Mutation and Phosphorylation Change the Oligomeric Structure of Phospholamban in Lipid Bilayers†

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Received August 6, 1996; Revised Manuscript Received December 30, 1996⊗

ABSTRACT: Phospholamban (PLB), a 52-residue protein integral to the cardiac sarcoplasmic reticulum, is a key regulator of the Ca pump. PLB has been shown to form pentamers in the denaturing detergent sodium dodecyl sulfate (SDS), but its oligomeric state in the natural environment of the lipid membrane remains unknown. In order to address this issue, we performed electron paramagnetic resonance (EPR) experiments on two types of lipid-reconstituted, recombinant PLB: wild type (WT PLB) and a mutant substituted with alanine at leucine 37 (L37A PLB), whose propensity to oligomerize in SDS is greatly diminished. The lipid used in reconstitution was dioleoylphosphatidylcholine (DOPC) doped with a phospholipid spin-label that detects protein contact. EPR spectroscopy was used to determine the fraction of the total lipid molecules in contact with PLB. Our results show that, in phospholipid bilayers, WT PLB is oligomeric (effective oligomeric size of 3.52 ± 0.71), while L37A PLB is monomeric (effective oligomeric size of 1.15 ± 0.15). Thus, the oligomeric states of these proteins in the lipid membrane are remarkably similar to those in SDS solution. In particular, the point mutation in L37A PLB greatly destabilizes the PLB oligomer. Phosphorylation of PLB by protein kinase A, which has been shown to relieve inhibition of the cardiac Ca pump, changes the lipid-PLB interactions, decreasing the number of lipids restricted by contact with protein. The results are consistent with a phosphorylation-dependent increase of the effective oligomer size of WT PLB from 3.52 to 5.34 and of L37A PLB from 1.15 to 1.91. These phosphorylation effects were abolished in a medium with a high ionic strength. We conclude that the oligomeric states of PLB in lipid membranes are in a dynamic equilibrium that is perturbed by phosphorylation due to reduced electrostatic repulsion among PLB protomers.

Biochemical and biophysical studies of the sarcoplasmic reticulum (SR)[1] Ca pump (Ca-ATPase) have established the importance of protein-protein interactions in controlling the enzymatic rate and the pumping mechanism (James et al., 1989; Mahaney & Thomas, 1991; Karon et al., 1995). Phospholamban (PLB) is a 52-amino acid regulatory transmembrane protein, residing in cardiac SR, that inhibits (Tada & Katz, 1982) and aggregates (Birmachu et al., 1993) the Ca pump. The N-terminal 26 amino acids constitute a highly charged cytoplasmic domain, containing phosphorylation sites for protein kinase A (PKA) (serine 16) and calcium/calmodulin-dependent protein kinase (threonine 17). Phosphorylation of either site reduces the net charge of the cytoplasmic domain from about +3 to +1 and abolishes the inhibition of the Ca pump (Wegener et al., 1989). Electrostatic forces appear to be important in the mechanism by which PLB regulates the pump (Chiesi & Schwaller, 1989; Xu & Kirchberger, 1989). Phosphorylation of PLB has also been shown to reverse the aggregation of the Ca pump, suggesting that the regulatory mechanism involves modulation of Ca pump self-association (Voss et al., 1994).

On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), PLB exhibits a distribution of oligomers ($1 \leq N \leq 5$) in which the pentamer and monomer are the most populated forms (Wegener & Jones, 1984; Jones et al., 1985; Fujii et al., 1986). It has been previously shown that the PLB oligomers are stabilized by noncovalent interactions in the extremely hydrophobic C-terminal transmembrane region, residues 27−52 (Wegener et al., 1986; Simmerman et al., 1986). On the basis of circular dichroism (Simmerman et al., 1989; Arkin et al., 1995) and infrared (Tatulian et al., 1995; Arkin et al., 1995; Ludlam et al., 1996) spectra, each of these C-terminal domains is proposed to form a single α-helix that traverses the lipid bilayer. The pentameric form of PLB has been proposed to be important for the Ca channel activity of PLB in planar bilayers (Kovacs et al., 1988) and for the mechanism of Ca pump regulation (Colyer, 1993; Arkin et al., 1995).

The effects of amino acid substitutions on the stability of the PLB pentamer in SDS solution have led to a model for a tightly packed coiled-coil pentamer (Simmerman et al., 1994; Arkin et al., 1994), in which the α-helical transmembrane domains of five monomers associate by intramembrane

† This work was supported by grants to D.D.T. (GM27906) and L.R.J. (HL06308 and HL49428) from the National Institutes of Health. J.M.A. was supported by a Predoctoral Fellowship from the American Heart Association, Indiana Affiliate.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: SR, sarcoplasmic reticulum; PLB, phospholamban; PKA, cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; L37A, replacement of Leu-37 with alanine; WT, wild-type; EPR, electron paramagnetic resonance; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycero-3-phosphocholine; OG, *n*-octyl β-D-glucopyranoside; ATP, adenosine triphosphate; MOPS, 3-(*N*- morpholino)propanesulfonic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; L/P, lipid to protein molar ratio; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CSU, catalytic subunit of PKA; TPX, methylpentene polymer.

