Direct Spectroscopic Detection of Molecular Dynamics and Interactions of the Calcium Pump and Phospholamban*  

DAVID D. THOMAS,1 LAXMA G. REDDY, CHRISTINE B. KARIM, MING LI, RAZVAN CORNEA, JOSEPH M. AUTRY,2 LARRY R. JONES,2 AND JOHN STAMM

Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455, USA
2Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

ABSTRACT: In order to test molecular models of cardiac calcium transport regulation, we have used spectroscopy to probe the structures, dynamics, and interactions of the Ca pump (Ca-ATPase) and phospholamban (PLB) in cardiac sarcoplasmic reticulum (SR) and in reconstituted membranes. Electron paramagnetic resonance (EPR) and phosphorescence of probes bound to the Ca pump show that the activity of the pump is quite sensitive to its oligomeric interactions. In cardiac SR, PLB aggregates and inhibits the pump, and both effects are reversed by PLB phosphorylation. Previous analyses of PLB’s oligomeric state were only in detergent solutions, so we used EPR and fluorescence to determine the oligomeric structure of PLB in its native state in lipid bilayers. Wild-type PLB is primarily oligomeric in the membrane, while the mutant L37A-PLB is monomeric. For both proteins, phosphorylation shifts the dynamic monomer-oligomer equilibrium toward oligomers, and induces a similar structural change, as indicated by tyrosine fluorescence; yet L37A-PLB is more effective than wild-type PLB in inhibiting and aggregating the pump. Fluorescence energy transfer shows that the Ca pump increases the fraction of monomeric PLB, indicating that the pump preferentially binds monomeric PLB. These results support a reciprocal aggregation model for Ca pump regulation, in which the Ca pump is aggregated and inhibited by association with PLB monomers, and phosphorylation of PLB reverses these effects while decreasing the concentration of PLB monomers. To investigate the structure of the PLB pentamer in more detail, we measured the activities of cysteine residues in the transmembrane domain of PLB, and recorded EPR spectra of spin labels attached to these sites. These results support an atomic structural model, based on molecular dynamics simulations and mutagenesis studies, in which the PLB pentamer is stabilized by a leucine-isoleucine zipper within the transmembrane domain.

Active calcium transport into the sarcoplasmic reticulum of cardiac muscle is catalyzed by the Ca pump (Ca-ATPase, SERCA2a) and regulated by phospholamban (PLB), a 52-amino-acid protein that inhibits the pump at submicromolar [Ca²⁺] until PLB is phosphorylated under beta-adrenergic stimulation (reviewed in Ref. 1). This is a complex regulatory mechanism in which dynamic protein-protein interactions are proposed to play crucial roles. For example, it has been proposed, on the basis of SDS-PAGE, that PLB is a pentameric complex in the membrane, that phosphorylation results in the dissociation of an inhibitory complex between PLB and the pump, and that

* This work was supported by grants from the National Institutes of Health to D.D.T. (GM27906) and L.R.J. (HL06308 and HL49428). L.G.R. and C.B.K. were supported by grants from the American Heart Association.

1 Phone: 612-625-0957; fax: 612-624-0632; e-mail: ddt@ddt.biochem.umn.edu

186
PLB regulates the oligomeric state of the pump. In order to test and refine these hypotheses, we have used spectroscopic probes to detect directly the molecular dynamics and oligomeric interactions of these two proteins in native and reconstituted membranes. This work was facilitated not only by the development of spectroscopic probe technology, but also by methods for functional co-reconstitution of PLB and the Ca pump, and by molecular genetic techniques that made possible the expression and purification of PLB and its mutants in mg quantities and the functional coexpression of PLB with the Ca-ATPase.

**OLIGOMERIC INTERACTIONS OF THE CA PUMP**

Our early work on protein and lipid dynamics in skeletal SR established principles that proved essential in understanding the more complex mechanism of regulation of calcium pumping in the heart. It had been shown that optimal activity of the skeletal muscle Ca pump appears to require a fluid lipid environment, but it was not clear how lipid affects the structure and dynamics of the pump. Therefore, we measured the rotational dynamics of the Ca pump on the microsecond time scale, using saturation transfer electron paramagnetic resonance (EPR) and time-resolved phosphorescence anisotropy (TPA), and we found that the Ca-sensitive ATPase activity of the pump correlated much better with rotational mobility of the pump protein than with other physical parameters, such as lipid fluidity. In particular, it was found that the Ca-ATPase could be strongly inhibited and aggregated by the addition of melittin, a cationic amphipathic peptide, without any significant change in lipid fluidity. Similar effects of Ca-ATPase inhibition and aggregation were observed with lidocaine, a cationic amphipathic local anesthetic, whereas general anesthetics tended to disaggregate and activate the pump. This inhibition, as in the case of other agents that aggregate the Ca-ATPase, was found to be correlated with stabilization of the E2 (low-Ca affinity) form of the Ca pump.

