Protein docking and steered molecular dynamics suggest alternative phospholamban-binding sites on the SERCA calcium transporter

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Edited by Roger J. Colbran

The transport activity of the sarcoplasmic reticulum calcium ATPase (SERCA) in cardiac myocytes is modulated by an inhibitory interaction with a transmembrane peptide, phospholamban (PLB). Previous biochemical studies have revealed that PLB interacts with a specific inhibitory site on SERCA, and low-resolution structural evidence suggests that PLB interacts with distinct alternative sites on SERCA. High-resolution details of the structural determinants of SERCA regulation have been elusive because of the dynamic nature of the regulatory complex. In this study, we used computational approaches to develop a structural model of SERCA–PLB interactions to gain a mechanistic understanding of PLB-mediated SERCA transport regulation. We combined steered molecular dynamics and membrane protein–protein docking experiments to achieve both a global search and all-atom force calculations to determine the relative affinities of PLB for candidate sites on SERCA. We modeled the binding of PLB to several SERCA conformations, representing different enzymatic states sampled during the calcium transport catalytic cycle. The results of the steered molecular dynamics and docking experiments indicated that the canonical PLB-binding site (comprising transmembrane helices M2, M4, and M9) is the preferred site. This preference was even more stringent for a superinhibitory PLB variant. Interestingly, PLB-binding specificity became more ambivalent for other SERCA conformers. These results provide evidence for polymorphic PLB interactions with novel sites on M3 and with the outside of the SERCA helix M9. Our findings are compatible with previous physical measurements that suggest that PLB interacts with multiple binding sites, conferring dynamic responsiveness to changing physiological conditions.

This article contains supporting information.

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Simulations of SERCA regulatory interactions

![Figure 1](image)

**Figure 1.** Exploring possible binding sites for phospholamban on different enzymatic states of SERCA. In this study, we examined the interaction of PLB with three different enzymatic states of SERCA. The three states are shown in a: the E1-like state of SERCA bound to PLB (purple) (PDB code 4KYT), the E2 calcium-free state (PDB code 3AR4), and the E1 calcium-bound state (PDB code 1SU4). The canonical inhibitory binding site is formed by helices M2, M6 (blue), and M9 (green). The two postulated alternative sites are binding to the outside of helix M9 (green) and M3 (red). The role of each state in the catalytic cycle of ATP-mediated SERCA transport is shown in b. c shows two methods for exploring PLB interactions with SERCA. Steered molecular dynamics simulations pull PLB away from a postulated binding site and quantitate the unbinding force. In contrast, membrane protein docking tests possible interaction sites and quantitates the binding energy.

and modeling (29) studies indicated that NKA regulatory partners, the FXYD family of proteins, bind to the outside of helix M9 (Fig. 1a, green). Another accessory binding site was suggested by electron crystallography of SERCA and PLB, which involves an interaction of PLB with transmembrane segment M3 of SERCA (Fig. 1a, red) (30–32). Like the canonical site, the putative novel sites undergo significant structural changes during the SERCA transport cycle (Fig. 1b).

These previous studies suggest novel regulatory complex configurations, but higher resolution models are required to reveal the mechanistic significance of these alternative modes of PLB binding. Computational methods offer a promising route to such high-resolution information about protein–protein interactions, especially when paired with experimental data. By virtue of their native environment embedded in a lipid bilayer, membrane protein interactions are among the most difficult to measure, model, and simulate. However, the interaction of SERCA with PLB is an excellent membrane protein complex for evaluation by computational structural biology methods. X-ray crystallography has mapped out nearly every step in the SERCA transport cycle (Fig. 1b), revealing the different conformations of the intermediate enzymatic states (26, 33). Moreover, a structure that reveals key aspects of the SERCA–PLB complex has been determined by X-ray crystallography (Fig. 1a, 4KYT) (12). This breakthrough was enabled by a mutant form of PLB that greatly increased its affinity and inhibitory potency. The degree to which this mutant recapitulates the regulatory complex structure of the WT protein is unknown. In addition, the crystal structure revealed an unexpected E1-like conformation of SERCA bound by PLB. How PLB may interact with other SERCA conformations is another unanswered question.

This wealth of structural information about the SERCA regulatory complex represents an excellent foundation for analysis. However, the complexity of the system creates challenges in capturing a full structural description of the regulatory complex. This structure comprises more than 1,000 residues embedded in a lipid bilayer environment and samples numerous conformational states. Methods for modeling protein–protein interactions are well-established for soluble proteins (34); however, progress for membrane proteins has been delayed because of the paucity of known structures (35) and difficulty accounting for the lipid bilayer. Applicable methods include molecular dynamics (MD), which simulates the time-evolved behavior of a macromolecular system using Newton’s equation of motion. Of the available computational techniques, molecular dynamics most accurately represents the lipid bilayer including atoms in each lipid molecule, but it is computationally expensive, even with enhanced sampling techniques. This limits the number of binding sites and modes of binding that can be evaluated. In contrast, protein docking aims to predict the best binding conformation between two macromolecular partners (36). Recently, a handful of protein–protein docking methods have been adapted to account for the membrane (37–39). Although these methods are faster to compute and can examine both where and how macromolecular partners bind in an unbiased manner, many of the membrane-adapted methods are so far validated only on a small data set. When performed together, molecular dynamics and protein–protein docking can provide complementary information about ideal binding conformations. To benefit from their respective advantages and minimize their limitations, we used a computational approach that integrates both methods. For molecular dynamics, we chose to employ steered molecular dynamics (SMD) (40): a well-established technique for examining protein–protein interactions by inducing receptor–ligand unbinding on an accessible time scale (Fig. 1c, Pulling). Importantly, SMD offers the opportunity to quantify the unbinding force required to extract PLB bound to a candidate site in the context of an all-atom lipid bilayer, with peak force taken as an index of the affinity of PLB for that site. For molecular docking, we used RosettaMPDock: a tool that combines soluble Rosetta protein–protein docking (41) with a membrane protein framework that accounts for the position, orientation, and physical chemistry of the lipid bilayer (37) (Fig. 1c,
Docking). We applied these techniques to model the relative binding of PLB to three conformations of SERCA (Fig. 1a) that represent major intermediates in the calcium transport cycle (Fig. 1b). Together, these techniques suggest where and how PLB binds to the SERCA pump, providing insight into the structural determinants of the regulatory interaction of PLB with different enzymatic states of SERCA.