S0006-2960(96)01955-1 CCC: $14.00

leucine-isoleucine zipper interactions (Simmerman et al., 1996). A point mutation in the proposed zipper region, in which Leu-37 was changed to Ala (L37A), drastically reduces the ability of PLB to form oligomers; this mutant exhibits mainly monomers on SDS gels (Simmerman et al., 1996). Nevertheless, L37A PLB has been shown to be functional when coexpressed with the Ca pump (Autry et al., 1994). Obviously, the quaternary structure of PLB in SDS is not necessarily the same as in the native membrane environment. Although SDS-PAGE provides a convenient and useful assay for some of the basic features of PLB, SDS solution is a relatively remote model of the *in situ* situation. To obtain information more closely related to the oligomeric structure of PLB in its native membrane environment, measurements should be performed in lipid bilayers in the absence of detergent.

Therefore, in the present study, we have used electron paramagnetic resonance (EPR) spectroscopy to determine the oligomeric state of recombinant purified PLB after reconstitution into phospholipid bilayers. Using a lipid spin-label that resolves EPR signals from boundary lipid (in direct contact with the protein) and the remaining bulk lipid, we were able to determine the number of lipids in contact with each recombinant protein and thus to calculate the number of PLB protomers in an oligomeric complex. These experiments were performed on both WT PLB and L37A PLB, with or without phosphorylation at Ser-16.

MATERIALS AND METHODS

Reagents and Solutions. 1-Acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycero-3-phosphocholine (14-PCSL), a phosphatidylcholine with the paramagnetic probe attached to the 14th carbon on one of the acyl chains, was synthesized according to Marsh and Watts (1982). Analysis by thin layer chromatography showed that this compound was pure and contained no detectable trace of free fatty acid. *N*-Octyl β-D-glucopyranoside (OG), PKA, adenosine triphosphate (ATP), and MOPS were purchased from Sigma (St. Louis, MO). Dioleoylphosphatidylcholine (DOPC) and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were obtained from Mallinckrodt (Paris, KY) and were of the highest purity available. All assays and EPR experiments were carried out in a buffer containing 100 mM KCl and 20 mM MOPS at pH 7.0 (buffer A), unless otherwise stated. The buffers and solutions used were saturated with nitrogen gas prior to use.

Sample Preparation and Assays. Recombinant WT PLB and L37A PLB were expressed and purified from baculovirus-infected Sf21 insect cells, as described previously (Reddy et al., 1995; Simmerman et al., 1996). The purified proteins were stored frozen (−70 °C) in a buffer containing 18 mM glycine, 88 mM MOPS, 5 mM DTT, and 0.92% OG (pH 7.2).

For PLB reconstitution into phospholipid bilayers, DOPC and 14-PCSL (100/1) were mixed in chloroform, and the solvent was removed under a stream of nitrogen gas so that the lipid was deposited in a thin layer at the bottom of the glass test tube. Chloroform traces were removed under vacuum. The lipid amount was calculated to obtain the desired lipid to protein molar ratio (L/P). The dried lipid sample was then rehydrated with 100 μL of a 1 mg/mL PLB solution containing 0.92% (w/v) of the nonionic detergent OG. For rehydration, the samples were gently vortex-mixed for 5 min and then incubated for 1 h at room temperature, during which time gentle mixing was repeatedly applied for 1 min at intervals of approximately 10 min. These samples were then diluted with 0.3 mL of buffer A and incubated for 15 min at room temperature. Finally, each sample was diluted to 3 mL in a buffer containing 20 mM MOPS and 0.3 M sucrose at pH 7.0 (sucrose buffer) and concentrated by centrifugation in a Beckman TL 100.3 rotor at 200000*g*, for 30 min, at 4 °C. The lipid-reconstituted PLB was recovered in the pellets. These PLB/lipid complexes were resuspended and homogenized in 100 μL of sucrose buffer, then loaded on a 25%/35%/40%/50% sucrose gradient, and centrifuged at 5 °C in a Beckman SW 50.1 rotor for 14 h at 200000*g*. The lipid-reconstituted PLB migrated as a single sharp band in the center of the 25% sucrose zone, indicating the formation of protein/lipid complexes of homogeneous L/P. No pellet was formed, indicating that lipid- and detergent-free PLB precipitates did not form. EPR spectroscopy of spin-labeled PLB reconstituted into lipid using this procedure showed that the N-terminal domain is exposed to the solvent, while the C-terminal domain is inserted deeply into the lipid bilayer (Cornea et al., 1995), confirming a model based primarily on sequence analysis (Wegener at al., 1986).

For the experiments requiring phosphorylation of PLB, the proteoliposome pellet (50 μg of PLB) was resuspended and homogenized in 180 μL of buffer B (50 mM MOPS, 10 mM $MgCl_2$, and 1 mM EGTA). The catalytic subunit (CSU) of PKA (10 picomolar international units) was added, and the reaction was initiated by the addition of 20 μL of a 10 mM ATP solution containing a trace amount of [γ-^{32}P]ATP. To allow the kinase and ATP to equilibrate in the multilamellar vesicles, the sample was subjected to three cycles of freezing and thawing in liquid nitrogen. The phosphorylation reaction was allowed to proceed for 120 min at 30 °C, and the sample was diluted with 2.8 mL of buffer A and then centrifuged at 200000*g* at 4 °C for 30 min. The control (unphosphorylated) sample was prepared in the same way, except that ATP was omitted. Since at subsolubilizing concentrations the partition coefficient of OG into lipid relative to water is 0.09 mM^{-1} (Paternostre et al., 1988), and the total dilution factor was 4500 in this preparation, the final OG concentration in the reconstituted PLB sample was calculated to be less than 7 μM, corresponding to less than 0.001 mol of OG per mole of lipid.