**EFFECT OF PHOSPHOLAMBAN ON OLIGOMERIC INTERACTIONS OF THE CA PUMP**

We used TPA to show that, at low [Ca\(^{2+}\)], the Ca pump is less mobile (more aggregated) in cardiac SR than in skeletal SR, and that phosphorylation of PLB in cardiac SR decreases pump aggregation as it relieves the PLB-induced inhibition of the Ca-ATPase, suggesting that the regulation of Ca pump aggregation plays an important role in the function of PLB in the heart (Fig. 1). We proposed that in the absence of phosphorylation, the cationic phospholamban pentamer binds to the anionic Ca pump and induces pump aggregation, resulting in inhibition (Fig. 1, top), but upon phosphorylation the cationic charge on PLB is greatly reduced, decreasing affinity for the pump and relieving inhibition (Fig. 1, bottom). In order to verify this mechanism in a more controlled system, we co-reconstituted PLB with the Ca pump in lipid bilayers (Fig. 2, bottom) and found that PLB inhibits the SERCA1 (skeletal) as well as the SERCA2 (cardiac) isoform of the pump, that this inhibition is reversed by either phosphorylation or PLB antibody (Fig. 3, bottom), and that PLB aggregates the pump, as indicated by TPA (Reddy and Thomas, unpublished).

**OLIGOMERIC INTERACTIONS OF PHOSPHOLAMBAN**

PLB was proposed to be a stable pentamer, based on mobility in SDS-PAGE, so we set out to determine the oligomeric state of PLB in a lipid bilayer. EPR spectra of spin-
FIGURE 1. Model for the regulation of the Ca pump (gray) by the phospholamban pentamer (black), based on phosphorescence anisotropy data showing that PLB phosphorylation disassociates and activates the Ca pump.4

FIGURE 2. Reconstitution of PLB in lipid vesicles without (top) and with (bottom) the Ca pump. The proteins are dissolved in the detergent C12E8 (left), lipid vesicles are added, and detergent removed, producing vesicles with proteins inserted (right).5
labeled phospholipids can be used to count the number of boundary lipids in contact with the protein surface, and thus to measure the perimeter of the protein, which is quite sensitive to its oligomeric state (Fig. 4). We combined PLB with a mixture containing detergent, phospholipid, and phospholipid spin label (14-P CSL), then removed detergent, resulting in reconstituted PLB in lipid vesicles (Fig. 2, top). We found that the

---

**FIGURE 3.** Oligomeric state on SDS-PAGE (top) and regulation of Ca-ATPase activity in reconstituted membranes (bottom) for WT-PLB (left) and mutant L37A-PLB (right), in the absence (−P) and presence (+P, equivalent to PLB phosphorylation) of PLB antibody.

**FIGURE 4.** Oligomeric state of PLB wild-type (WT) and PLB mutant L37A reconstituted in lipid bilayer membranes, determined by using EPR and a phosphatidylcholine spin label (14-P CSL) to quantitate the number of restricted (boundary) lipids (gray) surrounding PLB (black).

17
number of boundary lipids per PLB protomer was much less than that expected for a monomeric α-helix, indicating that PLB is oligomeric in a lipid bilayer. In contrast, the number of boundary lipids observed for L37A-PLB (a monomer on SDS-PAGE, as shown in Fig. 3, top right) was precisely the number expected for a monomer (Fig. 4). These results indicate that the oligomeric states of these proteins in lipid are similar to those observed on SDS gels (Fig. 3). Despite the very different oligomeric states of these proteins, phosphorylation shifts the dynamic monomer-oligomer equilibrium toward oligomers for both proteins in lipid bilayers and causes a similar structural change in the cytoplasmic domain of PLB, as detected by tyrosine fluorescence. Thus the pentameric form does not seem to be required for the key structural changes of PLB. In fact, the monomeric L37A is even more effective than the pentameric wild-type PLB in inhibiting the Ca pump reversibly (Fig. 3, bottom). Thus it seems unlikely that a (presumably oligomeric) PLB channel plays a significant functional role.

