Synthetic Null-Cysteine Phospholamban Analogue and the Corresponding Transmembrane Domain Inhibit the Ca-ATPase†

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ABSTRACT: Chemical synthesis, functional reconstitution, and electron paramagnetic resonance (EPR) have been used to analyze the structure and function of phospholamban (PLB), a 52-residue integral membrane protein that regulates the calcium pump (Ca-ATPase) in cardiac sarcoplasmic reticulum (SR). PLB exists in equilibrium between monomeric and pentameric forms, as observed by SDS−PAGE, EPR, and fluorescence. It has been proposed that inhibition of the pump is due primarily to the monomeric form, with both pentameric stability and inhibition dependent primarily on the transmembrane (TM) domain. To test these hypotheses, we have studied the physical and functional properties of a synthetic null-cysteine PLB analogue that is entirely monomeric on SDS−PAGE, and compared it with the synthetic null-cysteine TM domain (residues 26−52). The TM domain was found to be primarily oligomeric on SDS−PAGE, and boundary lipid spin label analysis in lipid bilayers verified that the isolated TM domain is more oligomeric than the full-length parent molecule. These results indicate that the stability of the PLB pentamer is due primarily to attractive interactions between hydrophobic TM domains, overcoming the repulsive electrostatic interactions between the cationic cytoplasmic domains (residues 1−25). When reconstituted into liposomes containing the Ca-ATPase, the null-cysteine TM domain had the same inhibitory function as that of the full-length parent molecule. We conclude that the TM domain of PLB is sufficient for inhibitory function, the oligomeric stability of PLB does not determine its inhibitory activity, and the three Cys residues in the TM domain are not required for inhibitory function.

Phospholamban (PLB)[1] is a 52-residue integral membrane protein that regulates the enzymatic activity of the Ca-ATPase in cardiac sarcoplasmic reticulum (*1*). This protein contains a single transmembrane (TM) helix, which forms homopentamers that are stable in SDS−PAGE (*2*), and spectroscopic analysis has verified that PLB is primarily oligomeric in lipid bilayers (*3*−*5*). Mutagenesis has been used to define the regions of this protein that are essential for pentameric stability. This was used by Simmerman et al. (*6*) who proposed a model for the PLB pentamer as a left-handed coiled-coil stabilized specifically by interactions between leucines (residues 37, 44, and 51) and isoleucines (residues 40 and 47). This interaction resembles the interlocking of the teeth of a zipper. Changing any of these zipper residues to Ala prevents pentamer formation. Mutating all three Cys residues (36, 41, and 46) to Ala decreases the stability of the pentamer, forming predominantly monomers on SDS−PAGE (*7*). This cysteine-to-alanine mutation also reduces the apparent calcium affinity of the Ca-ATPase (*8*). However, mutation of Cys 36 and Cys 46 to Ala, leaving Cys 41 intact, results in a mixture of pentamer and monomer on SDS PAGE (*9*). Compared with the other two Cys residues in WT-PLB, Cys 41 has much lower reactivity to thiol-reactive reagents (*9*). These results indicate that Cys 41 is critical to the stability of the pentamer and may be involved in interhelical interactions (*6*, *9*).

Recent results suggest that the pentameric form of PLB is not required for inhibition of the Ca-ATPase. Indeed, since some mutations that destabilize the pentamer in SDS (*6*) and in lipid bilayers (*3*) have higher inhibitory activity than wild-type (*10*, *11*), and since phosphorylation of PLB stabilizes

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[1] Abbreviations: PLB, phospholamban; WT, wild-type; Ala-PLB, replacement of Cys 36, 41, 46 in PLB with alanine; TM-Ala-PLB, isolated transmembrane domain of PLB with Cys 36, 41, 46 changed to alanine; L37A, replacement of Leu-37 in PLB with alanine; C41L, replacement of Cys-41 in PLB with leucine; ATP, adenosine triphosphate; DBU, 1,8-diazobicyclo-[5.4.0]undec-7-ene; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EPR, electron paramagnetic resonance; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; Mops, 3-(*N*-morpholino)propanesulfonic acid; NADH, β-nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; OG, octylglucoside; PAC, *p*-alkoxybenzyl ester; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; *t*Boc, *tert*-butyloxycarbonyl; O*t*Bu, *tert*-butyl ester; Trt, triphenylmethyl; PAGE, polyacrylamide gel electrophoresis; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycerol-3-phophocholine; PEG-PS, poly(ethylene glycol)-polystyrene (graft support); SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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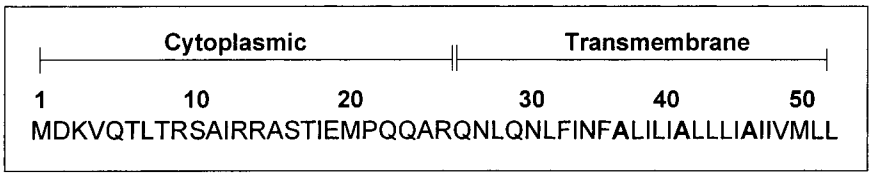