SDS-PAGE of lipid-reconstituted PLB was conducted according to Laemmli (1970), using a 10 to 20% acrylamide gradient in the resolving gel. Lipid-reconstituted PLB (3 μg) was loaded in a total volume of 15 μL. The SDS concentration in the sample, before loading on the gel, was 5%. The sample was not boiled. After electrophoresis, the gel was stained with Coomassie Blue, dried, and processed for autoradiography. The oligomeric distribution of PLB on the gel was determined from a digitized densitometric scan. Phosphorylation levels were measured with a 445SI Molecular Dynamics phosphorimager and by scintillation counting of the PLB bands cut from the gel. The phosphorylation level of the reconstituted PLB was 69 ± 10% [standard error of the mean (SEM), $n = 5$], similar to that of the lipid-free

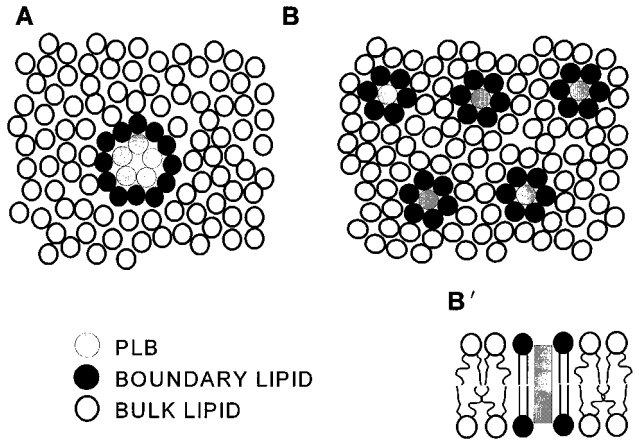

FIGURE 1: Determining the oligomeric size of a bundle of cylindrical (α-helical) transmembrane protomers in a lipid bilayer membrane, by measuring the protein perimeter available for interaction with the lipid phase. The number (N_B) of lipids per protomer that can come in contact with protein (boundary lipids) is much smaller for an oligomeric (e.g., pentamer-forming) protein (A) than for a monomeric protein (B). The hydrocarbon chains of lipids that are in contact with the relatively rigid transmembrane protein body are more restricted (less mobile) than those of the bulk lipid (B′), enabling these two populations to be resolved and quantitated by EPR spectroscopy using spin-labeled lipids.

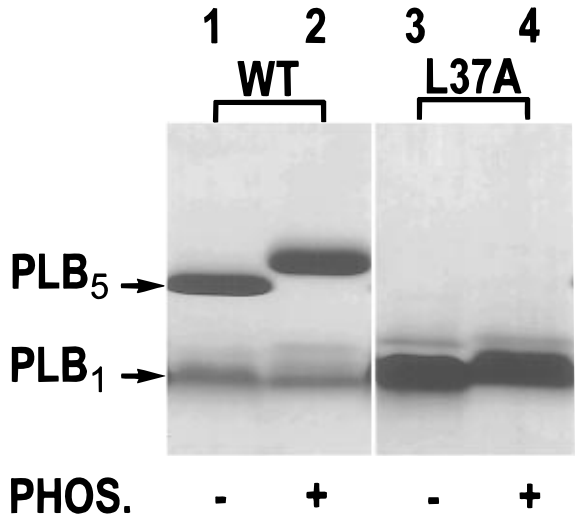

FIGURE 2: SDS-PAGE of lipid-reconstituted PLB was carried out using 4% acrylamide in the stacking gel and a 10 to 20% acrylamide gradient in the resolving gel, stained with Coomassie Blue. Exhaustive phosphorylation was carried out after reconstitution in DOPC. WT PLB (lane 1) is predominantly pentameric (PLB_5), with a significant monomeric component (PLB_1). L37A PLB (lane 3) is predominantly monomeric, with a minor dimeric component. Phosphorylation (PHOS.) causes a clear mobility decrease for WT PLB (lane 2) and a less pronounced but reproducible shift for L37A PLB (lane 4).

PLB controls, and it remained unchanged throughout the time range of our experiments.

The PLB concentration was determined by the amidoblack method, as previously described (Schaffner & Weissman, 1973). The molar concentration of lipids in the reconstituted PLB preparations was determined by measuring total phosphate (Chen et al., 1956). The values of L/P, determined for the final EPR samples, were in good agreement (±5%) with the L/P values in the initial mixtures.

EPR Spectroscopy. EPR was carried out with an X-band Bruker ESP-300 spectrometer equipped with a Bruker ER4201 cavity. Spectra were digitized with the spectrometer's built-in microcomputer using Bruker OS-9 acquisition software, then downloaded to an IBM-compatible microcomputer, and analyzed with software developed by R. Bennett. Pellets obtained by the centrifugation of reconstituted PLB were used in the EPR measurements without any further dilution. Each sample (typically 10 μL) was contained in a 1 mm diameter methylpentene polymer (TPX) capillary. The sample temperature was controlled to within 0.1 °C with a nitrogen gas-flow temperature controller and monitored with a digital thermometer using a Sensortek (Clifton, NJ) IT-21 thermocouple microprobe inserted into the top of the sample capillary, such that it did not interfere with data acquisition. The spectra were acquired using 100 kHz field modulation with a peak-to-peak modulation amplitude of 2 G. The microwave power was set to provide a microwave field intensity at the sample (H_1) of 0.07 G, usually 3−4 mW (Squier & Thomas, 1986).

