A Structural Model of the Complex Formed by Phospholamban and the Calcium Pump of Sarcoplasmic Reticulum Obtained by Molecular Mechanics

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Phospholamban (PLN) is an intrinsic membrane protein of 52 amino acids that modulates the activity of the reticular Ca^{2+} ion pump. We recently solved the three-dimensional structure of chemically synthesized, unphosphorylated, monomeric PLN (C41F) by high-resolution nuclear magnetic resonance spectroscopy in chloroform/methanol. The structure is composed of two α-helical regions connected by a β turn (Type III). We used this structure and the crystallographic structure of the sarcoplasmic reticulum calcium pump (SERCA) recently determined by Toyoshima and co-workers and modeled into its E_2 form by Stokes (1KJU) or by Toyoshima (1FQU). We applied restrained and unrestrained energy optimizations and used the AMBER molecular mechanics force field to model the complex formed between PLN and the pump. The results indicate that transmembrane helix 6 (M6) of the SERCA pump is energetically favored, with respect to the other transmembrane helices, as the PLN binding partner within the membrane and is the only one of these helices that also permits contact between the N-terminal residues of PLN and the critical cytosolic binding loop region of the pump. This result is in agreement with published biochemical data and with the predictions of previous mutagenesis work on the membrane sector of the pump. The model reveals that PLN does not span the entire width of the membrane, that is, its hydrophobic C-terminal end is located near the center of the transmembrane region of the SERCA pump. The model also shows that interaction with M6 is stabilized by additional contacts made by PLN to M4. The contact between the N-terminal portion of PLN and the pump is stabilized by a number of salt and hydrogen-bond bridges, which may be abolished by phosphorylation of PLN. The contacts between the cytosolic portions of PLN and the pump are only observed in the E_2 conformation of the pump. Our model of the complex also offers a plausible structural explanation for the preference of protein kinase A for phosphorylation of Ser16 of PLN.

KEYWORDS:

enzyme models · molecular modeling · noncovalent interactions · protein kinases · protein structures

Introduction

Free Ca^{2+} ions in the myoplasm control the contraction and relaxation of muscles. The sarcoplasmic reticulum (SR) calcium pump (SERCA), a 110-kDa protein that belongs to the family of P-type adenosine triphosphatases (ATPases),[1] removes Ca^{2+} ions from the myoplasm and works in association with a plasma membrane Na^+/Ca^{2+} ion exchanger and Ca^{2+}-releasing channels in the SR membrane to maintain the appropriate calcium level in the cell. In cardiac muscles the activity of the Ca^{2+} pump is modulated by β-adrenergic agonists, which regulate contractile force and muscle relaxation.[2] These effects are mediated by the phosphorylation of a small amphipathic SR protein called phospholamban (PLN) by two kinases.[3, 4] PLN is an intrinsic membrane protein of 52 amino acids that interacts with the cardiac, slow-twitch, and smooth muscle isoforms of the SERCA pump and keeps them in an inhibited state. Phosphorylation of Ser16 by the cAMP-dependent protein kinase (PKA; AMP = adenosine monophosphate),[3] or of Thr17 by a calmodulin-dependent kinase,[4, 5] or of both these residues, causes PLN dissociation from the ATPase and thereby relieves the inhibition.

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Cross-linking experiments by James et al.[6] with a photoaffinity labeling probe showed that Lys3 in the cytoplasmic domain of PLN binds to a region of the Ca^{2+} pump (residues 397 – 402) just downstream of its phosphorylation site (Asp351). The efficiency of cross-linking was significantly reduced by Ca^{2+} ions or when PLN was phosphorylated. These results indicated that inhibition is brought about by electrostatic interactions that involve basic residues in the N-terminal, cytosolic domain of PLN and acidic residues near the active site of the pump, that is, the ATP binding site and the phosphorylated aspartate. Alternatively, Ca^{2+} binding could induce structural changes of SERCA in its PLN binding domain and phosphorylation of PLN could result in destabilization of its cytosolic helix. This view was corroborated by mutagenesis experiments.[7] These experiments confirmed that the sequence 397KDDKPV402 next to the phosphorylation domain of the pump was critical for interaction with PLN, which stresses the importance of charged side chains in the interaction of the two proteins. Mutation of charged amino acids in the N-terminal portion of PLN, for example, Glu2, Arg9, or Arg14, to Ala further emphasized the importance of polar interactions between the two proteins.[8] In addition, recent mutational screening experiments[9, 10] pointed to the importance of hydrophobic interactions between the transmembrane domains of PLN (domain II) and helix 6 (M6) of the Ca^{2+} pump.

PLN can exist in the membrane in monomeric and in pentameric forms.[11] The detection of pentameric forms in isolated PLN preparations led to the suggestion that pentameric PLN could form a calcium channel in an artificial phospholipid bilayer system, which implies that PLN could mediate Ca^{2+} leakage from SR[12–15]. However, Shannon et al.[16] reported no differences in Ca^{2+} leakage from the SR when control mice were compared to PLN knockout mice or to mice transgenic for the monomer-forming mutant C41F, which suggests that there is no significant role for a PLN-mediated Ca^{2+} leak through a pentameric PLN channel. This conclusion has been validated by recent reports, which indicate that the active form of the protein is monomeric.[9, 17] We recently solved the structure of the monomeric form of synthetic PLN (C41F) in chloroform/methanol by two-dimensional homonuclear NMR spectroscopy.[18] The results showed that the protein is composed of two helical portions spanning residues 4 – 16 and 21 – 49, connected by a short β turn (Type III), which includes one of the two phosphorylation sites (Thr17). This structure is in agreement with earlier studies that used either circular dichroism spectroscopy[19] or NMR spectroscopy on portions of PLN.[20, 21] These studies provided evidence that PLN has both α-helical and β-sheet (or random coil) components and is not exclusively α helical, as recently suggested by Smith et al.[22] The flexibility of the β turn would enable PLN to interact simultaneously with helix M6 of the SERCA pump and with a critical cytoplasmic portion of the pump (Lys397 – Val402).[7]