FIGURE 1: Amino acid sequence of Ala-PLB (1−52) and TM-Ala-PLB (26−52).

the pentamer, it has been proposed that the monomeric form of PLB is the inhibitory moiety, and depolymerization of the pentamer is essential for its regulatory function (*3*, *10*, *12*). This hypothesis received direct support from the finding, obtained from fluorescence energy transfer experiments, that the Ca-ATPase protein stabilizes the monomeric form of PLB (*4*). However, monomer formation is not the only factor involved in the inhibitory function of PLB and its mutants. For example, while L37A-PLB, which is more monomeric on SDS−PAGE than WT-PLB, is a super-inhibitor, i.e., shows stronger inhibition of the ATPase than wild-type (WT-PLB) at 25 °C, C41L-PLB exhibits no inhibitory function despite having a similar level of monomer as WT-PLB on SDS−PAGE at 25 °C (*13*). The cytoplasmic domain (residues 2−25) does not inhibit the Ca-ATPase (*14*), but transmembrane domain fragments consisting of residues 28−47 (*15*) or 30−52 (*16*) do inhibit the Ca-ATPase, with results similar to those of the full-length PLB; inhibition is observed only at submicromolar Ca, resulting in a decrease in the apparent Ca affinity.

In the present study, we set out to test the hypothesis that the TM domain determines both the oligomeric state of PLB and its inhibitory activity. We focused on an analogue of PLB in which all three Cys residues (residues 36, 41, and 46, all in the TM domain) were substituted to Ala; this has been shown to be monomeric on SDS−PAGE (*7*) and to have inhibitory activity (*8*). We prepared full-length Ala-PLB and its TM domain (TM-Ala-PLB), using Fmoc solid-phase peptide synthesis techniques. The oligomeric states were determined in detergent solution by SDS−PAGE and in lipid bilayers by using EPR and a phosphatidylcholine spin label. The synthetic peptides were then co-reconstituted with the Ca-ATPase in lipid vesicles, and Ca-ATPase inhibition assays were performed. These results were compared to those of WT-PLB.

MATERIALS AND METHODS

Peptide Synthesis and Purification. The general methodology followed earlier descriptions from one of our laboratories (*17*−*20*). Starting with Fmoc-Leu-PAC−PEG-PS resin (initial loading 0.18 mmol/g), the 52 and 27 residue Ala-PLB and TM-Ala-PLB (Figure 1) were assembled by automated Fmoc solid-phase synthesis on a Milligen 9050 automated peptide synthesizer. Acidolyzable side-chain protecting groups were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg; N^{w}-triphenylmethyl (Trt) for Asn and Gln; *tert*-butyl esters (O*t*Bu) for Asp and Glu; *tert*-butyloxycarbonyl (*t*Boc) for Lys; and *tert*-butyl ethers (*t*Bu) for Ser, Thr, and Tyr. Fmoc removal was achieved with 20% piperidine and 2% DBU in NMP (6 min from Leu 51 to Leu 37; 10 min from Ala 36 to Leu 31; 13 min from Asn 30 to Met 1). HBTU/HOBt/DIEA couplings (4 equiv each over resin-bound amine) involved 7 min of preactivation time and the following reaction times: 60 min from Leu 51 to Leu 37; 90 min from Ala 36 to Leu 31; 120 min from Asn 30 to Met 1. Upon completion of chain assembly, the peptide-resin was stored at −20 °C. To deprotect and cleave the peptide from the resin (200 mg each time), first an Fmoc removal step was carried out, followed by treatment with 2 mL of freshly prepared reagent K: 82.5% TFA, 5% phenol, 5% thioanisole, 2.5% 1,2-ethanedithiol, 5% water (*21*), for 4 h at 25 °C. The cleavage mixture was filtered, and the resin was washed with 2 mL of the same cocktail. The combined filtrates were concentrated under N_2, and 30 mL of diethyl ether was added at 0 °C. The precipitated peptide was collected by centrifugation, and washed three times with 30 mL of cold diethyl ether. The crude peptide was dissolved in 5 mL of TFA and purified by HPLC on a C-18 column (Vydac, 218TP54; 5 μm, 300 Å, 4.6 × 250 mm) that had been equilibrated with 95% water, 2% acetonitrile, and 3% 2-propanol. Peptide elution was achieved with a linear gradient to a final solvent composition of 5% water, 38% acetonitrile, and 57% 2-propanol (*22*). Fractions containing peptides were lyophilized to yield 24 mg of TM-Ala-PLB (22% yield based on starting resin) and 26 mg of Ala-PLB (12% yield based on starting resin). Wild-type PLB (WT-PLB) was kindly provided by Edward McKenna, Merck Research Laboratories, who synthesized PLB (1−52) using an Applied Biosystems 430A synthesizer. The peptides were dissolved in methanol/chloroform 2:1 at a final concentration of 1−2 mg/mL, as quantified by amino acid analysis.