Oligomer Size Calculation. We calculated the size of the intramembrane oligomers from the number of lipids in contact with the transmembrane portion of the protein (Figure 1), as measured by EPR of spin-labeled phospholipids. The transmembrane domain of each protomer, for both WT PLB and L37A PLB, is thought to consist of a single α-helix (see the introductory section), which has approximately the same diameter as a phospholipid (Figure 1). If these protomers associate into oligomers, the number of lipids in contact with protein (boundary lipid) decreases, and this results in a smaller fraction of spin-labeled phospholipids restricted by contact with protein. This method, carried out in lipid bilayers without detergent and without chemical modification of the protein, has been validated for a number of membrane proteins and provides a sensitive measure of protein perimeter and the oligomeric state in the membrane (Mahaney et al., 1992; Marsh, 1993). Details of the method will be described below in conjunction with Results.

RESULTS

SDS−PAGE. As reported previously (Simmerman et al., 1996), WT PLB forms mainly pentamers (Figure 2, PLB_5), with a significant monomeric component (PLB_1), whereas the point mutant L37A PLB forms mainly monomers, with a minor dimeric component (Figure 2, lane 3). It has been established (Wegener et al., 1986) that the pentameric form of PLB undergoes a phosphorylation-dependent decrease in mobility (Figure 2, lanes 1 and 2); for the monomeric band of WT PLB and for the mutant L37A PLB, this gel shift is reproducible but substantially smaller (Figure 2, lanes 3 and 4). These phosphorylation-dependent mobility shifts are not due to changes in oligomeric size (Wegener et al., 1986). Results from a PLB tyrosine fluorescence study indicate that phosphorylation causes a conformational change of PLB in SDS (Li et al., 1995). Quantitative scans of the gels showed no significant or reproducible effect of phosphorylation on the oligomeric distribution of either WT PLB or L37A PLB. The oligomeric distribution of PLB on gels is quite sensitive to the gel conditions (e.g., protein concentration, acrylamide concentration, SDS concentration, temperature, and pH), so it is unclear what set of conditions (and what oligomeric distribution) might actually correspond to the native state of the protein in a lipid bilayer membrane. Therefore, we carried out quantitative studies of PLB's oligomeric state in lipid bilayers, in the absence of SDS.

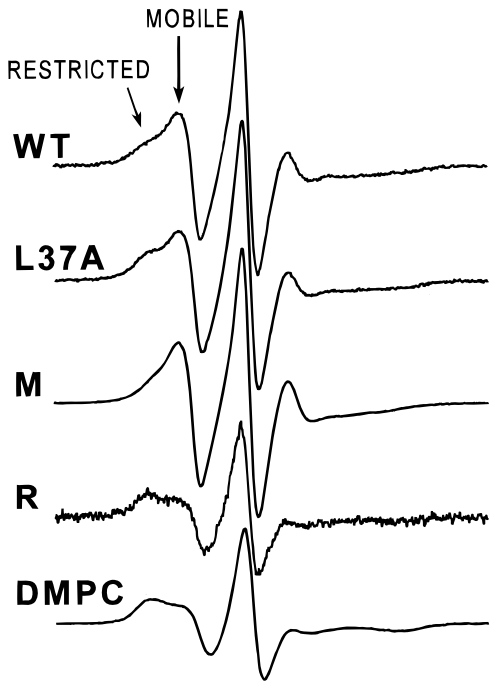

FIGURE 3: Composite spectral analysis. EPR spectra of 14-PCSL in PLB/DOPC complexes containing WT PLB (WT) and L37A PLB (L37A), recorded at 0 °C, exhibit contributions from two distinct populations of lipids (see Figure 1). Each composite spectrum S (WT or L37A) was analyzed using methods described in detail previously (Mahaney et al., 1992). R is the restricted component (corresponding to boundary lipid). M is the mobile component (corresponding to bulk lipid), obtained from a protein-free sample of DOPC at 0 °C. DMPC is the spectrum of 14-PCSL incorporated into sonicated DMPC liposomes, at 6 °C. Spectra were normalized to the same spin concentration prior to analysis.

Method of Boundary Lipid Analysis by EPR. The EPR spectra of 14-PCSL in PLB/DOPC membranes containing either WT PLB or L37A PLB exhibit contributions from two distinct populations of lipids (Figure 3). Each composite spectrum S (WT or L37A in Figure 3) was analyzed using a method described in detail previously (Mahaney et al., 1992), as a linear combination of two single-component spectra, $S = xR + (1 - x)M$, where R is the restricted component (corresponding to the boundary lipid), and M is the mobile component (corresponding to the bulk lipid). Spectrum M (Figure 3), obtained from a protein-free sample of 14-PCSL in DOPC, was subtracted from the composite spectrum (Figure 3, WT or L37A), varying the subtracted fraction $(1 - x)$ until a single-component spectrum corresponding to restricted motion (Figure 3, R) was obtained. The criterion for this end point was that spectrum R must match the spectrum of the spin-label in dimyristoylphosphatidylcholine (Figure 3, DMPC) in the gel phase. The temperature T used to obtain the DMPC spectrum was varied, and it was found that only a narrow range of T (6 ± 1 °C) and x values resulted in an acceptable fit between the residual spectrum R and spectrum DMPC (Figure 3). An alternative method, in which two composite spectra (e.g., WT and L37A in Figure 3) are subtracted from each other to obtain the end point spectra M and R (Sankaram et al., 1989), gave equivalent results for x, the mole fraction of restricted (boundary) lipid. The number of boundary lipids per protomer is $N_B = xN_T$, where N_T is the total number of lipids (bulk and boundary) per protomer.

