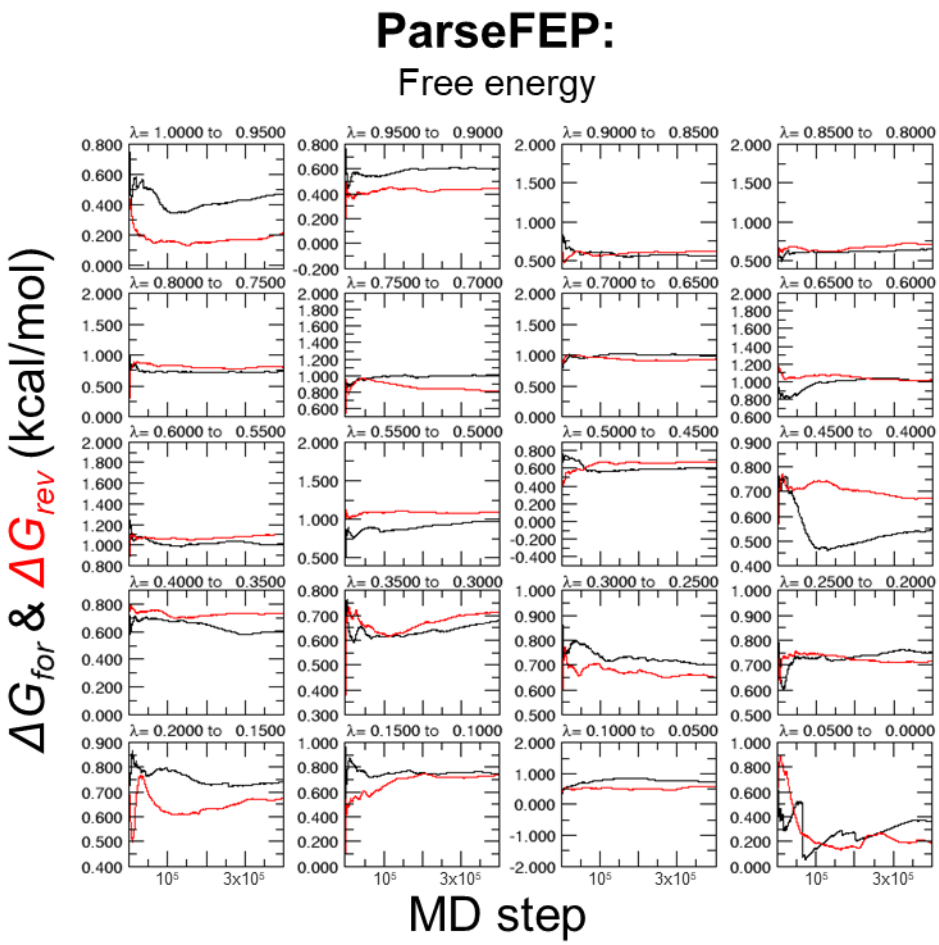
PLN variant	Equilibration time	SERCA-PLN from monomer ΔΔG (kcal/mol)	SERCA-PLN from pentamer ΔΔG (kcal/mol)
L37A	10 ns	-0.65 ± 0.04	-2.28 ± 0.02
	25 ns	0.67 ± 0.02	-0.58 ± 0.02
	40 ns	-1.64 ± 0.01	-2.90 ± 0.05
	50 ns	1.64 ± 0.01	-0.58 ± 0.08
L43A	10 ns	-1.33 ± 0.02	-2.85 ± 0.04
	25 ns	1.44 ± 0.03	-0.63 ± 0.06
	40 ns	0.81 ± 0.01	-10.98 ± 0.04
	50 ns	0.04 ± 0.02	-2.18 ± 0.07
L44A	10 ns	-1.41 ± 0.01	-4.64 ± 0.07
	25 ns	0.25 ± 0.01	-3.03 ± 0.06
	40 ns	-1.03 ± 0.01	-3.86 ± 0.07
	50 ns	1.04 ± 0.02	-2.77 ± 0.05
I47A	10 ns	0.01 ± 0.02	-2.58 ± 0.05
	25 ns	0.47 ± 0.01	-1.57 ± 0.05
	40 ns	-0.14 ± 0.01	-0.85 ± 0.05
	50 ns	1.31 ± 0.03	-1.65 ± 0.06
L51A	10 ns	-0.75 ± 0.01	-3.19 ± 0.06
	25 ns	-0.69 ± 0.01	-1.85 ± 0.05
	40 ns	0.30 ± 0.02	-2.02 ± 0.08
	50 ns	-0.97 ± 0.02	-1.80 ± 0.06
Average ΔΔG (kcal/mol)		-0.03	-2.20
Total ΔΔG (kcal/mol)		-0.63	-41.81