SR-Vesicles. SR vesicles were prepared from the fast-twitch skeletal muscle of New Zealand white rabbits (*23*). The SR vesicles were purified on a sucrose gradient, and the Ca-ATPase was purified using a reactive-red affinity column (*24*).

Analysis of Peptide Size and Composition. SDS−PAGE was performed using 16.5% Tris/Tricine gel (Bio-Rad). The peptide samples from the stock methanol/chloroform 2:1 solution were dried overnight. 20 μL of 1% SDS was added to the samples which contained 5−60 μg of WT-PLB, Ala-PLB, or TM-Ala-PLB. For SDS−PAGE, samples contained 20 μL of Tricine sample buffer (*25*) with 1.5% final SDS concentration.

Mass spectrometry was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) using a crystallized matrix of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid).

Ca-ATPase/PLB Co-Reconstitution. The method used for the functional reconstitution of Ca-ATPase with PLB has been described (*4*, *26*). PLB or its analogues were dried and solubilized in 240 μL of chloroform containing 2.4 mg of lipids (DOPC/DOPE, 4:1). The solvent was dried under nitrogen. The dried film of lipid and PLB was hydrated with 120 μL of 25 mM imidazole, pH 7.0, by vortexing thoroughly followed by a brief sonication. The resulting

vesicles, containing lipid and PLB, were made to 20 mM imidazole, pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 10% glycerol. Then, 4.8 mg of β-OG was added, followed by 60 μg of purified Ca-ATPase. The final volume was adjusted to 300 μL with buffer. The detergent was then removed by incubation with 120 mg of wet Biobeads (Bio-Rad) for 3 h at room temperature. The Ca-ATPase/PLB lipid vesicles were separated from Biobeads and assayed immediately.

ATPase Activity Measurements. Ca-ATPase activity was assayed by an enzyme-linked assay (*27*), performed in microtiter plate wells. Each assay was done in triplicate at different calcium concentrations in a volume of 175 μL. Between 1 and 3 μg of Ca-ATPase (5−15 μL of vesicles) was added to a buffer containing 1 mM phosphoenol pyruvate, 5 mM ATP, 0.4 mM NADH, 100 IU of pyruvate kinase, 100 IU of lactate dehydrogenase, and 1−2 μg /mL calcium ionophore (A23187). SR vesicles were added to start the assay, and the absorbance of NADH was monitored at 340 nm to determine the rate of ATP hydrolysis. All assays were performed at 25 °C in a Thermomax microplate reader (Molecular Devices).

EPR Spectroscopy. EPR spectra were acquired with a Bruker ESP-300 X-band spectrometer using a Bruker ER4201 cavity, and they were digitized with the built-in microcomputer using Bruker OS-9-compatible ESP 1620 acquisition software. Conventional EPR spectra were obtained using 100 kHz field modulation (peak-to-peak amplitude 1 G), 0.1 G microwave field intensity, 100 G sweep width, 40 ms filter time constant, and 40 s scan time. Signal averaging was carried out until signal/noise was greater than 20, which typically required about 10 scans (400 s). Samples were contained in glass capillaries, at a PLB concentration of approximately 1 mg/mL. The sample temperature was controlled to within 0.1 °C with a nitrogen gas flow-temperature controller and monitored with a Sensortek Bat-21 digital thermometer using an IT-21 thermocouple probe inserted into the top of the capillary, such that it did not interfere with spectral acquisition.