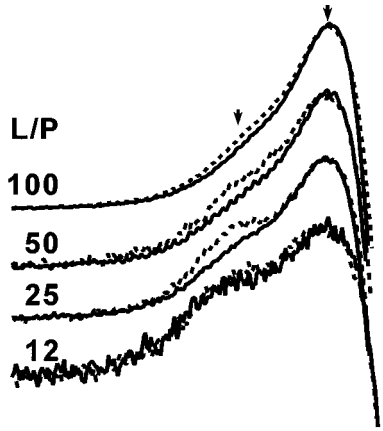

FIGURE 4: Low-field region of the EPR spectra of 14-PCSL in DOPC proteoliposomes containing WT PLB (continuous lines) and L37A PLB (dotted lines). L/P ranged from 12 to 100, as indicated, and the temperature was 0 °C. From left to right, arrowheads point at the spectral features corresponding to restricted and mobile populations of probes, respectively.

To optimize the resolution of boundary lipids, and underscore the differences between the two PLB species, we present here only the EPR spectra acquired at 0 °C. For composite spectra acquired at temperatures higher than 10 °C, the composite spectral analysis described above was hindered by less resolution between the mobile and restricted spectral components, presumably due to rapid exchange between the bulk and boundary lipid populations (Davoust & Devaux, 1982). As an alternative to DOPC, we have performed these measurements with PLB reconstituted in native SR lipids (data not shown). The results obtained in DOPC and SR lipids were consistent, but the latter provided lower precision, due to the broader line widths and hence worse resolution, perhaps due to the heterogeneous lipid composition. Even in pure DOPC bilayers, the 14-PCSL spectrum shows a hint of a restricted component at 0 °C (Figure 3, spectrum M, shoulder on low-field peak) but the splitting of this component is substantially less than that of the boundary lipid signal, so there is not a significant effect on the precision of the boundary lipid quantitation.

Effect of Mutation on the Number of Boundary Lipids. Typical EPR spectra of 14-PCSL in DOPC proteoliposomes of WT and L37A PLB at L/P = 25 are shown in Figure 3 (spectra WT and L37A). Although spectral features corresponding to the motional regimes of the bulk and boundary lipids are present throughout the entire EPR spectrum, they are most distinctly resolved in the low-field region (Figure 4). Clearly, the relative intensity of the restricted component increases as the density of PLB in the membrane increases (L/P decreases). This trend is exhibited by both WT and L37A PLB and confirms that the motion of the spin-label is hindered due to its interaction with the membrane protein. The relative fraction of the boundary lipid (motionally restricted) component is clearly larger for L37A PLB (Figure 4, dotted lines) than for WT PLB (Figure 4, solid lines) at L/P values ranging from 25 to 100, whereas at an L/P of 12 or lower, the traces are virtually identical (Figure 4, bottom traces). These results suggest that WT PLB inserts in the membrane as a larger oligomeric assembly than L37A PLB.

Effect of Phosphorylation. To ensure the equivalent treatment of samples, phosphorylation of Ser-16 of PLB was performed and quantified after lipid reconstitution. For both WT PLB and the mutant L37A PLB, phosphorylation results

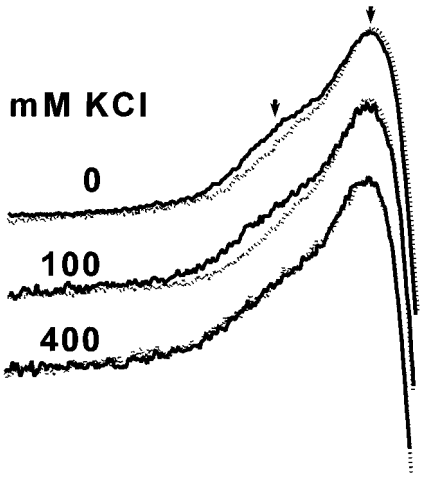

FIGURE 5: Phosphorylation effects. Low-field region of the EPR spectra of 14-PCSL in DOPC proteoliposomes containing unphosphorylated (continuous lines) and phosphorylated WT PLB (dotted lines). L/P was 25, and the temperature was 0 °C. The measurements were done in 20 mM MOPS at pH 7.0 in the presence of 0, 100, and 400 mM KCl, as indicated. From left to right, arrowheads point at the spectral features corresponding to restricted and mobile populations of probes, respectively.

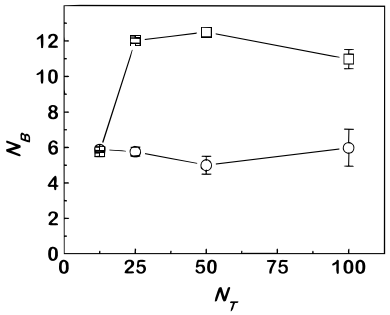

FIGURE 6: Number of boundary lipids per protomer (N_B), determined from each composite EPR spectrum (as described in Materials and Methods and in Figure 3) of WT PLB (○) and L37A PLB (□), as a function of the total number of lipids per protomer (N_T). Error bars represent SEM from four to six experiments.

in a decreased fraction of boundary lipid relative to the unphosphorylated samples (e.g., the spectra labeled 0 and 100 mM KCl in Figure 5).