The detailed mechanism by which PLN interacts with the SERCA pump to modulate its activity is not yet understood. To obtain structural details on the interaction between the two proteins we used their 3D structures to model their complex by application of energy minimization calculations in vacuo with the AMBER force field.[23] We initially used the sites of interaction between the two proteins suggested by mutational experiments as constraints. The results provide evidence that transmembrane helix M6 of the pump is indeed energetically favored as the binding partner for the intramembrane C-terminal helix of PLN, which still permits contact between the N-terminal residues of PLN and the critical cytosolic loop of the pump (Lys397-Asn-Asp-Lys-Pro-Ile402 in SERCA1 of skeletal muscle, Lys397-Asp-Asp-Lys-Pro-Val402 in SERCA2 of the cardiac or slow twitch muscle isoform). Thus, the results offer compelling support to the conclusions and predictions made from the binding and mutagenesis data, and provide a structural model for further studies.

Results

The interaction energies obtained by energy optimization of the molecular complexes of PLN and SERCA carried out by using molecular mechanics with the parameterization of the AMBER force field[23] (see the Materials and Methods section) are given in Table 1. The E_2 conformation of SERCA as modeled by Stokes (1KJU)[24, 25] was taken as the structural model. Energy minimization of PLN at various sites on the E_2 conformation of SERCA showed that helix M6 is the most favorable partner for binding of the intramembrane domain of PLN and binding gives a complex that also retains the cytosolic interaction with the loop region of the pump (see Figure 1). As a result of these calculations we observed that the C-terminal portion of PLN does not cross the membrane completely, but is fixed in the center of the transmembrane region in order to accommodate the interaction with helix M6 of the SERCA pump and, simultaneously, to bridge the distance of more than 60 Å to the critical loop region of SERCA around Lys400. This rather surprising result of our calculations is plausible when one considers that the C-terminal sequence of PLN contains exclusively hydrophobic amino acids and is discussed in detail in the Discussion section.

Table 1. *Comparison of structures obtained from the energy optimization of phospholamban (PLN) when its transmembrane region is in contact with the respective transmembrane helices of the SERCA pump.*[a]

helix	$E_{interaction}$	E_{intra}	E_{estat}	E_{vdW}	E_{Hbond}
M1[b]	− 3278.6	+ 1073.6	− 3567.2	− 729.3	− 93.3
M2[b]	− 3999.5	+ 727.6	− 3900.7	− 730.1	− 96.2
M3[b]	− 3948.4	+ 564.8	− 3672.3	− 750.6	− 90.4
M5[b]	− 4402.4	− 688.7	− 4323.7	− 742.2	− 138.1
M6[b]	− 4279.0	− 669.0	− 4018.3	− 810.9	− 118.8
M6[c]	− 4320.4	− 234.7	− 4161.4	− 282.8	− 110.9
M6[d]	− 4287.8	− 636.4	− 4070.6	− 752.3	− 101.7
M8[b]	$\gg 0$	$\gg 0$	− 3448.5	$\gg 0$	> 0
M9[b]	− 4120.0	− 582.8	− 3804.9	− 788.7	− 109.2

[a] The interaction energy ($E_{interaction}$) is given in kJ mol^{-1} and comprises the internal bonded energy terms of PLN (E_{intra}) and the nonbonded energies, namely electrostatic (E_{estat}), van der Waals (E_{vdW}), and hydrogen bonding (E_{Hbond}), within PLN and between PLN and SERCA. [b] SERCA model of D. Stokes[24] (PDB entry 1KJU).[25] [c] SERCA model of Toyoshima (PDB entry 1FQU).[30] [d] SERCA model of Toyoshima (PDB entry 1EUL).[26]

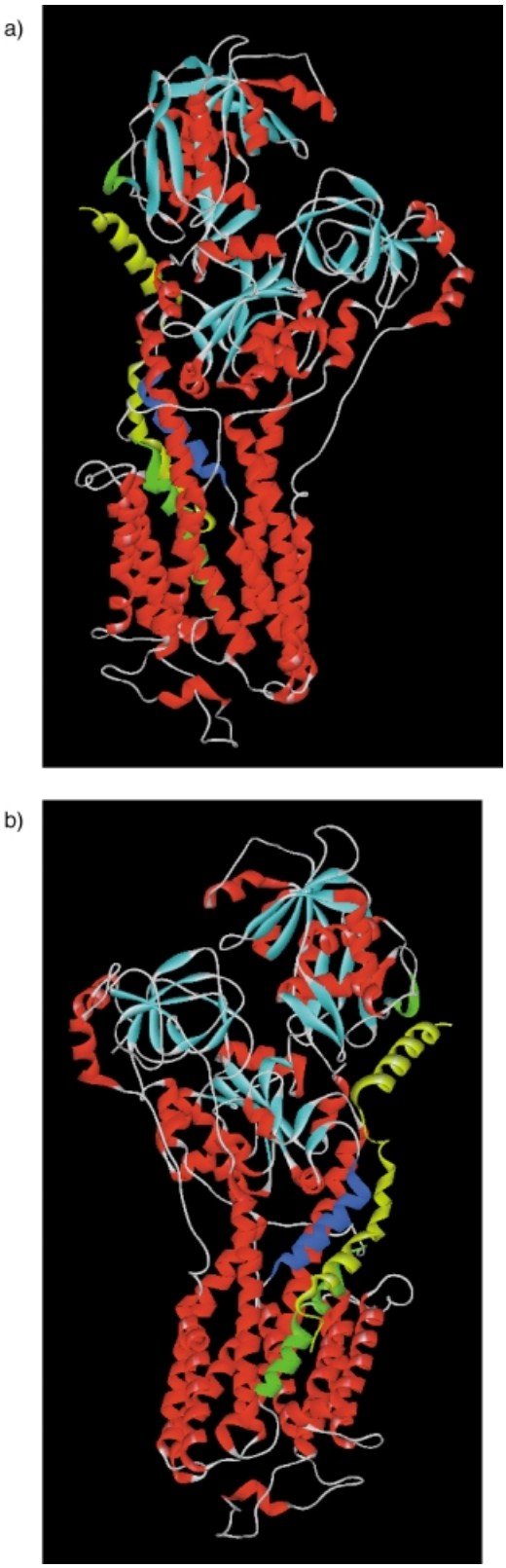

Figure 1. *AMBER force field optimization of a complex of PLN (yellow) with SERCA indicated helix M6 (green) of SERCA in the E_2 form to be the most favorable interaction site in the transmembrane part of the protein. a) Common orientation of SERCA according to Toyoshima;[26] b) changed orientation rotated by 180° around a vertical axis. The part of the M4 helix facing the cytosol, which also provides contacts to PLN, is colored dark blue. Lys397–Ile402 are also colored green.*