Results

To test whether PLB may bind to putative alternative sites, we performed steered molecular dynamics and protein–protein docking of PLB to different conformations of SERCA. These conformations are sampled as SERCA progresses through the catalytic cycle (Fig. 1b), binding calcium ions from the cytoplasm and transporting them into the lumen of the sarcoplasmic reticulum. In this study, we examined three key SERCA conformations (Fig. 1a): 1) the calcium-free E1-like state bound by PLB (represented by 4KYT); 2) the calcium-free E2 state (represented by 3AR4) (42), which is characterized by a more open PLB-binding groove compared with 4KYT; and 3) the calcium-bound E1 state (represented by 1SU4) (43). The starting structure of the PLB TM domain was taken from the PLB–SERCA complex 4KYT. The cytoplasmic domain of PLB was not detected in this X-ray structure and was omitted from the SMD simulations. Previous biochemical studies have demonstrated that the transmembrane domain is sufficient for binding and inhibition of SERCA (44).

Steered molecular dynamics simulations

To perform SMD simulations, the SERCA residue Cα atoms positions were first restrained by applying a spring force to each Cα atom, preventing significant deviations from the starting position. Pulling force was applied uniformly to Cα residues of PLB, with force directed away from the center of mass of the candidate site on the SERCA TM domain. Fig. 2a quantifies the force applied to PLB (versus time) for three repeated simulations of PLB interacting with the canonical cleft of 4KYT beginning with the original orientation of the X-ray crystal structure (PDB code 4KYT). As the simulation progressed, the number of interacting residues decreased until the interaction was broken. As pulling progressed, force increased to a maximum and then rapidly declined to a nonzero plateau. The peak force represents the point at which the PLB–SERCA complex ruptured and was quantified as an index of binding affinity. The plateau force was due to viscous drag of the lipid bilayer after the loss of the protein–protein contacts.
For the equilibrated PLB–SERCA crystal structure (4KYT), we determined a peak force of 1,292 ± 69 kJ/mol/nm (error is standard deviation for $n = 3$). The simulation was then repeated for four different PLB orientations, rotating the helix around its long axis by 90°, 180°, and 270°. Simulations of alternative axial orientations showed ~30% lower peak force (Table 1 and Fig. 2, b and c), which is consistent with the native orientation of PLB in the X-ray crystal structure being the most stable configuration. $z$-score analysis is given in Table S1.

### SMD of PLB docked to putative alternative sites

We performed SMD experiments comparing PLB bound to the canonical M6/M9 site with PLB bound to alternative sites to M3 or to the outside of M9, with three repeated trajectories simulated for each orientation (Fig. S1). The average peak force associated with the alternative sites was reduced by ~50% compared with the canonical site. Binding to M3 or the outside of M9 was modestly improved by axial rotation of the PLB transmembrane domain by 270° or 90°, respectively (Table 1 and Fig. 2, b and c), but rupture forces were still lower than those observed for various axial orientations of PLB at the canonical site. The data suggest that PLB binds best to the M6/M9 site in the E1-like conformation captured in the SERCA–PLB crystal structure. When bound to the canonical site, PLB interacts with side chains of SERCA helices M4, M6, and M9. In contrast, when docked to the outside of M9 in the manner of binding of FXYD proteins with M9 of the Na,K-ATPase (28, 29), PLB had interactions with M9 only, which accounts for the lower rupture force of this configuration.

### SMD of different SERCA conformations

Next, we measured the relative binding of PLB to different conformations of SERCA to test how PLB affinity might change as a result of SERCA structural transitions during the catalytic cycle. Repeated SMD simulations revealed that peak rupture force for PLB bound to the canonical binding site (Fig. 2, b and c, and Fig. S2) of the calcium-free E2 state (3AR4) structure was 34% lower than that observed for the E1-like state (4KYT). Interestingly, the rupture force for PLB bound to the hypothetical alternative site outside M9 in a 270° orientation (Fig. 2d) was slightly improved for E2 compared with the binding of PLB to the outside of M9 of the E1-like structure. Thus, because of improved binding to the outside of M9 and worsened binding to the canonical M6/M9 site, the two alternative sites then showed similar maximal rupture forces ($838 \pm 108$ and $848 \pm 29$ kJ/mol/nm, respectively). The calcium-bound E1 structure (1SU4) yielded a peak force that was 33% lower than that observed for E1-like state (Fig. 2e and Fig. S3). E1-Ca also showed a decrease in the rupture force deficit between the M6/M9 site and the proposed site outside M9 compared with the E1-like structure (4KYT). The magnitude of the difference in rupture forces observed for 4KYT (calcium-free) versus 1SU4 (calcium-bound) is in harmony with our previous physical measurements that suggested a 41% difference in apparent binding for the SERCA–PLB complex in low- and high-calcium conditions (18). Fig. 2c reports the $z$ score for each binding site on each SERCA conformation. This value is a statistical measure that conveys how many standard deviations a particular data point lies from the mean. Thus, Fig. 2c shows that the binding of PLB to the M6/M9 canonical site in 4KYT is significantly better than PLB binding to other sites. For other SERCA enzymatic states, M9 becomes a plausible alternative. Thus, we compare the binding values within particular SERCA enzymatic state rather than between enzymatic states.