Preparation of Boundary Lipid Samples. The peptides were reconstituted into dioleoylphosphatidylcholine (DOPC) vesicles that contain 1% of a spin-labeled phosphatidylcholine derivative, 14-PCSL (kindly provided by Derek Marsh), where the position-14 carbon of one of the acyl chains contains a spin label (doxyl group).

Spectral Analysis. EPR spectra of 14-PCSL were analyzed to determine the number of boundary lipids around the protein, as described previously (*3*, *28*). EPR spectra of this lipid spin label are sensitive to the rotational motion of the acyl chains. 14-PCSL incorporated in protein-free lipid vesicles yields EPR spectra that contain primarily a single mobile spectral component. With the addition of a transmembrane protein to the vesicles, some of the acyl chains become more motionally restricted, presumably due to steric interactions with the protein. EPR can resolve these two motional components, corresponding to bulk (not interacting with the protein) and boundary (interacting) lipid, and determine their mole fractions. A linear combination of model spectra is used to determine the relative mole fractions of the two motional components (*3*, *28*). When this is used in conjunction with the protein concentration, the number of boundary lipids (N_B) per transmembrane helix is calculated. An increase in the number of boundary lipids per protein, with the number of total lipids per protein unchanged, indicates a decrease in the degree of oligomerization (*3*). A smaller protein oligomer will have more of its surface area exposed to lipid, thus restricting more of the lipid acyl chains up to a maximum of approximately 6 boundary lipids around a solitary α-helix per leaflet of the bilayer for a total of approximately 12 total boundary lipids (*3*). Thus, the number of protomers (transmembrane helices) N per oligomer can be calculated (*3*).

Table 1: Amino Acid Analysis of TM-Ala-PLB and Ala-PLB[a]

	theoretical amino acid		synthetic amino acid	
amino acid	TM	full	TM	full
Asx	3	4	3.2	4.3
Glx	2	6	1.7	5.9
Ser	0	2		2.0
Thr	0	3		2.9
Ala	3	6	3.0	6.0
Arg	0	4		4.1
Val	1	2	0.6	1.9
Met	1	3	0.4	3.2
Phe	2	2	1.9	2.7
Ile	6	8	4.7	7.4
Leu	9	10	10.0	10.4
Lys	0	1		0.64
Pro	0	1		1.1

[a] Purified TM-Ala-PLB and Ala-PLB was hydrolyzed in 6 N HCl, at 110 °C, for 24 h. The data were normalized to Ala.

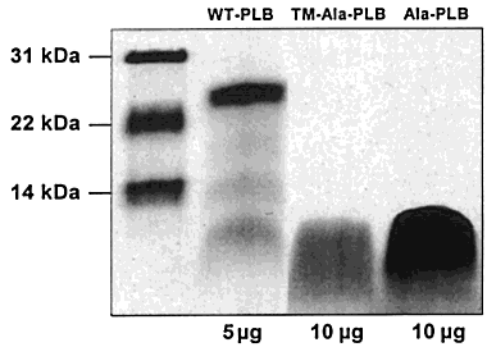

FIGURE 2: SDS−PAGE of synthetic PLB analogues at 25 °C.

RESULTS

Characterization of TM-Ala-PLB and Ala-PLB. Mass spectrometry for the TM domain of Ala-PLB yielded a molecular mass value of 3088.0 Da $[M + K]^+$, in agreement with the predicted value of M, 3049.9 Da. MS analysis of Ala-PLB corresponded to the expected molecular mass of 5919.5 Da $[M + H]^+$ (calculated 5918.4). Amino acid analysis (Table 1) was also consistent with the expected compositions for both peptides. The small deviations from the predicted values of Val and Ile can be attributable to incomplete hydrolysis of the hydrophobic TM-Ala-PLB. In addition, Met is partially destroyed during acid hydrolysis. Sequence analysis of the purified Ala-PLB analogue for seven cycles was consistent with the expected structure (Figure 1).