Close inspection of the interaction between PLN and M6 showed that the hydrophobic C-terminal residues of PLN are located within the membrane and generate the following contacts: Leu802 (M6) to Phe41 (of PLN), Leu797 to Ile47 and Leu51. An additional contact not included in the initially imposed constraints between residues of PLN and helix M6 of SERCA (see the Methods section) is established between Thr805 (M6) and Leu37. Contacts of PLN with Val795 and Val798 were, however, not observed due to the conformations of these side chains in Stokes' E_2 model[24, 25] as well as in the crystallographic structure of the E_1 form (PDB entry 1EUL);[26] the side chain of Val798 is turned sideward, and that of Val795 points inwards towards the pump itself. The situation for Phe809 is similar as its side chain is mostly shielded by the neighboring transmembrane helices of SERCA. Thus, no corresponding contacts to residues of PLN are possible. These observations partially disagree with the model proposed by Asahi et al.,[10] who suggested that residues Val795, Leu802, T805, and Phe809 are located on the side of the M6 helix facing the transmembrane helix of PLN. Attempts to prove this model correct by cross-linking experiments failed,[10] therefore it cannot be excluded that some of the mutational experiments that led to the proposed model could be interpreted differently. The N-terminal residues of domain II of PLN, if docked to M6, also made contacts to M4, for example, Phe32 and Ile33 made contacts to Ala320 and Leu321, and Leu37 and Ile40 to Thr317. Apparently, the interaction between the C-terminal helix of PLN (domain II) and helices M4 and M6 of the pump occurs throughout the length of the helices. This result concurs with a narrower winding of the PLN helix between Asn27 and Ile33, which shows an $n+3$ hydrogen bonding pattern. Toyoshima et al. reported that M4 and M6, which participate in the formation of a high-affinity Ca^{2+} binding site, are separated by a loop region into a cytosolic and a luminal domain.[26] Interestingly, cross-linking experiments by Rice et al.[27] under conditions that favor the E_2 conformation of the pump provided evidence for contacts between a number of residues of M4 and M6, which in our model are also in contact with PLN. This is remarkable, as there are several hydrophobic residues located at the top of helix M4 that stick out of the transmembrane region, for example, Leu321 and Ala320. These residues are, however, not exposed to solvent in our model because of their contacts with PLN.

After completion of our calculations, Jones et al.[28] reported cross-linking experiments to study the direct interaction between PLN and the E_2 onformation of SERCA in which Asn30 of PLN was replaced by a cysteine residue. The authors provided evidence that the only residue of SERCA cross-linked to Cys30 of PLN was the native Cys318 of the SERCA pump. These results are compatible with our model calculations since the linking agent 1,6-bismaleimidohexane used by Jones et al. is able to span a distance of around 10 Å between the side chains of the two cysteine residues, which corresponds to a distance of up to 14.7 Å between the C_α atoms of the two linked cysteine residues. In our model we determined a distance of 14.2 Å between the C_α atom of Asn30 of PLN and that of Cys318 in SERCA, which is in very good agreement with the experimental constraints reported by Jones et al.[28] In the complex in which PLN is docked to

helix M6 (and stabilized by M4) all energy terms are minimal when compared to all other investigated complexes, except the complex in which PLN was docked to helix M5. In the complex with M5, PLN adopts a totally unfavorable conformation (see below). All other structures have significantly higher energies, in particular with respect to the electrostatic and van der Waals terms. These structures are also unfavorable as a result of geometric requirements; the Lys400 – Asp2 contact can only be maintained without significant structural changes of PLN when its intramembrane region is in contact with M6 and M4. Docking of PLN to the exposed M2 helix resulted in unwinding of the cytosolic helix to cope with the increased distance to SERCA. Conversely, docking of PLN to the M9 helix conserves the PLN cytosolic helix only if the Asp2 – Lys400 contact is lost (Figure 2 a) and PLN adopts a helical shape over its entire length. This effect was also observed when PLN was docked to the M3 helix at the opposite side of the ATPase. Docking of PLN to helix M5 resulted in a favorable energy, but displaced PLN out of the membrane region, which resulted in a random coiled shape (Figure 2 b). This effect was caused by favorable electrostatic interactions that result from the compact conformation adopted by PLN, which includes the formation of non-natural, internal salt bridges between the side chains of Arg14 and Glu19, and between Lys3 and the carboxylate group of the terminal Leu52. Random coil conformations were also observed when trying to dock PLN to helix M1.

Docking of PLN to helices M7 or M10 was not investigated since these helices do not contain residues that affect the PLN-dependent properties of the SERCA pump upon mutation. Furthermore, their distance from PLN would not permit interaction between Asp2 of PLN and Lys400 of the pump. A recent publication by Asahi et al.[29] described an interaction between domain IB of PLN, that is residues 21 – 30, and residues in the loop region of the SERCA pump that connects transmembrane helices M6 and M7. This interaction is discussed in detail below. Docking of PLN to helix M8 yielded significantly higher energies for all individual energy terms than for the other complexes (see Table 1).