Overall, the data indicate that there is a clear preference for the E1-like PLB-bound state captured by the crystal structure. However, for other enzymatic states, PLB may bind with similar affinity to the canonical site and the putative site on the outside of M9. This result is broadly compatible with the hypothesis of diverse modes of interaction between PLB and SERCA.

### Membrane protein–protein docking of the SERCA–PLB complex

Our molecular docking strategy involves two steps: 1) a global-docking step to explore possible PLB-binding sites in an unbiased manner and 2) a local docking step to optimize possible SERCA–PLB binding poses in the context of the lipid bilayer. We required two steps because there are currently no global docking strategies that account for the membrane. For step one, we used fast-Fourier transform (FFT)–based global docking (45) to identify possible binding sites on different enzymatic states of SERCA (represented by structures 4KYT, 3AR4, and 1SU4). For each structure, the FFT method identified 30–50 overall docked SERCA–PLB complexes, ranked by an energy function that uses desolvation and electrostatic energy terms, and filtered based on the requirement for PLB to span the membrane in the correct orientation. Using these criteria, we obtained eight SERCA–PLB complexes for the calcium-free E1-like state of SERCA (4KYT) (Fig. 3, a and d), five for the calcium-free E2 state (3AR4) (Fig. 3, b and e), and six for the calcium-bound E1 state (1SU4) (Fig. 3, c and f).

Several docking solutions included PLB bound to the M6/M9 canonical site, while the remaining solutions included PLB docked at the M3 accessory site. This result contrasts with the SMD experiments, which showed no appreciable affinity of PLB for this site. Moreover, whereas the SMD experiments suggested some affinity of PLB for the putative binding site on the outside of helix M9, the unbiased protein–protein docking experiments did not yield M9-docked structures among the most favorable solutions. Additional comparison of the complementary methods is provided under “Discussion.”

### Table 1

| SERCA state                  | Binding location | Mean rupture force (kJ/mol/nm) |
|------------------------------|------------------|--------------------------------|
| E1-like calcium-free (4KYT)  | M3               | 546 ± 53                       |
|                              | M6/M9            | 1,292 ± 69                     |
|                              | M9               | 686 ± 46                       |
| E2 calcium-free (3AR4)       | M3               | 544 ± 20                       |
|                              | M6/M9            | 848 ± 29                       |
|                              | M9               | 658 ± 40                       |
| E1 calcium-bound (1SU4)      | M3               | 606 ± 49                       |
|                              | M6/M9            | 871 ± 25                       |
|                              | M9               | 682 ± 32                       |

*Simulations of SERCA regulatory interactions*

$z$-score analysis is given in Table S1.
Docking of different axial orientations of PLB

After obtaining initial docked SERCA–PLB complexes, we used RosettaMPDock (37) to optimize high-ranking PLB orientations within the membrane environment. RosettaMPDock is an adaptation of the RosettaDock (41) protocol that locally searches for docked structures in two stages. First, the algorithm performs a coarse-grained search with random rigid-body rotations and translations. Then an all-atom stage includes side-chain optimization and minimization along side-chain torsion degrees of freedom. RosettaMPDock uses an implicit model to represent the bilayer, meaning the heterogeneous lipid bilayer is represented as a continuous medium rather than individual “explicit” solvent molecules. In the context of this study, an important consequence of an implicit model is that we cannot capture interactions between the protein and specific lipids. Therefore, any displacement of PLB relative to SERCA is due to changes in the protein conformation and any interchain interactions.

For each of the 19 SERCA–PLB docked complex-starting structures, we generated 5,000 high-resolution candidate structures. The resulting structures were ranked by PLB angle of rotation around its long axis, tilt angle relative to the membrane normal, and the ΔDG of binding to SERCA. Importantly, RosettaMPDock follows the premise that the observed complex is almost always in a low free-energy state (46). Therefore, the resulting complexes represent snapshots rather than a thermodynamic ensemble or dynamics of SERCA–PLB interactions in energy minima. A summary of the strongest interactions of PLB with each structural state of SERCA is provided in Table 2, and the full list of SERCA–PLB interactions is provided in Table S2.