**EFFECT OF THE Ca PUMP ON OLIGOMERIC INTERACTIONS OF PHOSPHOLAMBAN**

Since the EPR boundary lipid method (discussed above) is not applicable in a membrane containing more than one protein, we developed a fluorescence energy transfer method, with probes specifically bound to PLB, to determine the oligomeric state of PLB in the membrane, in the presence and absence of the Ca pump (Fig. 5). In this method, two separate populations of PLB were labeled with fluorescent donors and acceptors, mixed in varying ratios in the absence and presence of the Ca pump, and reconstituted in lipid bilayers (Fig. 2). Quantitative analysis of the energy transfer data showed that PLB is primarily oligomeric in the membrane, but that a small but significant fraction of the PLB protomers is monomeric. In the presence of the Ca pump,

![Diagram](image)

**FIGURE 5.** Fluorescence energy transfer method of analyzing oligomeric structure of labeled PLB. PLB labeled with donors (D) and acceptors (A) shows no energy transfer in the monomeric state (left) but shows complete energy transfer in an oligomer (right).
the monomeric fraction substantially increases, indicating that the Ca pump depolymerizes PLB by binding preferentially to the monomeric form.\textsuperscript{20}

**RECIPROCAL AGGREGATION MODEL FOR Ca PUMP REGULATION**

The above results support a model in which PLB and the cardiac Ca pump undergo reciprocal changes in oligomeric state in response to PLB phosphorylation, as illustrated in Figure 6. In the absence of PLB phosphorylation (top), PLB is in a dynamic equilibrium between monomers and oligomers (probably pentamers), and the Ca pump is inhibited and aggregated due to the binding of PLB monomers. Electrostatic repulsion probably destabilizes the PLB oligomer, and electrostatic attraction probably stabilizes pump-PLB interactions, which screen electrostatic repulsion between pump molecules. After phosphorylation, the PLB pentamer becomes stabilized, probably due at least in part to reduced electrostatic repulsion, decreasing the concentration of PLB monomers and thus allowing the pump molecules to become dissociated and activated. The mechanism for the correlation between pump aggregation and inhibition is not known, but may involve the TM domain of PLB binding to the pump and stabilizing the E2 conformation, which correlates with pump aggregation and inhibition.\textsuperscript{21}

![Figure 6. Reciprocal aggregation model of Ca pump (gray) regulation by PLB (black) phosphorylation. Before phosphorylation (top), PLB is in a dynamic equilibrium between pentamers and monomers, and the Ca pump is aggregated and inhibited by monomeric PLB. After PLB phosphorylation (bottom), PLB pentamers are more stable, reducing the concentration of monomers, disaggregating and activating the Ca pump.](image-url)
FIGURE 7. Atomic structural models of (A) Adams et al. and (B) Karim et al. for the transmembrane domain of the PLB pentamer. Top: schematic top view of helix packing in the pentamer, showing the seven helical positions predicted for the packing of helices into a left-handed coiled coil (3.5 residues per turn). Bottom: atomic model of two of the helices (side view), showing the view from the inside (IN) and outside (OUT) of the pentamer, highlighting the location of the three cysteine sulfur atoms (indicated by residue number), and the leucine (gray) and isoleucine (black) residues that are important for pentamer stability. The two models are energetically equivalent, but model B is most consistent with our experimental results.
STRUCTURAL MODEL FOR THE PHOSPHOLAMBN PENTAMER

We investigated the relative reactivities of the three Cys residues (36, 41, and 46) in the transmembrane domain of PLB and several PLB mutants. We found that Cys 41 is much less reactive than Cys 36 and Cys 46 to sulfhydryl reagents, and that labeling Cys 41 disrupts the PLB pentamer. This suggested that Cys 41 is not accessible to the surface of the PLB pentamer, and that this residue is at or near the interface between helices in the pentamer. This seemed inconsistent with the atomic model proposed previously by Adams et al., based primarily on molecular dynamics simulations (Fig. 7A). In that model, the helices pack together so that helical positions g and d are in contact, which would make Cys 41 (at helical position e) the most exposed of the three cysteine residues (Fig. 7A, top), in disagreement with our results. As shown at the bottom of Figure 7A, that model also failed to produce the close packing between key leucine side chains (residues 37, 44, and 51, all at helical position a) and isoleucine side chains (residues 40 and 47, all at helical position d) that had been predicted to form a leucine/isoleucine zipper, on the basis of site-directed mutagenesis. Therefore, we constructed an alternative model (Fig. 7B), refined by molecular dynamics simulation, in which the helical positions a and d are in contact. The resulting model (Fig. 7B) was energetically equivalent to that of Adams et al. (Fig. 7A), but it is more plausible, since it does show clearly the predicted leucine/isoleucine zipper, and it predicts that Cys 41 is the least accessible of the three cysteine residues, in agreement with our experimental results. This work illustrates the importance of experimental constraints in evaluating molecular models for helix packing.

CONCLUSIONS

The dynamic regulation of protein-protein interactions is key to the understanding of Ca pump regulation by phospholamban in the heart. Although much further research is needed to test rigorously the models shown in Figures 6 and 7, it is clear that direct measurement of protein dynamics and interactions, using site-specific spectroscopic probes, will continue to be crucial to the elucidation of molecular mechanisms in this and other membrane systems.

ACKNOWLEDGMENTS

We thank R. Bennett for technical assistance, and H. Kutchai for his contributions to the work on Ca pump oligomeric interactions.

REFERENCES