The Toyoshima research group also proposed a model (1FQU) for the E_2 form of SERCA.[30] In order to apply this structure to model a 1:1 complex of SERCA with PLN, the structural similarity between the two E_2 conformations, 1KJU[24, 25] produced by Stokes and 1FQU[30] by Toyoshima, was analyzed. The root mean square (RMS) deviation between the two structures was calculated by using all backbone atoms of the residues in the sequences 86 – 115 (M2), 291 – 315 (M4), 788 – 810 (M6), and 931 – 959 (M9) of the transmembrane region of SERCA. These sequences are either proposed as PLN binding sites or are adjacent to such sites. While these regions are structurally very similar in both models, the overall RMS deviation of all heavy atoms is 3.8 Å. This deviation is mainly caused by the elongated shape of the cytosolic loop region of the 1FQU structure as compared to the Stokes model, 1KJU.

Optimization of a model of PLN docked to the M6 region of 1FQU shows that the PLN cytosolic helix can tolerate displacement in the loop region of SERCA. The transmembrane helix is almost unchanged, while the coil region (Ile18 – Gln22) is somewhat different when compared to the optimization carried out with Stokes' coordinates. The hydrogen bond interactions between PLN and the SERCA pump are listed in Table 2 for both models. Most interactions are formed between the same

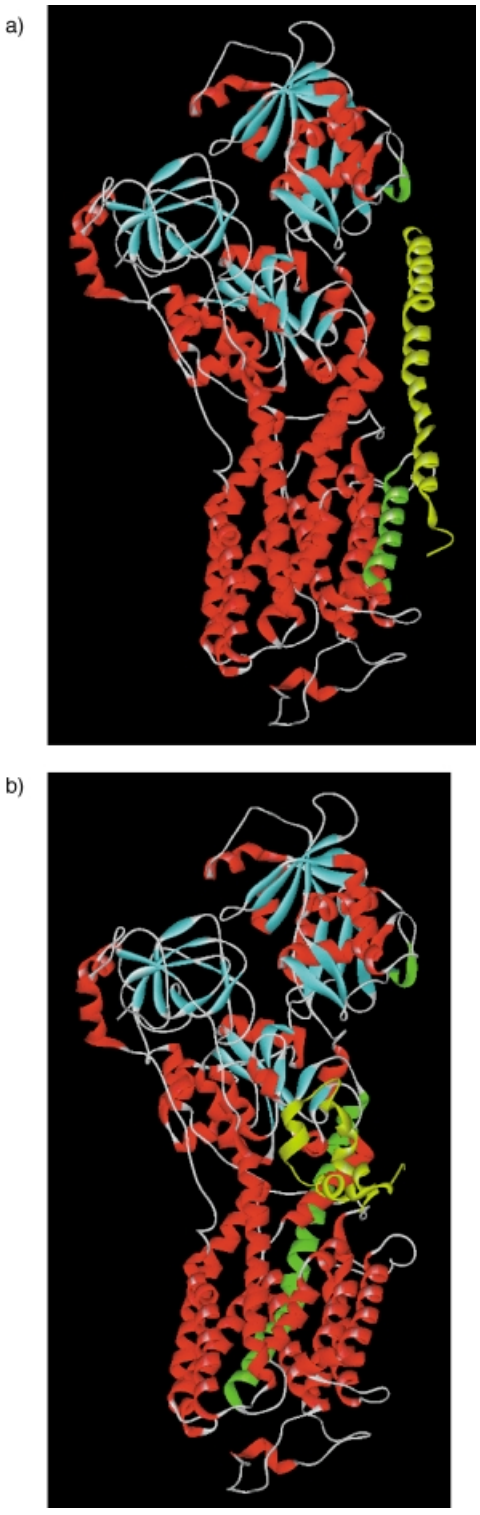

Figure 2. *a) Docking of PLN (yellow) to helix M9 (green) of SERCA resulted in loss of contact to the cytosolic loop (green). b) Optimization near helix M5 (green) yielded a random-coiled structure of PLN.*

Table 2. *Hydrogen bonding interactions between phospholamban and two models of the E_2 form of the SERCA pump in the cytosolic region.*[a]

SERCA Stokes[b]	Phospholamban		SERCA Toyoshima[c]
Leu462-O	Arg9-NH1	Arg9-NH2	Leu462-O
Glu392-OE2	Ser10-OG	Tyr6-OH	Glu392-OE1
Lys464-NZ	Ser10-OG	Ser10-OG	Lys464-NZ
Glu432-OE2	Arg13-NH2	Arg13-NH1	Glu429-O
Glu432-O	Arg14-NH2	Arg14-NE	Glu432-O
		Arg14-NH2	Lys431-O
Ala331-O	Gln22-NE2	Gln22-NE2	Ala327-O
		Gln22-NE2	Lys329-O
Arg324-NH1	Gln29-OE1	Gln29-O	Arg324-NH1
Arg324-NH2	Gln29-O		

[a] The interaction data resulted from force field energy minimization when the transmembrane helix of phospholamban is modeled in contact with helix M6 of SERCA. Those contacts that are within hydrogen bonding distance between the corresponding heavy atoms (3.5 Å) are listed. The atoms are named according to the nomenclature used in the PDB files. [b] Coordinates from D. Stokes[24] (PDB entry 1KJU).[25] [c] Coordinates from Toyoshima (PDB entry 1FQU).[30]

residues of PLN and SERCA, while the exact binding partners (backbone or side chain atoms) differ in some cases. The differences in the hydrogen bonding pattern of the cytosolic portion of SERCA are caused by the different side-chain conformations of the two SERCA models.

The formation of a salt bridge between Lys400 and Asp2 of PLN was initially hampered since all atoms of the SERCA model were kept frozen during the optimization, which caused the side chain of Lys400 to remain oriented in an unfavorable direction, pointing away from PLN. In order to permit the formation of such a bridge in subsequent optimizations, the side chain of Lys400 was allowed to move freely in both structural models. This resulted in the formation of a salt bridge in both cases (see Figure 3). The elongated portion of the pump in the model by Toyoshima (1FQU) is reflected by a longer salt bridge than with the Stokes model (4.4 Å between the nitrogen atom of the Lys400 side chain and the carboxylic oxygen atom of Asp2, as compared to 2.6 Å with Stokes' model).