For the E1-like state of SERCA (4KYT), we observed two SERCA–PLB complexes at the M6/M9 site with strong binding affinity with rotational axial angles of 6° and 267°. Both structures exhibit a strong binding funnel (Fig. 4a), meaning that the conformations with the lowest ΔDG of binding are concentrated at a specific PLB axial rotation angle. Further, both z scores indicated strong and specific interactions, with z_all of −4.3 for the 6° PLB orientation and −5.2 for the 267° orientation. These docked complexes are represented in Fig. 4b in red and blue, respectively. The first orientation was closest to the SERCA–PLB crystal structure (4KYT) with an all-atom root-mean-squared deviation between PLB from the
crystal structure and PLB from the model of 0.89 Å (Fig. 4b). Fig. 4c shows a helical wheel representation of PLB, highlighting key residues in the interface with SERCA for the 6° (red) and 267° (blue) orientations and residues that are common to the interfaces of both orientations (orange). The docking results implicating this face of the PLB helix are in harmony with previous biochemical and cross-linking studies (47, 48) and are consistent with the interpretation of the SERCA–PLB co-crystal X-ray structure (12). Global docking also identified a PLB rotational orientation that interacted with the M3 accessory site, although the apparent binding affinity was weaker than those observed for binding to the M6/M9 canonical site (Fig. S5). M3-binding yielded interface energy of −45.2 Rosetta energy units (REU), and both z scores indicated weaker binding (Table 2 and Table S2). Note that the Rosetta energy unit is an arbitrary unit that approximates a kcal mol⁻¹. Overall, the binding affinity for the M6/M9 is only modestly better (21% improvement in REU) than the M3 site, suggesting that M3 is a reasonable alternative interface for PLB.

**Docking of PLB to different enzymatic states of SERCA**

The global docking results were also consistent with a multiplicity of interactions between PLB and other conformations of SERCA. Interestingly, there were two strong binding interactions between PLB and the E2 conformation. The first was PLB binding to M6/M9 at 321° with a binding affinity of −49.3 REU (Fig. S6, a–c), and the second was PLB interacting with the alternative M3 site at 334° with a binding affinity of −45.1 REU (Table 2 and Fig. 5, a and d). Although the gap between binding affinities to the canonical cleft and M3 site was 10 REU for the E1-like–PLB state (4KYT), the gap narrowed in the E2 (3AR4) state to only 4 REU. Further, the interaction z scores were suggestive of a significant interaction with this site.

Finally, we also examined the affinity of PLB for different sites of the calcium-bound structure of SERCA (1SU4). Overall, we found that PLB bound the most tightly to the M6/M9 canonical site for two rotation angles: 6° and 25° (Fig. S6, d–f). Both sites exhibited strong binding funnels with $z_{all}$ scores of −6.1 and −2.8, respectively. Interestingly, the first conformation was the same binding angle as the low energy E1-like M6/M9 structure, suggesting that face of the helix encodes an important sequence for SERCA interaction. PLB also bound to the M3 site at an angle of 280° (Fig. 5, b and e), a similar angle for which moderate binding was predicted for docking to the E1-like structure. These data emphasize the role of M3 as an important site for PLB binding.

**Comparison of WT–PLB and PLB4 binding to SERCA**

To investigate the contribution of specific key side chains to the energetics of the PLB–SERCA regulatory complex, we repeated the protein–protein docking experiments with the quadruple mutant PLB4 (N27A, N30C, L37A, and V49G) that was used to obtain the 4KYT X-ray crystal structure (Table S3). This superinhibitory mutant binds more avidly than the WT sequence (49). Interestingly, PLB4 docking solutions did not include significant binding to the alternative site on SERCA helix M3. At the canonical M6/M9 site, we observed similar docking for WT–PLB and PLB4, with a narrow binding funnel and interface energies ranging between −54 and −57 REU (Table S3 and Fig. 6a), but the interface energy was slightly more favorable for docking of PLB4. In addition, there were fewer favorable orientations for PLB4 (Fig. 6a) than for WT (Fig. 4a). The data suggest that the mutations that enhance PLB binding to SERCA increase the specificity of the interface with regard to the range of favorable axial rotational angles of PLB and the preference for the M6/M9 cleft of SERCA. We noted that the SERCA-binding interface was different from WT, and surprisingly, several of the mutated residues faced the bilayer rather than the SERCA–PLB interface. Only the N27A and V49G mutant residues participated in direct interactions with SERCA (Fig. 6, b and c).

**Discussion**

The goal of this study was to test the hypothesis that PLB may interact with alternative binding sites on SERCA and determine how the population of the alternative sites by PLB may shift with the transporter’s changing structural pose. The motivation for the present computational study comes from consideration of previous physical measurements: 1) SERCA–PLB binding affinity changes with SERCA conformational changes (18), but PLB remains bound to SERCA throughout the enzymatic cycle (18, 21, 22); 2) cryo-EM studies showed...
densities attributable to PLB near SERCA TM helix M3 (31, 50); and 3) X-ray crystallography of an analogous transporter, NKA, showed its cognate regulatory peptide bound to the outside of TM helix M9. We investigated modes of SERCA–PLB interaction through a combined strategy of hypothesis-driven steered molecular dynamics and unbiased protein–protein docking. Overall, the data support the hypothesis of SERCA regulatory complex structural polymorphism, where PLB can bind to multiple binding sites in any of several preferred orientations. Thus, the PLB–SERCA quaternary structure can be considered a “fuzzy complex” (51, 52), characterized by static structural disorder. The selectivity of PLB for the multiple, leniently defined binding sites changes with SERCA conformational transitions during the calcium transport cycle. The results provide new insight into the diverse modes of interaction for the PLB–SERCA regulatory complex.