Current structural models of PLB suggest a topology of the charged cytosolic residues that allows electrostatic interactions to modulate PLB oligomerization. To test this hypothesis, we performed boundary lipid measurements in buffers of different ionic strengths. The effect of phosphorylation on the boundary lipid fraction was maximal at low ionic strengths (buffer A lacking KCl) and in the physiological range (buffer A with 100 mM KCl) but was completely abolished at high ionic strengths (buffer A with 400 mM KCl) (Figure 5).

The mobile and restricted spectral components were resolved and quantified by digital spectral analysis in order to determine the mole fraction x of restricted lipids (Figure 3). Increasing the total number of lipid molecules per PLB protomer (N_T) resulted in a decrease of x, indicating a direct association of the restricted lipids with the protein. For each PLB species, the number of restricted (boundary) lipids per protomer (N_B) was essentially independent of N_T (Figure 6), consistent with a constant protein perimeter that is available for contact with the lipid phase. The only exception was a lower value of N_B for L37A PLB at a very low N_T, but this

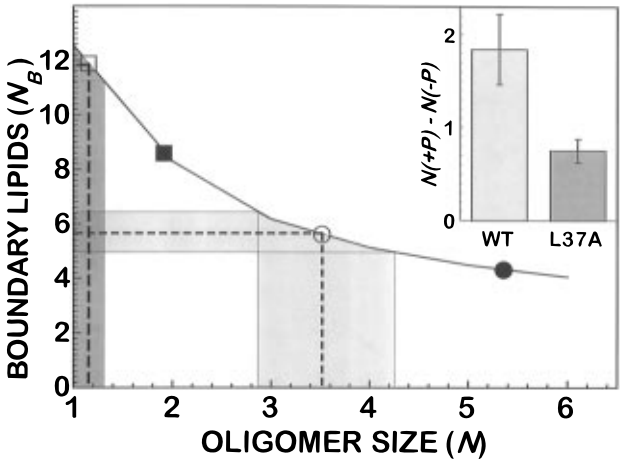

FIGURE 7: Calculating the size of PLB oligomers from boundary lipid data. The continuous curve represents the calculated number of boundary lipids per PLB protomer (N_B) vs the number (N) of circularly assembled transmembrane α-helices. These theoretical transmembrane α-helices were modeled as vertical circular cylinders 10 Å in diameter, equal to the diameter of phospholipid molecules (Marsh, 1993). We measured N_B (as described in Figures 3 and 6, varying N_T from 25 to 100) for WT and L37A PLB (circles and squares, respectively), both unphosphorylated (open symbols) and phosphorylated (closed symbols). SEM for the unphosphorylated proteins was calculated from six separate experiments and is indicated by the shaded areas of the main graph. The inset demonstrates that the effect of phosphorylation on N is significant for both PLB species. $N(-P)$ and $N(+P)$ are the effective oligomeric sizes before and after phosphorylation. The difference $[N(-P) - N(+P)]$ was calculated for each experiment, and the mean was calculated from four separate experiments for each PLB species. This pairwise analysis optimized precision (minimized SEM, as shown by the error bars).

is expected due to protein crowding at low L/P values (Peelen et al., 1992).

To determine the number of protomers per oligomer (N) from N_B, we assumed that each PLB protomer passes through the membrane as a single α-helix, with the same (approximately 10 Å) diameter as a phospholipid (Marsh et al., 1993), and that any PLB oligomers are circular bundles of α-helices (Figure 1), as proposed previously (Simmerman et al., 1986) and supported by structural analysis and modeling of PLB in solution (Simmerman et al., 1989, 1996; Adams et al., 1995). Using this model and the measured N_B, we found an effective oligomer size N of 3.52 for WT PLB and 1.15 (a monomer) for L37A PLB (Figure 7). Phosphorylation increased the effective oligomer size N for both PLB species, from 3.52 to 5.34 for WT PLB and from 1.15 to 1.91 for L37A PLB (Figure 7). These phosphorylation effects were eliminated by an increased ionic strength (Figure 5).

DISCUSSION

The prevailing molecular model for the regulation of calcium transport into cardiac SR depicts the PLB pentamer with the Ca pump in an inhibitory complex that is disrupted upon PLB phosphorylation (Wegener et al., 1984; Jones et al., 1985; James et al., 1989; Voss et al., 1994). The measured channel activity of PLB (Kovacs et al., 1988), together with its pentameric state, has been included in some regulation models (Arkin et al., 1995). However, mutants of PLB that are predominantly monomeric on SDS−PAGE (e.g., L37A PLB) are still regulatory when coexpressed with

Table 1: Oligomeric State of Wild-Type (WT) and Mutant (L37A) PLB, as Detected by EPR or SDS-PAGE[a]

PLB species	EPR boundary lipid				SDS-PAGE	
	$N(-P)$[b]	$\frac{N(+P)}{N(-P)}$	$X^{mon}(-P)$[c]	$\frac{X^{mon}(+P)}{X^{mon}(-P)}$	$X^{mon}(-P)$	$\frac{X^{mon}(+P)}{X^{mon}(-P)}$
WT	3.52 (0.71)	1.52 (0.32)	0.17 (0.03)	0.00 (0.04)	0.35 (0.21)	0.90 (0.20)
L37A	1.15 (0.15)	1.66 (0.22)	0.84 (0.03)	0.09 (0.07)	0.87 (0.05)	1.00 (0.30)