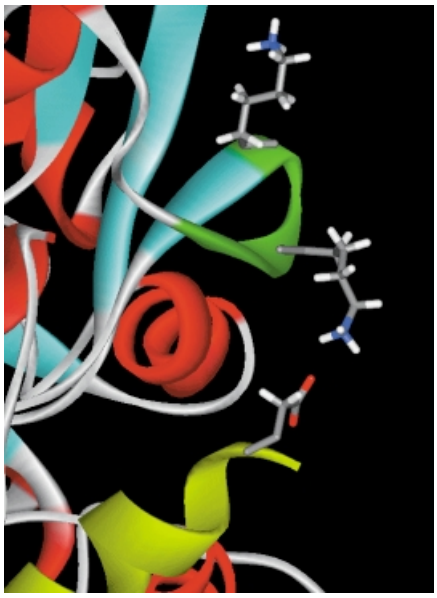

Figure 3. *A close-up view of the salt bridge between the side chains of Lys400 (SERCA) and Asp2 (PLN) in the M6 complex shown in Figure 1. The side chain of Lys397 points upwards, away from Asp2.*

Docking of PLN to the E_1 conformation of the SERCA pump (1EUL)[26] was also investigated by using either the X-PLOR[31] or AMBER force field[23] (see the Methods section for details). In both cases, the intramembrane helix of PLN bound to the M6 helix. The intramembrane helices of PLN are also very similar in shape and position relative to M6 for both the E_2 and E_1 conformation of the ATPase (AMBER results). However, the contacts between PLN and the SERCA pump described above for the cytosolic region were either not observed (AMBER force field calculations; see Figure 4) or could only be observed by distortion of the C-terminal domain of PLN from the ideal structure of an α helix (simulated annealing; see Figure 4), which caused partial loss of contact to M6. This result indicates that only the E_2 conformation of the pump permits the necessary simultaneous contacts between the two critical regions of the two proteins, which is in agreement with similar conclusions drawn by Asahi et al.[29]

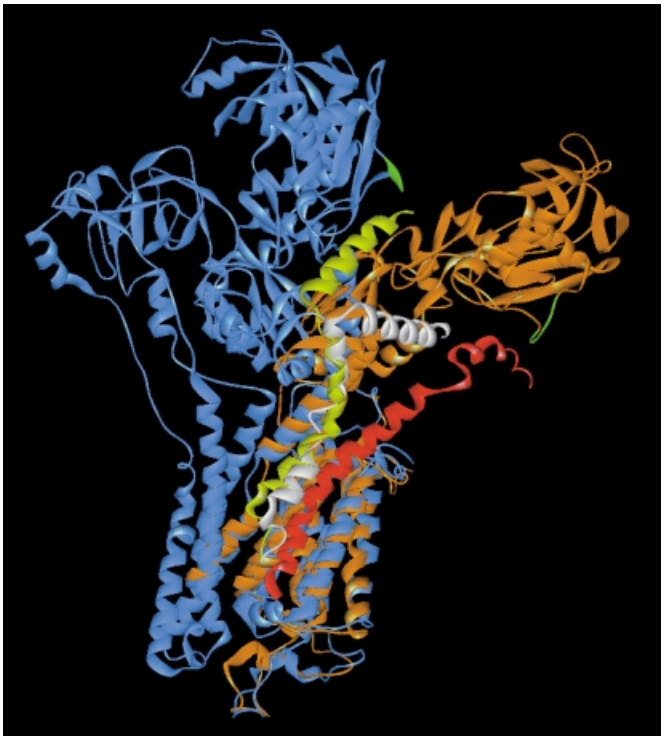

Figure 4. *Comparison of the results obtained from docking of PLN to the E_2 (blue) and E_1 (orange) forms of SERCA. Contact to the cytosolic loop (green) is only retained in the simulated annealing simulation at the expense of distortion of the helical shape of PLN (red). In the AMBER force field calculations, PLN is in contact with helix M6 in the E_2 (yellow) and the E_1 (white) form of SERCA, but fails to contact the cytosolic loop (green) in the E_1 conformation.*

Discussion

The model presented here has led to several interesting findings and suggestions/conclusions. 1) Helix M6 (in combination with M4) of the SERCA pump is energetically the most favorable binding partner for PLN. The interaction between the C-terminal

helix of PLN (domain II) and helices M4 and M6 of the pump occurs throughout their length; the extensive interaction between hydrophobic residues of the N-terminal part of PLN domain II and various residues of M4 and M6 as described above, prevent the exposure of hydrophobic residues of PLN domain II to the aqueous solvent. 2) The amino acid C-terminal residues of PLN, which are all hydrophobic, are located within the membrane, that is, PLN does not traverse the membrane completely. This finding is significant since transmembrane domains are normally fixed to both sides of the membrane by polar residues, which are missing at the C-terminal end of phospholamban. In this context, it is interesting to note that sarcolipin, the phospholamban-analogous modulator protein of skeletal muscle sarcoplasmic reticulum,[32] contains polar residues on both sides of the transmembrane region. We applied the program developed by the group of von Heijne[33] to predict transmembrane topology of given protein sequences in phospholamban and sarcolipin. The results indicated that sarcolipin traverses the full width of the membrane, in contrast to phospholamban, which ends within the membrane. This outcome is in agreement with recent structural studies of sarcolipin in a lipid environment.[34] 3) The anchoring of PLN within the membrane without crossing its entire width, spans the distance between the N-terminal end residues (for example, Asp2, see below) of PLN and the binding loop Lys397 – Ile402 of the SERCA pump (the distance between Phe809 of M6 at the cytosolic surface of the membrane and Lys400 is about 45 – 50 Å). 4) Hydrophobic residues in the N-terminal portion of domain II of PLN are in contact with hydrophobic residues of the cytosolic domain of helix M4, which protects the PLN residues from solvent exposure. This may contribute to the stabilization of the intramembrane location of PLN, but may also couple the functions of M4 and M6 in a cooperative manner, since their intramembrane polar residues contribute to the formation of the two high-affinity Ca^{2+} binding sites. This view is corroborated by the observation that the orientations of the side chains of Glu309 (M4), Gln796, and Asp800 (M6), which are stabilized by a network of hydrogen bonds in the E_1 conformation of the pump,[26] are significantly changed in the E_2 conformation and may thus be controlled by the coupled interaction of PLN with M4 and M6. Interaction between PLN and the M4 and M6 helices of the SERCA pump may also lead to stabilization of these helices, which would result in restriction of the dynamics of M4 and M6 and have an important effect on Ca^{2+} binding to the high-affinity sites, as suggested by Tatulian et al.[35] 5) The simultaneous interaction of PLN with transmembrane regions and the cytosolic loop region Lys397 – Ile402 of the pump is only possible if the pump is in the E_2 conformation. 6) The model offers a plausible structural explanation for the preference of PKA for Ser16 of PLN (see below).