Structural determinants of the SERCA–PLB regulatory complex

The most important point of comparison for the present results is with the high-resolution structure of SERCA co-crystallized with PLB (4KYT) (12). Although that structure did not resolve the PLB cytoplasmic domain, it did reveal details of an interface between the PLB transmembrane domain and SERCA. PLB was found at the expected location, in the canonical binding cleft comprising SERCA helices 2, 6, and 9. Residues that were identified as important for the PLB–SERCA interaction in that structure and previous biochemical studies (47, 48) also emerged as key elements in the SMD and unbiased

Figure 5. High-resolution models of PLB interaction with the M3 accessory site in the E1 and E2 enzymatic states of SERCA. Molecular docking identified PLB interaction with the M3 helix of the 3AR4 (E2) and 1SU4 (E1-2Ca) enzymatic states of SERCA. a and b show a ranking of PLB orientations by axial rotation and binding energy, with the top 5% scoring points shown in blue and red for 3AR4 and 1SU4, respectively. c, helical wheel diagram showing PLB interface residues: side chains only interacting with 3AR4 are shown in blue, positions only interacting with 1SU4 are shown in red, and mutual positions are shown in orange. d and e show structural models for PLB interactions with 3AR4 and 1SU4, respectively. PLB is highlighted in red or blue, and a 2× zoomed representation of the transmembrane domain is shown to the right of the full SERCA model.

Figure 6. Interactions between SERCA and the PLB4 variant. Protein–protein docking identified interactions between the PLB4 variant (N27A, N30C, L37A, and V49G) and the canonical cleft of the E1-like state of SERCA. a, ranking of PLB4 orientations by axial rotation and binding energy with the top 5% scoring points shown in red, b, structural model of PLB4 interaction with the canonical cleft of 4KYT. PLB4 is highlighted in red with mutation positions colored in light blue. A 2× zoomed representation of the transmembrane domain is shown to the right of the full SERCA model. c, helical wheel diagram showing PLB4 interface residues: side chains only interacting with 3AR4 are shown in blue, positions only interacting with 1SU4 are shown in red, and mutual positions are shown in orange. d and e show structural models for PLB interactions with 3AR4 and 1SU4, respectively. PLB is highlighted in red or blue, and a 2× zoomed representation of the transmembrane domain is shown to the right of the full SERCA model.
protein–protein docking analysis performed here. Key binding residues identified by biochemistry and crystallography include Asn27, Asn34, Leu1, Asn34, Leu35, Ile38, Leu42, Ile45, and Val49 (asterisks indicate residues mutated to create the superninhibitory mutant PLB4) (10). Those residues were the majority of the interface observed by docking SERCA E1-like conformers with the most favorable PLB rotational orientations (Fig. 4c). Those residues also partially overlapped with other PLB–binding interfaces observed from protein–protein docking (Fig. 5c) and SMD. The other major surface of PLB that repeatedly appeared in docking experiments was the upper left quadrant of the helical wheel (Figs. 4c, 5c, and 6c). Engagement of this surface was frequently associated with a significantly different binding position for PLB, suggesting movement of the regulatory peptide through either an upward shift in the membrane (Fig. 5, d and e) or translocation to a completely different alternative site (Fig. 2e). The involvement of residues near the top of the helical wheel was also favored for the E1 calcium-bound conformation of SERCA (1SU4; Fig. 5e), consistent with the concept that SERCA conformational changes alter the nature of the binding of PLB or, alternatively, that different modes of PLB binding are important for different enzymatic states in the SERCA transport cycle. The data may reconcile apparently contradictory studies of the calcium dependence of chemical cross-linking and FRET experiments. PLB–SERCA FRET is maintained in high calcium, suggesting that the complex is intact (18, 21, 22), but cross-linking of PLB is lost in high calcium (13, 14, 48, 53). If the rotational angle and vertical register of PLB changes with the E2–E1 transition, it is not surprising that cross-linking of specific residues is greatly diminished.

In this regard, it is instructive to compare the results of docking of WT PLB to SERCA with docking of the high-affinity, superninhibitory quadruple mutant PLB4. The latter interacts very avidly with SERCA (13) and provided a sufficiently stable regulatory complex to achieve co-crystallization for X-ray studies (12). Plots of the interface energies of PLB rotational orientations show similar landscapes, with PLB4 funnels (Fig. 6a) appearing at similar axial angles as those manifested by WT–PLB (Fig. 4a). However, the WT sequence yields more deep funnels (Fig. 4a and Fig. S4), suggesting that the WT helix has more docking options that are energetically acceptable. Therefore, we hypothesize that PLB mutations that caused tighter binding to SERCA (47) and yielded well-ordered structures for crystallization (12) tailored PLB to bind in a specific orientation to a specific (canonical) binding site of a specific SERCA conformer.