[a] The oligomeric size of PLB was measured either by EPR (Figure 7) or by SDS-PAGE (Figure 2). [b] $N(-P)$ and $N(+P)$ are the effective oligomeric sizes of PLB before and after phosphorylation, assuming that all PLB protomers are in symmetrical oligomers of equal size. [c] $X^{mon}(-P)$ and $X^{mon}(+P)$ are the mole fractions of monomers out of total PLB protomers, before and after phosphorylation, assuming a mixture of monomers and pentamers (WT) or a mixture of monomers and dimers (L37A). Each value represents the mean and SEM (in parentheses) for four or five measurements. The result after phosphorylation (+P) is given as the ratio to the value before phosphorylation (−P), since the pairwise analysis of these two values for each sample produced optimal precision (minimal SEM).

the Ca pump (Autry et al., 1994; Toyofuku et al., 1994), suggesting that the pentameric structure is not required for PLB function.

The pentameric form of PLB has previously been demonstrated only in SDS micelles during PAGE. In the present study, the recent availability of two well-characterized, highly purified recombinant PLB species, WT and L37A PLB, shown to form pentamers and monomers in SDS, respectively, has allowed us to probe, for the first time, the oligomeric structure of the protein in phospholipid bilayers. This EPR method, in which spin-labeled lipids are used to measure the intramembrane protein perimeter, has been used several times previously to analyze the oligomeric structures of membrane proteins (Mahaney et al., 1992; Peelen et al., 1992; Marsh et al., 1993). In the EPR boundary lipid technique, the spectroscopic probe is present only in trace amounts in the lipid phase of the protein/lipid complex and is, therefore, likely to perturb neither protein-protein nor lipid-protein interactions. This technique does not have the resolution to determine the detailed distribution of oligomeric species present in the membrane, but it is quite sensitive to changes in the oligomeric distribution that change the exposed perimeter of the protein complex. We selected DOPC for PLB reconstitution because this lipid has been shown to be an ideal replacement of the native SR lipid with respect to the activity and molecular dynamics of Ca-ATPase (Caffrey & Feigenson, 1981; Lee, 1991; Cornea et al., 1994), the enzyme regulated by PLB.

Our EPR measurements of oligomeric state (Figure 7) show that, in DOPC bilayers, WT PLB is oligomeric, with an average oligomer size between 3 and 4, while the replacement of a single leucine with alanine, to form L37A, almost completely destabilizes this oligomer, resulting in an average oligomer size of 1.15. These results are qualitatively consistent with results from SDS-PAGE (Figure 2), where WT PLB and L37A PLB are present primarily as pentamers and monomers, respectively (summarized in Table 1). It is remarkable that the membrane oligomeric states of these two proteins in the lipid bilayer resemble those observed in SDS solution so closely. The analysis in Figure 7 assumes a single effective oligomeric size N, since the EPR measurement cannot resolve a more complex distribution. SDS-PAGE data (Figure 2), which does resolve different oligomeric species, suggests that we should consider a model in which WT PLB is a mixture of pentamers and monomers, and L37A PLB is a mixture of dimers and monomers. In fact, this model is quite consistent with the observation that the effective oligomeric size N is less than 5 (3.52 ± 0.71) and greater than 1 (1.15 ± 0.15) for WT PLB and L37A PLB, respectively (Table 1). On the basis of this model, from the number of boundary lipids per protomer (N_B) measured by EPR (Figures 6 and 7), we can calculate the mole fraction of total PLB protomers that are distributed as monomers (X^{mon}) using the following relation:

$$X^{mon} = \frac{N_B - N_B^{olig}}{N_B^{mon} - N_B^{olig}}$$

where N_B^{olig} (4.32 for a pentamer, 8.28 for a dimer) and N_B^{mon} (12.56) are the theoretical numbers of boundary lipids per protomer. These values are tabulated in Table 1. The calculated mole fraction of monomers X^{mon} for WT PLB in DOPC is 0.17 ± 0.08. For L37A PLB, X^{mon} is 0.84 ± 0.14, assuming a monomer-dimer equilibrium, or 0.95 ± 0.07, assuming a monomer-pentamer equilibrium.

Thus, while most previous models considered PLB to be a stable pentamer in membranes, despite the coexistence of monomers and pentamers in SDS-PAGE, our results suggest that the PLB pentamer is no more stable in lipid bilayers than in SDS, with monomers and pentamers in equilibrium with each other even in the lipid bilayer. Another striking point is that our results with L37A PLB constitute the first direct physical evidence for a transmembrane α-helix that exists predominantly as a monomer in a lipid bilayer.

Phosphorylation of PLB at Ser-16 causes a change in PLB-lipid interaction, decreasing the number of boundary lipids that are in contact with the protein (Figure 5, Figure 7 inset). The most plausible interpretation of the diminished boundary lipid fraction after PLB phosphorylation is an increase of the effective PLB oligomeric size N. For WT PLB, we calculated an N increase from 3.52 to 5.34 (Figure 7), corresponding to a decrease in the fraction of monomers X^{mon} from 0.17 to 0.00 (Table 1). That is, after phosphorylation, PLB is completely pentameric. Similarly, phosphorylation of L37A PLB shifts N from 1.15 to 1.91, corresponding to a decrease in X^{mon} from 0.84 to 0.08 (completely dimeric, Table 1), assuming a monomer-dimer equilibrium (as suggested by SDS-PAGE), or from 0.95 to 0.55, assuming a monomer-pentamer equilibrium. These results suggest that there is a *dynamic equilibrium* of the intramembrane PLB oligomeric species, with phosphorylation shifting the equilibrium from monomers to oligomers. This phosphorylation effect depends almost entirely on electrostatic interactions (Figure 5), so the most likely cause of the increased PLB aggregation caused by phosphorylation (Figure 5, Figure 7,