Although the results of our calculations are in excellent agreement with published data on the interaction between PLN and the SERCA pump, a caveat is appropriate. The "complex" formed between PLN and SERCA presented here is a model based on molecular mechanics calculations and we are aware that such a model has limitations. On the other hand, our model is supported by the available biochemical data on the interaction between the two proteins (including some recent cross-linking studies by Jones et al.[28]). This model may thus stimulate discussion on the molecular mechanism by which PLN regulates the SERCA pump and may lead to further experiments since a high resolution structure of a PLN/SERCA complex is not yet available.

The model presented here is based on the assumption that a 1:1 complex is formed between the PLN monomer and the SERCA pump. This assumption is compellingly supported by a number of studies in which the formation of PLN pentamers was prevented without loss of inhibitory activity (Kimura et al.,[9] Asahi et al.,[10] Autry et al.;[17] see also, Young et al.[36]). At variance with this result, a recent study in which PLN was cocrystallized with the SERCA pump[37] instead interpreted the cryoelectron microscopy low-resolution structure made by using difference mapping as a 2:1 pump/PLN complex. Although the study failed to identify the transmembrane helix of PLN involved, it suggested that PLN enters the membrane close to the M3 helix of the pump. According to the model presented here, this process would not permit contact of the N-terminal portion of PLN with the Lys397-Asn-Asp-Lys-Pro-Ile402 loop region of the pump.

The calculated interaction energies for the various SERCA – PLN complexes show a striking preference for interaction of the intramembrane portion of PLN with the M6 helix of SERCA (stabilized by further contacts to M4), which is in agreement with mutational data that suggested a leucine-zipper-like interaction in this region. Since there are no polar contacts within this intramembrane segment, this interaction energy reflects mainly the favorable van der Waals energy of the M6 complex. Conversely, the electrostatic and hydrogen bonding energies of the polar residues in the cytosolic portion of PLN are the significant terms that favor the M6 complex for stabilization of the interaction between the cytosolic portions of the two proteins. In support of this view, the helical structure of the cytosolic portion of PLN was only preserved in its full length when PLN was docked to M6. This observation indicates that among the transmembrane helices M4, M5, M6, and M8, that contribute to the formation of high-affinity Ca^{2+} binding sites, only M6 provided optimal interaction with PLN (supported by simultaneous contacts to the domain of helix M4 that faces the cytosol). Even when docked to the adjacent helices M2 and M9, PLN had to unfold part of the cytosolic helix to retain both transmembrane and Asp2 – Lys400 contacts. Unfolding was even more pronounced for the other complexes, and was reflected in high energy terms in comparison with the M6 complex. Accordingly, the hydrogen bond interactions were affected by the choice of helix as well.

Similar results to those described above were obtained for the E_2 structural model of Toyoshima (1FQU),[30] despite the elongated shape of the cytosolic loop region in the E_2 conformation; the optimized PLN is evidently able to adapt to these domain movements. The differences between the two SERCA models seem to be less important for the stability of the cytoplasmic helix of PLN than the relative docking position of PLN in the transmembrane region of the SERCA pump.

Toyofuku et al.[8] argue that the negatively charged amino acid in position 2 of PLN is of special importance for interaction with

the pump. There is an aspartate residue in this position in our modeled structure of the PLN/SERCA pump complex since the chemical synthesis of PLN[38] used to obtain the three-dimensional structure was based on the sequence of canine PLN.[39] It has meanwhile become known that, with the exception of the dog and pig sequences,[39, 40] all known PLN sequences contain a glutamate residue at position 2. Interestingly, the length of the proposed salt bridge between Asp2 of PLN and Lys400 of the SERCA pump is somewhat greater than that of an ideal salt bridge. A glutamate residue in position 2, however, could compensate for this distance with its additional CH_2 group (see Figure 3). In contrast, contact between Asp2 and Lys397 in the cytosolic PLN binding loop is less likely than with Lys400, since Lys397 is located on the far end of this loop (see Figure 3), too distant for formation of a stable salt bridge with Asp2.