Oligomeric Sizes of PLB Derivatives on SDS−PAGE. We compared WT-PLB with the transmembrane domain of Ala-PLB (TM-Ala-PLB) and the full-length Ala-PLB on SDS−PAGE at 25 °C (Figure 2). WT-PLB runs primarily as a pentamer, with faint bands apparent at approximately 6 kDa

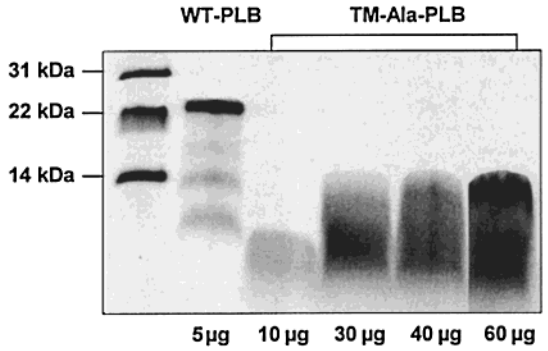

FIGURE 3: SDS−PAGE of synthetic WT-PLB and TM-Ala-PLB at 25 °C.

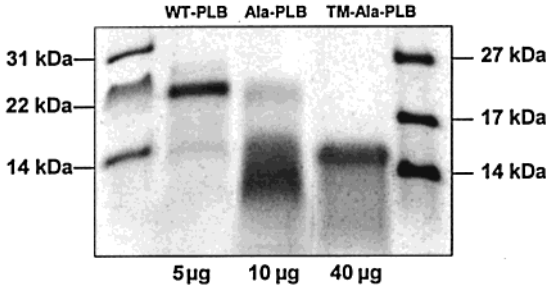

FIGURE 4: SDS−PAGE of WT-PLB, Ala-PLB, and TM-Ala-PLB at 4 °C.

(monomer), 12 kDa (dimer), and 18 kDa (trimer). TM-Ala-PLB (3 kDa) as well as Ala-PLB (6 kDa) run as monomers. Thus, replacement of all three transmembrane Cys residues with Ala greatly destabilizes the PLB pentamer in SDS solution.

The TM domain of Ala-PLB (TM-Ala-PLB) forms oligomers (probably pentamer) on SDS−PAGE when more than 10 μg of peptide is loaded on the gel (Figure 3), even though the full-length Ala-PLB is monomeric under the same conditions (not shown). This result demonstrates that the thiol groups of cysteine are not required for pentamer formation in the TM domain, even though they appear to be required in the full-length molecule.

Figure 4 compares the mobility on SDS−PAGE of Ala-PLB and its transmembrane domain (TM-Ala-PLB) at 4 °C. At low temperature, both species form oligomers. However, the full-length Ala-PLB remains predominantly monomeric, while its transmembrane domain is predominantly oligomeric (probably pentameric as compared to standards). This result suggests that the cytoplasmic domain destabilizes the PLB pentamer.

Oligomeric Sizes of PLB Derivatives by Boundary Lipid EPR. Figure 5 shows the low-field portion of the EPR spectra of 14-PCSL in DOPC bilayers containing PLB and its derivatives. The spin-label in these experiments is located at the 14th carbon on one of the two acyl chains, so any effects on the EPR spectra must be due to interactions deep in the hydrocarbon phase of the vesicles. The spectral positions having greatest sensitivity to the difference between the bulk and boundary components are marked by dashed arrows, so differences in these two regions indicate differences in overall oligomeric state. WT-PLB and TM-Ala-PLB show little difference, while the difference between these two and Ala-PLB is clearly significant. Since the EPR spectra indicate two distinct components, bulk and boundary

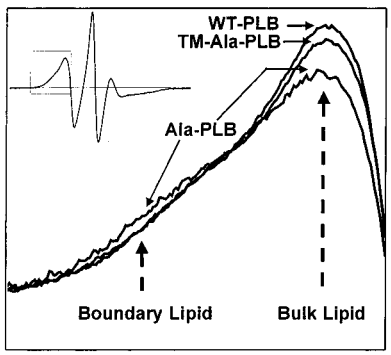

FIGURE 5: Low-field region (total 20G) of the EPR spectra of 14-PCSL in DOPC vesicles containing Ala-PLB, TM-Ala-PLB, and WT-PLB at 0 °C. The molar ratio of lipids per PLB protomer was 100. (Inset) Full 120 G WT-PLB spectrum with magnified region indicated by the box.