**Alternative modes of binding of PLB to SERCA**

Binding to M3 of SERCA has been observed for both PLB (31) and SLN (54) and is hypothesized to play a role in regulating the maximal activity (V_max) of SERCA (31, 55). Here, protein docking studies identified transmembrane segment M3 in both the E1–2CA and E2 states of SERCA as a potential interaction interface. The docked complexes are most similar to our model for PLB (31) where SERCA residues such as Val269 and Trp272 appear to stabilize the interaction. In the recent model for the SERCA–SLN complex (50), SLN sits directly along M3, although residues such as Val269 are predicted to interact in all cases. Of the docked complexes between SERCA and the WT PLB monomer, one of the solutions (model 6) is similar to the previously described MD simulations of the SERCA–PLB pentamer complex based on electron crystallography (31). The docked complex is not identical to the previous complex, although this is likely due to the PLB pentamer used in the prior docking and MD simulations. In addition, that previous study used the human PLB sequence (in which residue 27 is a lysine), whereas the present docking analysis follows the X-ray crystallography study in using the canine PLB (with an asparagine at position 27). Nonetheless, the PLB monomer in the docked complex (Fig. 5) interacts with M3, but it is located more toward transmembrane segment M1 of SERCA, and it is shifted upward by one turn of the helix. The interaction interface between SERCA and PLB is similar in the two models, with key residues such as Phe32 and Ile45 of PLB and Lys26 and Tyr272 of SERCA contributing. In the docked complex, Asn34 interacts with Glu258. This fits with the previous conclusion that electrostatic interactions, at least in part, draw PLB into this region of SERCA. The negatively charged residues include Asp224 and Glu255 on M3 and additional residues on M1 (e.g. Asp59 and Glu58). Finally, it is interesting to note that the protein docking of the PLB4 variant used in the X-ray crystal structure of the SERCA–PLB complex (12) did not yield a docked complex at the M3 accessory site of SERCA. Only the WT PLB yielded satisfactory docking to M3 of SERCA. As discussed above, the results suggest the mutations increased the selectivity of PLB4 binding to the canonical binding site on M6.

**Complementary approaches for interrogating membrane protein complexes**

Steered molecular dynamics and protein–protein docking provided complementary perspectives in evaluating PLB–SERCA binding interactions. SMD offers an all-atom view of SERCA’s behavior in a biologically realistic membrane, quantifying rupture forces as an index of protein–protein binding affinity. On the other hand, protein–protein docking provides an unbiased strategy. RosettaMPDock, in particular, is one of a few specialized docking methods that can efficiently and inexpensively explore new conformations while considering the physical properties of the surrounding lipid bilayer. Overall, the results from the two types of experiments were in harmony. Most notably, both suggested that the canonical binding cleft is the most favorable site for PLB binding.

On the other hand, the methods provided different insights into the interactions between PLB and alternative sites and the binding of PLB to different SERCA conformers. Both SMD and protein–protein docking simulations suggest that M3 is a plausible alternative site. Docking yielded a favorable PLB orientation at 298° binding with weak-to-moderate affinity (Fig. 5). By SMD, the best M3 binding is seen for the 180° and 90° orientations, yielding a rupture force that was 1 or 2 standard deviations from the mean rupture force, respectively. Interestingly, the 180° orientation showed relatively weak affinity by SMD (1 standard deviation above the mean). This configuration did not appear among high-scoring docking results, suggesting that it
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is not a preferred orientation. In contrast, there was an 80° PLB orientation with weak binding affinity (~35 REU). We regard the 80° value (from docking) and 90° value (from SMD) as representing a similar possible orientation of PLB bound to M3. Another difference between the experiments is that the 298° orientation observed in docking was not tested by SMD, because those experiments systematically evaluated fixed PLB orientations at 90° intervals. The identification of a favorable “in-between” orientation at 298° demonstrates the added value of an unbiased docking strategy for identifying new quaternary arrangements that may be important for regulating SERCA transport function.

Conversely, SMD provided insight into a possible interaction that was not detected by docking. SMD experiments showed that binding to hypothetical site on the outside of M9 was relatively strongly site- and orientation-specific for PLB in the E1-like calcium-free (3AR4) conformation of SERCA. The 180° and 270° orientations of PLB bound to M9 yielded rupture forces that appeared equally favorable to the canonical cleft. However, despite this apparent site selectivity, it was a moderately weak binder overall, as compared with all other interactions. Thus, the site was not ranked highly enough to be discovered by protein–protein docking. On the basis of the SMD experiments, we suspect that the outside of M9 may be populated by PLB for some conformations of SERCA, such as the E2 experiments, we suspect that the outside of M9 may be populated by protein–protein docking. On the basis of the SMD experiments, we suspect that the outside of M9 may be populated by PLB for some conformations of SERCA, such as the E2 calcium-free state represented by 3AR4 (Fig. 2). An analogous interaction is that of the Na,K-ATPase, which binds FXYD proteins on the outside of M9 of the NKA α (catalytic) subunit. This regulatory complex also benefits from the contribution of additional contacts between the FXYD extracellular residues with the NKA β subunit (28), accounting for the apparently stable occupation of that position in the crystal structure. Overall, the disparity between the SMD and docking results underscores the value of complementary approaches for evaluating metastable interactions that may be biologically significant. A possible future step is to use global docking poses as input to SMD to further integrate information from these approaches.

Challenges for modeling of protein–protein interactions in the membrane

In this study, we applied two complementary strategies to investigate a physiologically important integral membrane interaction. This dual strategy enabled the computational feasibility of exploring the wide protein–protein interaction space for a large target (>1,000 residues) while also considering the context of a heterogeneous membrane environment. The specialized membrane protein structural biology methods used in this study to explore membrane protein–protein interactions are still in their infancy. In particular, there are many open questions about the biophysical forces driving protein–protein interactions in the nonpolar membrane environment. With this, these specialized methods require improvement in several areas. For instance, the protein–protein docking program kept the backbone of both SERCA and PLB fixed. However, it is likely that PLB bends or straightens to enhance shape complementarity with its binding partner. This is especially notable because transmembrane membrane helices are notorious for kinks and curvature (56). Programs for incorporating backbone flexibility have emerged for soluble proteins (57, 58), and these developments will soon translate to membrane proteins. Another challenge is incorporating the effect of bilayer deformations induced by the protein that influence the landscape of available protein–protein complex conformations (59). Further, the all-atom membrane in SMD only included one lipid type, whereas biological membranes include hundreds of different lipid types (60). Although building membrane models with complex lipid compositions is computationally expensive because of long equilibration time, improved computing capabilities will make these models within reach.