Mutation-sensitive hydrogen bond contacts made by Arg9, Arg13, and Arg14 with the SERCA pump[8] were found in both models. Arg9 forms a hydrogen bond to an oxygen atom in the peptide backbone (Leu462) while Arg13/14 form contacts to Glu432 of the pump in both structures and, in addition, to Glu429 in the complex with the 1FQU structure. Further mutational experiments (mutation of Glu432 to Ala and/or of Glu429 to Ala) would establish whether those residues are involved in PLN/SERCA interactions. Such interactions were not found when PLN was docked to the pump in the E_1 conformation,[26] where a significant portion of the cytosolic helix of PLN (residues 12 – 24) is outside van der Waals interaction distance of the PLN-binding cytosolic loop of the SERCA pump. Likewise, an Asp2 – Lys400 salt bridge was not detected with the E_1 model, which is in line with the observation that PLN only inhibits the SERCA pump in its E_2 conformation. According to the model presented here, interaction of the transmembrane portion of PLN would be possible for both conformations of the pump. This outcome is in agreement with reports by Xu et al.[25] and Hua et al.[30] that the major differences between the E_1 and E_2 conformations of the pump concern its cytoplasmic domains, especially the movements of the N and P domains, which result in a displacement of up to 50 Å. In contrast, the transmembrane domain movements between the two conformations are confined to a few Angstroms.[25] Thus, dissociation of the transmembrane portions of the two proteins does not appear to be necessary for the reaction cycle of the pump.[29]

In a recent paper, Asahi et al.[29] suggested putative interaction sites between PLN and residues of the cytoplasmic pump loop that protrudes between helices M6 and M7 (L67) of SERCA. The computational results of the present study show longer extension of the intramembrane helix of PLN than in Asahi's study so that the interaction of Asn27 or Asn30 with Asp813 of the pump would require unfolding and/or bending of the intramembrane helix in this region.[29] In the present model, however, the nearest neighbors of Asn27 and Asn30 are Lys328 and Arg324, respectively, and the helical shape in this region of PLN is conserved. This information could be of interest since residues Arg324 and Lys328 are positioned between the charged residues of helix M4, which participate in the formation of the high-affinity calcium binding site, and Asp351, which is phosphorylated during the reaction cycle and extends the cooperative coupling of PLN, as discussed. The distances between Asn810 and Arg822 and the corresponding residues of PLN (Asn27 and Asn30)[29] are larger than 4 Å. Introduction of apolar residues (that is, alanine residues) into the region between residues 21 and 30 might thus cause at least partial unfolding, permitted by the increased flexibility of PLN. This would allow further contacts with the pump and eventually lead to enhanced interaction.

The absence of a membrane and of a solvent during optimizations and energy calculations implies descreening of

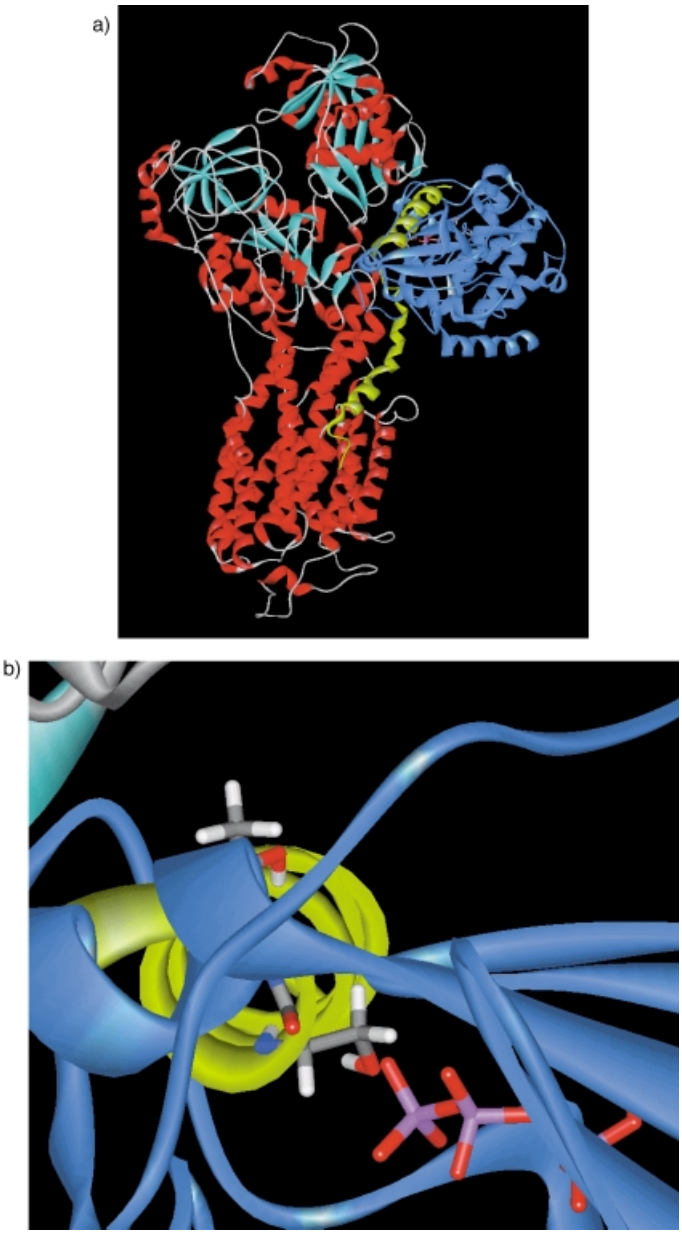

Figure 5. *a) Putative site of interaction of the catalyic subunit of PKA (shown in dark blue; PDB file 1ATP[43]) with Ser16 of PLN (shown in yellow) from our model complex with PLN optimized onto the M6 helix of SERCA in its E_2 conformation. b) A close-up view of the putative interaction site. PLN Ser16 is in close contact with the PKA-bound ATP molecule (β- and γ-phosphate groups shown in purple and red) while PLN Thr17 points away from the ATP molecule. This model indicates that phosphorylation of only Ser16 is possible since Thr17 is not accessible to PKA.*

the dominant electrostatic interactions between SERCA and PLN. This descreening is partially compensated by the use of a distance-dependent dielectric constant. Descreening predominantly affects interactions in the solvent-exposed cytosolic region, while in the transmembrane portion the solvent-independent van der Waals interactions are responsible for the formation of helix – helix interactions. The calculations thus show a somewhat increased energetic separation between the various SERCA – PLN complexes (see Table 1), which simplifies their ranking.