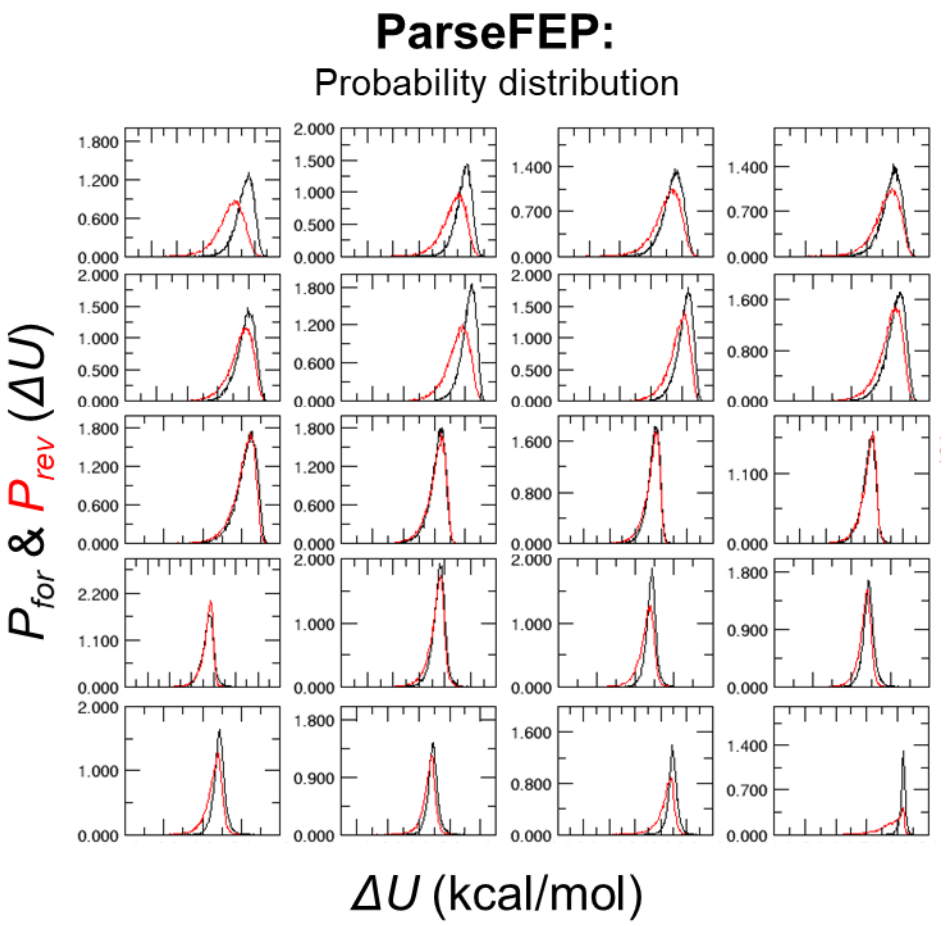
Supplemental Figure 1 (Figure S1): The apparent calcium affinity (K_{Ca}) of SERCA in the absence and presence of wild-type PLN and alanine substitutions of residues Ile^{18} to Leu^{52}. The black vertical line indicates the K_{Ca} for SERCA alone (negative control) and the grey vertical line indicates the K_{Ca} for SERCA in the presence of wild-type PLN (positive control). The K_{Ca} values for residues Ile^{18} to Ile^{33} were determined in the present study, and the K_{Ca} values for residues Asn^{34} to Leu_{52} have been previously reported (15,29). Except for the most severe loss-of-function variants, N34A and I38A, all K_{Ca} values are statistically significant compared to the negative control, SERCA in the absence of PLN ($p < 0.01$). Asterisks (*) indicate K_{Ca} values that are statistically significant compared to the positive control, SERCA in the presence of wild-type PLN ($p < 0.01$).



Supplemental Figure 2 (Figure S2): Scatter plots of *ΔΔG* (kcal/mol) versus ΔK_{Ca} for alanine substitutions of PLN and the thermodynamic cycle from the PLN monomer (grey circles) and PLN pentamer (black circles). In this figure, the ΔK_{Ca} values for alanine substitutions of PLN were obtained from Kimura et al. (*Journal of Biological Chemistry*, 1998, 273:14238-41). This latter study used canine PLN and there were differences in the ΔK_{Ca} values obtained in their study compared to our studies. Nonetheless, notice the better fit between the *ΔΔG* and ΔK_{Ca} values for the alanine substitutions Asn^{27}-Ala (N27A) and Ile^{40}-Ala (I40A), as well as other gain of function variants, using the thermodynamic cycle for formation of the SERCA-PLN complex from the PLN pentamer (some examples are indicated by red arrows). The different quadrants are labeled as in Figures 5 and 6.



Supplementary Figure 3 (Figure S3): Example graphical output of ParseFEP obtained for the reversible alchemical transformation of phospholamban (PLN) residues to alanines (residues Ile^{18} to Leu^{52}). The alchemical transformation employed a 20-window (λ) stratification strategy. Time-evolution of the free energy for the forward (black solid line) and reverse (red solid line) transformations are shown for a representative residue of PLN (Leu^{39}-Ala) in the SERCA-PLN complex. In the plots shown, free energy (ΔG_{for} & ΔG_{rev}) is plotted versus the MD step in the simulations. Each window (λ width 0.05) consisted of 1 x 10^5 equilibration and 5 x 10^5 production steps of 1 fs each. The cumulative simulation time for each alanine alchemical FEP calculation was 24 ns.



Supplementary Figure 4 (Figure S4): Example graphical output of ParseFEP obtained for the reversible alchemical transformation of phospholamban (PLN) residues to alanines (residues Ile18 to Leu52). The alchemical transformation employed a 20-window (λ) stratification strategy. Histograms of the probability distributions for the forward (black solid line) and reverse (red solid line) transformations are shown for a representative residue of PLN (Leu^{39}-Ala) in the SERCA-PLN complex. For each window (λ width 0.05 in previous figure), the probability distribution (P_{for} & P_{rev}) is plotted versus the difference in potential energy (ΔU; kcal/mol). In the free-energy perturbation method, the free-energy difference between the reference and the target states is expressed as an equilibrium average, ΔU, within each window of the simulation.

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