Implications for our understanding of SERCA–PLB interactions

This study represents a shift for our understanding of regulation of cardiac calcium handling. The reasonable first assumption about the regulatory complex of SERCA with PLB was that there was a simple, specific, lock-and-key mechanism in which PLB occupies a single, stringent binding site on a specific SERCA conformation, assuming a fixed orientation in a well-defined interface. Previous physical measurements have gradually undermined this simplistic model. FRET measurements suggested additional binding interfaces characterized by different binding affinities (18). Additional interfaces were also demonstrated by cryo-EM (31, 50). Moreover, a number of nonspecific hydrophobic peptide sequences were shown to functionally inhibit SERCA (61), indicating a broader specificity than was previously appreciated. The present results may reconcile these previous findings, revealing that PLB can bind SERCA at three alternative sites using several different favorable orientations. The preference among these sites and orientations is determined by the enzymatic poise of the structurally dynamic SERCA pump, which may explain ligand-dependent changes in site-specific chemical cross-linking of PLB to SERCA (13, 62). The structural details revealed by the present study suggest new binding interfaces that could be explored with cross-linking, mutagenesis, or other physical experiments. Finally, the results reveal a possible mechanism for the gain-of-function of PLB4, a superinhibitory mutant of PLB (49).

Based on the present observations, we propose that PLB equilibrates between different modes of binding. Some of these mode changes are minor shifts of PLB within a binding pocket (e.g. rotation about the PLB long axis or normal translations in the bilayer), whereas others involve translocation to completely different sites (e.g. to the outside of M9 or to M3). Based on the increased apparent specificity of the superinhibitory PLB4 mutant for the M6/M9 site, we conclude that the canonical binding mode mediates functional inhibition, and we presume that the other sites are noninhibitory, or possibly even stimulatory (4, 31, 55). Thus, PLB transitions between different structural poses may deliver different functional outcomes as is appropriate for the varying demands of different physiological conditions.
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Materials and methods

Preparation of starting structures

The SERCA–PLB complex was modeled from crystal structures of SERCA1a, the skeletal muscle isoform for which many conformations are available (26). This isoform has high homology to the cardiac-specific calcium pump, SERCA2a, and the recently published first structure of SERCA2a showed that the known PLB-binding site is highly conserved between these isoforms (63). Here we generated models starting from three different SERCA1a structures: 1SU4 (43), representing an E1 calcium-bound state; 3AR4 (42), representing an E2 calcium-free state; and 4KYT (12), representing a calcium-free state bound to PLB. The latter calcium-free X-ray crystal structure was designated an E2 structure, but the conformation resembles an E1 structure and is referred to as “E1-like” in the present study. In that X-ray crystal structural solution, the cytoplasmic domain of PLB was not observed. In addition, 4KYT contains structure of mutated PLB; for experiments that simulate interaction of WT–PLB with SERCA, we mutated the residues back to the native sequence. Each resulting SERCA–PLB structure was energy-minimized. As a measure of quality of M9-bound PLB models, we examine how the resulting complex compared with the NKA–PLM complex. To evaluate the quality of the M3-bound models, we compared the models with the overall structure of the low-resolution cryo-EM structures of PLB–SERCA co-crystals.

Steered molecular dynamics simulations

We examined transmembrane domain residues 22–52 of PLB docked in the canonical binding cleft of 4KYT or the equivalent site on 1SU4 or 3AR4. To test the possibility of PLB binding to the putative alternative sites on SERCA, we created a model of the PLB bound to M9 using as a guide the NKA co-crystal structure with a FXYD protein (28, 29). PLB was also docked to M3 to test a possible interaction hypothesized from cryo-EM studies of SERCA–PLB co-crystals (31, 32). Additional models were generated to represent different relative orientations of PLB with respect to the binding interface on SERCA, rotating PLB around its long axis by 90°, 180°, and 270°.

All-atom MD simulations were carried out with the CHARMM 36 force field (64–66) and TIP3P water model (67). Energy minimization was performed on the crystal structures using the steepest descent method for 1,000 steps, and then each model was embedded into a 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) lipid bilayer and solvated in a rectangular water box size with dimensions 130 Å × 130 Å × 160 Å. Na+ and Cl− ions were added to the solution to neutralize the charge of the system and to produce an ion concentration of 150 mM. The particle mesh Ewald method (68, 69) was used to calculate the long-range electrostatic interactions, and a cutoff of 12 Å was used for the short-range interactions. van der Waals interactions were reduced to 0 by switch truncation applied from 8 to 12 Å. Simulations were carried out with an integration time step of 2 fs. To reach the target temperature (300 K) and pressure (1 bar), the Berendsen method was used with relaxation times of 0.1 ps (70). After 1-ns equilibration, the production run was performed in the NPT ensemble using the Nose–Hoover thermostat (71, 72) and the Parrinello–Rahman barostat (73, 74) with relaxation times of 1.0 ps. The atomic coordinates of the trajectories were saved every 1 ps. The SMD runs were carried out for 5 ns each.