The results for the complexes that involve helix M6 and the cytosolic loop, which contains the sequence Lys397-Asn-Asp-Lys-Pro-Ile402, should stimulate further studies on the positioning of PKA for phosphorylation of Ser16 in PLN. Figure 5a shows a hypothetical ternary complex of the docked SERCA/PLN model presented here with the catalytic subunit of cAMP-dependent protein kinase (PKA). As can be seen from the figure, PKA is only able to access Ser16 (Figure 5b) if the pump is in the E_2 conformation. The spatial position of the cytosolic loop in the E_1 conformation, in which it is closer to the membrane surface, would block access to PKA.[44]

Methods

Molecular mechanics energy minimizations were carried out by using the parameterization of the AMBER force field[23] as implemented in the program HYPERCHEM.[41] A distance-dependent dielectric constant ($\varepsilon = r$) without cut-off was used throughout all calculations for the evaluation of electrostatic interactions.

Two different sets of coordinates were used for the E_2 form of the SERCA pump: the theoretical model of Toyoshima (PDB entry 1FQU),[30] as well as alternative coordinates provided by D. Stokes.[24] These coordinates refer to PDB entry 1KJU.[25] The two coordinate sets were independently modeled by Stokes and Toyoshima and co-workers, respectively, by fitting the high-resolution structure of the E_1 form of SERCA into the low-density electromagnetic map of the E_2 form, followed by subsequent geometry regularization. Both models should be viewed as equally valid solutions of this problem. The coordinates of the SERCA pump were kept frozen throughout all our minimizations. The NMR spectroscopy structure (PDB entry 1FJK)[18] was used for PLN.

In the case of the E_1 conformation the coordinates of the X-ray structure (PDB entry 1EUL)[26] were superimposed onto Stokes' coordinates of the E_2 form by using all backbone atoms of residues in the ranges 86 – 115 (M2), 291 – 315 (M4), 768 – 810 (M6), and 931 – 959 (M9) for the fit, in a similar process to that used for the overlay of the two different sets of coordinates of the E_2 form of the SERCA pump. This superimposition resulted in an RMS deviation of 4.7 Å of the atoms used for the fit.

Starting geometries for each SERCA – PLN 1:1 complex were generated by manual translation of PLN to within a distance of about 5 Å from the intended transmembrane region of SERCA. The vacuum energies referred to as interaction energies in the text contain the internal energy of PLN and the interaction energy between PLN and SERCA according to the AMBER force field employed. PLN was pulled towards different contact residues in SERCA by energy optimization with additional harmonic constraint forces between the corresponding residues. The following initial harmonic constraints were applied to dock the M6 helix: A leucine-zipper-like interaction between Phe809, Thr805, Leu802, and Val795 of the M6 transmembrane helix of SERCA and Ile33, Leu37, Ile40, and Ile47 of PLN with a desired distance of 3 Å and a force constant of 23.3 kJ mol^{-1} Å^{-2}. An additional constraint of the same magnitude was used in the cytoplasmic region between Lys400 and Asp2. This cytosolic contact was also applied during initial optimization with M1, M2, M8, and M9, and was subsequently switched off. No constraint was used for docking to M3, M4, and M5 because these helices are located on the opposite side of Lys400 from PLN when viewed perpendicular to the membrane surface.

Initial energy minimizations were performed up to a gradient norm of below 0.418 kJ mol^{-1} Å^{-1} by using the steepest descent algorithm. The above-mentioned constraints were switched off in subsequent energy minimizations to a gradient norm of below 0.0418 kJ mol^{-1} Å^{-1} by the conjugate gradient method of Polak and Ribiere.[42] Several optimizations were carried out at each helix starting from different geometries and only the best energy is reported in Table 1. The geometry of PLN optimized on M6 of the Stokes model was employed as the starting structure for the minimizations with the SERCA model reported by Toyoshima (PDB entry 1FQU)[30]. The geometry optimizations were performed on a 1-GHz Pentium III PC. Each optimization required about 3 – 4 days.

A simplified simulated annealing protocol that uses the program X-PLOR[31] was applied to dock PLN to the SERCA pump in the E_1 conformation. The simulation consists of three parts: 6500 steps of 5 fs at 2000 K, 5000 steps of 5 fs linearly decreasing the temperature to 1000 K, and 2000 steps of 5 fs linearly decreasing the temperature to 100 K. The energy of the resulting structure was minimized (200 steps POWELL minimization). The standard force field used for generation of structures under the influence of experimental restraints was modified to meet the demands of the docking procedure: the backbone structure of the E_1 conformation was fixed by application of strong harmonic potentials (41.81 kJ mol^{-1} Å^{-2}) to the positions of all Cα, C, or N atoms of the SERCA pump. The PLN structure was defined by application of a set of experimental NOE data.[18] Further distance restraints between the SERCA pump and PLN were applied to bring the side chains of Asp2 and Lys3 of PLN within 5 Å of the side chains of the amino acids in the loop aa 397 – 402 of SERCA. The C-terminal domain of PLN at aa 33 – 47 was docked to the M6 helix of SERCA at aa 795 – 809 by similar upper-limit distance restraints of 5 Å.

Docking of PKA onto the binary complex of SERCA and PLN by computational techniques would be prohibitively complex. Instead, the model shown in Figure 5 was generated interactively by manual positioning of the active site of PKA close to PLN Ser16 while avoiding atom contacts closer than 1 Å.

Supporting information: Stereo figures of Figure 1b and Figure 5b, a figure comparing the docking results for PLN docked against 1JKU.pdb and 1IWO.pdb, and a PDB file containing the structure of PLN, which shows the most favorable interactions with the SERCA model of Stokes.[24, 25]

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[44] Note added in proof: After submitting this paper Toyoshima and Nomura published the crystal structure of an E2 form of the SERCA pump (*Nature* **2002**, *418*, 605) which was stabilized by the specific inhibitor thapsigargin (PDB code IWO). PLN does not seem to interact with the SERCA pump appropriately in the presence of thapsigargin (see Ref. [28]), but we have nevertheless modeled PLN into this new structure using M6 as the template. We applied the same procedure and the same constraints we had used for our optimal structure (Figure 1 of the paper). The results were less satisfactory, as can be seen by comparing Figure 6 provided in the Supporting Information with Figure 1 b of the paper.

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