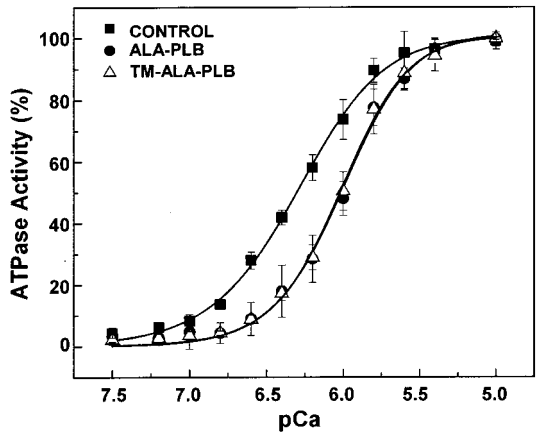

FIGURE 6: Inhibition of Ca-ATPase by (●) Ala-PLB ($n = 9$) and (△) TM-Ala-PLB ($n = 9$), at [PLB]/[Ca-ATPase] = 10. (■) Ca-ATPase only ($n = 9$).

lipid (*28*), the differences we see must arise from changes in the mole fractions of the two components. N_B, the number of boundary lipids per PLB protomer, was determined as the mean ± standard deviation ($n = 3$). N, the apparent number of protomers per oligomer (assuming a homogeneous population of oligomers) was calculated from N_B as described previously (*3*). TM-Ala-PLB ($N_B = 6.0 \pm 0.6$, average oligomer size $N = 3.3 \pm 0.4$) is very similar in oligomeric state to WT-PLB ($N_B = 5.6 \pm 0.5$, $N = 3.5 \pm 0.4$) (*3*), which has been shown to be primarily oligomeric in the membrane under these conditions (*3*). However, when the cytoplasmic domain of Ala-PLB is present, the protein is less oligomeric ($N_B = 7.8 \pm 0.3$, $N = 2.2 \pm 0.5$). We conclude that Ala-PLB is less oligomeric than WT-PLB and TM-Ala-PLB, in lipid bilayers (Figure 5) as well as in SDS solution (Figures 2−4).

Inhibitory Function of PLB Derivatives. Figure 6 shows the effects of Ala-PLB and its TM domain on Ca-ATPase activity as a function of Ca^{2+} concentration, measured in reconstituted membranes at a peptide/DOPC/ATPase molar ratio of 10:5500:1. Both Ala-PLB and Ala-TM-PLB decrease the activity of the Ca-ATPase below pCa 5.5, resulting in an increase in K_{Ca} (the calcium concentration, in pCa units, required for 50% activation). Ala-PLB and TM-Ala-PLB shifted K_{Ca} by -0.29 ± 0.03 (from 6.30 to 6.01) (Figure 6), which is similar to the effect of WT-PLB (from 6.63 to 6.31) (*16*). Thus, TM-Ala-PLB has the same inhibitory activity as

both WT-PLB and Ala-PLB. These results indicate that the TM domain of PLB is the critical component for inhibition of the pump, and they suggest that the oligomeric form of PLB is active.

DISCUSSION

We synthesized Ala-PLB and its transmembrane domain, in which the three Cys residues were replaced with Ala (Figure 1, Table 1). SDS−PAGE indicated that the TM domain of Ala-PLB is primarily pentameric, with an apparent molecular weight of 15 kDa (Figure 4), in contrast to the full-length Ala-PLB, which is mainly monomeric (6 kDa) (Figure 4).

Boundary lipid EPR experiments (Figure 5) on WT-PLB and TM-Ala-PLB yielded similar values for the level of oligomerization in DOPC bilayers, $N = 3.5 \pm 0.4$ and 3.3 ± 0.4, respectively, calculated from geometric considerations (*3*). The value calculated for WT-PLB differs from that for 100% pentamer (i.e., $N = 5$), presumably due to the presence of an equilibrium between monomer and pentamer in the lipid, as was found previously (*3*−*5*). We conclude that TM-Ala-PLB is in a similar equilibrium in lipid, since the predominant form on SDS−PAGE is probably pentamer (Figure 4).