To test the reproducibility of the experiments, three independent SMD runs were performed for each orientation of PLB. Pulling force was applied with a spring constant of 1,000 kJ/mol/nm with a constant pulling velocity of 0.001 nm/ps (75, 76). Increasing the pulling speed increases the rupture force (77) but does not change the relative “stickiness” of candidate-binding sites. Hence, this approach is useful for comparing the relative affinities of different binding modes. For these computationally intensive experiments, we found that a pulling speed of 0.001 nm/ps is an ideal compromise that allows sufficient time for side-chain and lipid motions. Force was applied to backbone atoms of PLB transmembrane residues (Asn27–Ile48), pulling PLB away from the candidate SERCA-binding site along line connecting the center of mass of the SERCA-binding site and the TM region of PLB. The orientations given represent different SERCA–PLB relative orientations, starting with the original orientation of PLB relative to SERCA (denoted here as 0°) and then with PLB rotated around its long axis by 90°, 180°, and 270°. During the SMD run, positions of SERCA Cα atoms were constrained and atoms of PLB were allowed to move only in the x–y direction (in the membrane plane) and not along the z axis (normal to the membrane plane). During SMD runs we recorded pulling force values as a function of time, recording the peak force (rupture force) as an index of the affinity of PLB for the candidate-binding site.

Protein–protein docking simulations

A search for SERCA–PLB interaction sites was performed using global and local docking. Each crystal structure was first refined using RosettaMPRelax (37) to erase artifacts from crystallization and prior binding ligands. To accomplish this, the protocol performs cycles of small backbone torsion moves followed by side-chain repacking and energy minimization of all torsion angles (ϕ, ψ, χ) (78). For each starting structure, 50 PLB orientations were generated, and the lowest-energy conformation was used as the next starting pose. Next, the ClusPro FFT-based rigid-body docking server (45, 79) was used to perform a global search for SERCA–PLB binding sites. Then the following criteria were used to filter solutions from the best-scoring clusters: 1) the conformers are in the correct topological orientation, with both SERCA and PLB N termini on the cytoplasmic side, and 2) the conformers span the membrane.

Following global docking, RosettaMPDock (37) was used to locally search for PLB-binding orientations. RosettaMPDock an adaptation of the RosettaDock (41) minimization protocol that locally searches for docked PLB orientations through two stages: 1) a coarse-grained stage to quickly identify favorable orientations and 2) an all-atom refinement stage that optimizes the rigid body position and side-chain rotamers. To account for the membrane environment, RosettaMPDock samples the protein–membrane orientation and represents the bilayer using an implicit membrane model. An implicit approach represents the solvent as a continuous medium rather than individual
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“explicit” solvent molecules. In this work, we used the Lazaridis implicit membrane model (IMM1/EEF1) (80, 81), which captures the membrane environment as three phases: an isotropic water phase, an isotropic hydrophobic phase, and an anisotropic phase corresponding to the interfacial region. The IMM1 model is 30 Å thick, which is similar to the thickness of the endoplasmic reticulum membrane (60).

For each starting pose, 5,000 PLB orientations were generated. Then the interface analyzer protocol (82) was used to compute two properties: 1) axial angle, the angle between the second principal axis of the 4KYT PLB conformation and the candidate PLB conformation; and 2) ΔΔGbind, the change in Rosetta energy when the chains are separated versus when they are in complex.

z-score analysis

To quantify the significance of each major mode of SERCA–PLB binding, we computed two scores that measure the similarity of binding conformations, with values of 0 indicating that the conformations are identical, and larger values corresponding to greater differences. The first score called zall evaluates the relationship of a particular binding conformation relative to the mean of all possible binding conformations. The second score, called zsite, computes the same quantity but relative to the mean of all structures interacting with a specific site (e.g. M3, M6/M9, or the outside of M9). For docking, the mean was computed from the top 5% of structures ranked by ΔΔG of binding.

Data availability

All data are available upon request. Please contact Prof. Seth L. Robia, srobia@luc.edu.

Author contributions—R. F. A., N. S., and S. L. R. conceptualization; R. F. A. and N. S. data curation; R. F. A., N. S., and J. G. software; R. F. A., N. S., and H. S. Y. formal analysis; R. F. A. and N. S. validation; R. F. A., N. S., and S. L. R. investigation; R. F. A. and N. S. visualization; R. F. A., N. S., and J. G. methodology; R. F. A. and S. L. R. writing-original draft; R. F. A., N. S., H. S. Y., J. J. G., and S. L. R. writing-review and editing; J. J. G. and S. L. R. supervision; J. J. G. and S. L. R. funding acquisition; J. J. G. and S. L. R. project administration.

Funding and additional information—This work was supported by the Loyola University Chicago Cardiovascular Research Institute. R. F. A. was funded by a Hertz Foundation Fellowship and a National Science Foundation Graduate Research Fellowship. J. G. was supported by National Institutes of Health Grant GM-078221. H. S. Y. and S. L. R. were supported by National Institutes of Health Grants HL-092321 and HL-143816. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase; PLB, phospholamban; REU, Rosetta energy unit(s); SMD, steered molecular dynamics; NKA, Na,K-ATPase; FFT, fast-Fourier transform; PDB, Protein Data Bank; TM, transmembrane.

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