inset) is the decrease in the net charge of the cytosolic domain (+3 to +1), which reduces repulsive interprotomeric electrostatic forces. This hypothesis is supported by the observation that phosphorylation affects oligomerization maximally at low ionic strengths, where screening of the cytosolic domain charges by solution counterions is minimized (Figure 5). These results are consistent with a previous report by Chiesi and Schwaller (1989), in which high ionic strength was found to decrease the effect of PLB phosphorylation on the calcium affinity of the Ca pump. While the fraction of monomers X^{mon} determined by EPR in lipid bilayers is in good agreement with that of SDS-PAGE in the absence of phosphorylation (Table 1), the change in oligomeric size due to phosphorylation is not detected under SDS-PAGE conditions (Figure 2), probably because the electrostatic effects are screened by SDS.

WT and L37A PLB have both been coexpressed with the Ca pump and found to regulate it in a similar manner (Autry et al., 1994). Each species inhibits the Ca pump at low calcium in the absence of phosphorylation, and this inhibition is relieved by an anti-PLB monoclonal antibody (2D12), which mimics the phosphorylation effects by relieving the inhibition of the Ca pump (Sham et al., 1991; Briggs et al., 1992). This result, combined with the present study showing that L37A PLB is mostly monomeric in its unphosphorylated (inhibitory) form, is in contradiction with the hypothesis that PLB pentamers, possibly acting as ion channels, are required for the inhibitory interaction between PLB and the Ca pump (Arkin et al., 1995; Colyer, 1993).

The results reported here indicate surprising new features for the mechanism of Ca pump regulation. Since L37A PLB, which regulates the Ca pump normally when coexpressed with the Ca-ATPase, is monomeric in its unphosphorylated state in lipid bilayers, *it is likely that the pentameric state of PLB is not required for inhibition*. Rigorous proof of this hypothesis will require measurements of the PLB oligomeric state under conditions in which PLB regulates the Ca-ATPase. We found that PLB phosphorylation, which relieves Ca pump inhibition in cardiac SR, promotes PLB oligomerization in lipid bilayers for both wild-type and mutant PLB. It is possible that this change in PLB oligomeric state is not crucial for function and that the key physical effect of phosphorylation is a decrease of the attractive forces between a positively charged protein and negatively charged sites on the Ca pump (Voss et al., 1994, 1995a,b). However, oligomerization of PLB may directly decrease inhibition, either because of the resulting decrease in the effective ratio of inhibitors to pumps (Figure 8) (assuming that monomers and oligomers are equally effective inhibitors) or because monomers are actually more potent inhibitors. The interplay of protein-protein interactions in the cardiac SR membrane is fascinating. Since PLB phosphorylation increases PLB aggregation but relieves Ca pump aggregation and inhibition (Voss et al., 1994), there is a reciprocal relationship between PLB aggregation and Ca pump aggregation (Figure 8).

Regulated equilibria of membrane protein associations have been reported previously (Yarden & Schlessinger, 1987; Gadella & Jovin, 1995) and may be crucial to the regulation of many membrane functions. This general hypothesis must be tested by direct measurement of protein oligomeric states in lipid bilayers.

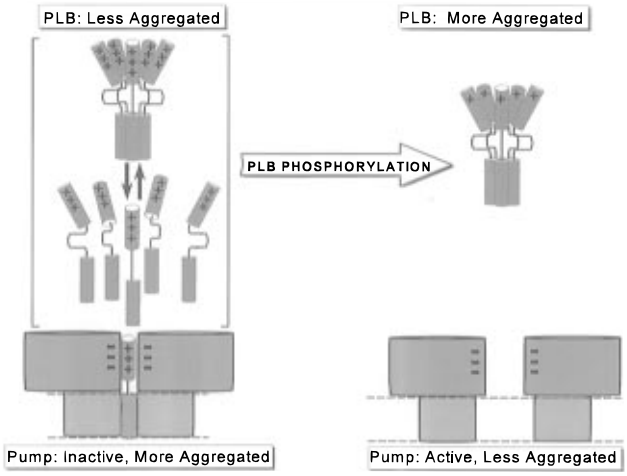

FIGURE 8: Model for the role of protein oligomerization in the regulation of the cardiac Ca pump by PLB phosphorylation. (Top) Phosphorylation of Ser-16 in PLB decreases the charge on its cytosolic domain (+3 to +1) and increases the propensity of PLB to self-associate into pentamers. (Bottom) PLB phosphorylation leads to activation and decreased aggregation of the Ca pump (Voss et al., 1994). This may be due to dissociation of the inhibitor from the Ca pump due to the reduced charge on PLB (Voss et al., 1995b) and/or to the decreased effective PLB concentration in the membrane caused by its increased aggregation.

ACKNOWLEDGMENT

We are grateful to Anne Walter for technical advice that proved extremely useful. We thank Howard Kutchai and Jim Mahaney for critically reading the manuscript. We also thank Edmund Howard, Nicoleta Cornea, and Roberta Bennett for excellent technical support.

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BI961955Q