There are two main possible sources of error in these boundary lipid experiments. The first arises from the geometric derivation of the relationship between the number of boundary lipids, N_B and the average oligomer size, N. The sensitivity of this method decreases with a corresponding increase in oligomer size (*3*). However, the relative changes between protein species are very reliable (*30*). A second source of error can arise from any possible differences in affinity for PLB between spin-labeled and nonspin-labeled phosphatidylcholines. Much previous work has shown that PC lipids have similar affinities for transmembrane helices, whether spin-labeled or not. In addition, all of the lipids cited in the literature that have affinities dependent upon the spin label have charged headgroups (*31*). Even if there is a perturbation due to the spin label, the same spin label was used in all experiments, so the relative differences between TM-Ala-PLB and Ala-PLB are still reliable.

It has been shown that a Leu/Ile zipper, involving leucines 37, 44, and 51, and isoleucines 40 and 47, is required for thermodynamic stability of the PLB pentamer (*3*, *6*). For TM-Ala-PLB, this zipper is sufficient to overcome the steric packing defects that are introduced by the three Cys-to-Ala mutations. However, in the full-length molecule, the cytoplasmic domain must introduce additional repulsive interactions that are sufficient to destabilize the pentamer. The most likely source of this destabilization is electrostatic repulsion due to the net charge of +3 in the cytoplasmic domain (Figure 7).

When Ala-PLB or its transmembrane domain, TM-Ala-PLB, were reconstituted with the ATPase in lipid bilayers, both PLB derivatives were found to decrease the apparent Ca-affinity (increase K_{Ca}) of the Ca-ATPase (Figure 6). The effects of the transmembrane domain Ala-PLB and the full-length Ala-PLB were similar to that of WT-PLB. This result supports the proposal that the TM domain is sufficient for Ca-ATPase inhibition, and helps to resolve a controversy in the literature. A report that a cytoplasmic domain fragment

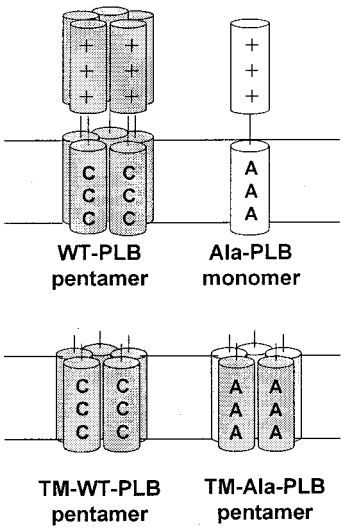

FIGURE 7: Schematic illustration of oligomeric states of the PLB derivatives in the present study.

of PLB inhibits the Ca-ATPase (*32*) was contradicted by subsequent reports (*14*, *26*). The present study shows that, in reconstituted lipid bilayers containing purified Ca-ATPase and lipid, the cytoplasmic domain has no effect on the inhibitory effect of Ala-PLB (Figure 6), suggesting that the transmembrane domain is the inhibitory domain of PLB, while the cytoplasmic domain plays its role in the reversal of inhibition upon phosphorylation. This result is in agreement with previous work (*16*).

Since TM-Ala-PLB has similar inhibitory activity and is more oligomeric than Ala-PLB, these results suggest that the inhibitory form of PLB is not necessarily monomeric. However, the oligomeric states were measured only in the absence of Ca-ATPase, and it is quite possible that the form of TM-Ala-PLB that binds to and inhibits the Ca-ATPase is the monomer, since it has been shown by fluorescence energy transfer that the Ca-ATPase depolymerizes PLB (*4*).

CONCLUSIONS

Although the substitution of all three Cys residues in PLB to Ala, producing Ala-PLB, results in substantial destabilization of the PLB pentamer, the TM domain of this molecule, TM-Ala-PLB, remains oligomeric and is probably a pentamer. Thus, although the Cys residues of WT-PLB contribute to the stability of the pentameric TM domain, the other attractive forces within the TM domain are sufficient to stabilize the pentamer. The cytoplasmic domain contributes repulsive forces, e.g., electrostatic repulsion between cationic residues that destabilize the pentamer. These are not sufficient to destabilize WT-PLB, but they are sufficient to destabilize Ala-PLB. TM-Ala-PLB has the same inhibitory activity as the full-length Ala-PLB, indicating that the cytoplasmic domain is not required for inhibitory activity. Since TM-Ala-PLB is more oligomeric than Ala-PLB, this result appears to contradict the model in which the monomeric species of PLB is the primary inhibitor of Ca-ATPase function. However, until the oligomeric state of TM-Ala-PLB is measured while bound to the Ca-ATPase, we cannot rule out that the species of TM-PLB bound to the pump is monomeric.

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