Biochemical and Biophysical Comparison of Native and Chemically Synthesized Phospholamban and a Monomeric Phospholamban Analog*

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Phospholamban (PLB) is an oligomeric membrane protein present in stoichiometric amounts associated with the Ca\textsuperscript{2+}-ATPase of cardiac sarcoplasmic reticulum (SR). PLB in its unphosphorylated state attenuates the catalytic activity of the Ca\textsuperscript{2+}-ATPase by reducing its apparent calcium sensitivity. Phosphorylation of PLB (1–3), treatment with antibodies directed against PLB (4–7), or mild trypsin proteolysis of PLB (8) reverses the decreased calcium sensitivity, leading to an increase in Ca\textsuperscript{2+}-ATPase activity at submicromolar Ca\textsuperscript{2+} concentrations. Co-reconstitution of PLB and Ca\textsuperscript{2+}-ATPase into liposomes (9, 10) or co-expression of the two proteins in COS cells (11) suggests that PLB itself is sufficient to modulate the Ca\textsuperscript{2+}-ATPase activity. Chemical cross-linking studies (12, 13) and co-expression experiments with Ca\textsuperscript{2+}-ATPase and site-directed PLB mutants (14) indicate that the NH\textsubscript{2} terminus of PLB is important for regulation of Ca\textsuperscript{2+}-ATPase activity and demonstrate the ability of monomeric PLB mutants to regulate Ca\textsuperscript{2+}-ATPase activity. Data supporting reversible and direct regulation of the calcium pump by PLB are abundant, yet the detailed mechanistic and structural requirements for PLB inhibition remain to be described.

The primary structure of PLB has been deduced from its cDNA (15). It is highly conserved among species, has an acetylated amino terminus, and consists of 52 amino acids with a predicted molecular mass of 6123 Da. Several secondary structure models (an example is shown in Fig. 1) have been proposed (13, 14, 16) with the following general features: (i) PLB is an amphipathic peptide with a hydrophilic NH\textsubscript{2} terminus, part of which is predicted to form an \(\alpha\)-helix whose exact length and position are uncertain, (ii) serine 16 and threonine 17 are the sites for protein phosphorylation by cAMP-dependent protein kinase (PKA) and calcium-calmodulin-dependent protein kinase, respectively, and (iii) the hydrophobic COOH-terminal amino acids (31–52) form a transmembrane \(\alpha\)-helical segment and are involved in protein oligomerization. The quaternary structure of PLB under non-denaturing conditions is assumed to be pentameric. PLB migrates as a homopentamer with an apparent M\textsubscript{o} of 28,000 in SDS-PAGE, which can be dissociated upon boiling into monomer subunits with an apparent M\textsubscript{o} of 6,000. Other oligomeric states are also detected. Pentameric complexes persist following trypsin cleavage of the NH\textsubscript{2}-terminal 26 amino acids of PLB (17), implying that the NH\textsubscript{2} terminus is not primarily responsible for association. Site-directed mutagenesis studies of PLB (11, 18–20) have identified numerous transmembrane amino acid residues that appear to be required for oligomerization. For example, leucine or serine replacement of the three cysteine residues located within the transmembrane domain at 5-amino acid intervals (36, 41, and 46) abolishes the protein’s oligomeric properties (11).

Detailed structural information about PLB is lacking because of the limited availability of highly purified PLB and its poor solubility. A variety of purification schemes have been employed using canine cardiac SR (21–24) with low PLB recoveries and sometimes disparate amino acid compositions. The low abundance of PLB in cardiac SR has led to other strategies for obtaining PLB for structural studies. PLB expressed in Escherichia coli spontaneously aggregated into pentamers, but ultimately PLB expression led to cell lysis and was not suitable for large scale production (25). Preliminary abstracts
added to the trifluoroacetic acid as a scavenger. Introduction of the NH₂-terminal acetyl group was carried out by coupling acetic acid (2 mM). Due to excessive swelling, half of the resin was removed following the coupling of Ala13, and the synthesis was continued. The final weight of the peptide resin (0.25 mmol) was 1.76 g. The resin was suspended in 3 ml of 1:1 (v/v) p-toluenesulfonic acid in an HF apparatus (Pierce Chemical Laboratories). The pH of the supernatant, and HF was condensed (30 ml). After stirring at 0–5 °C for 1.5 h, the reaction was evaporated. The residue was triturated with ether, filtered, and washed with additional ether. The filtered residue was extracted three times with 50 ml each of 1:1 acetic acid/H₂O and the filtrate lyophilized to yield 620 mg of crude product.

Crude PLB (300 mg) was dissolved in 20 ml of a 4:3:3 mixture of formic acid/H₂O/isopropanol alcohol and purified on an Asahipak® gel (Asahi Chemical Industries), ODP-200, 20-μm HPLC support cartridge (47 × 300 mm) PrepPak © (Waters). A step gradient (100-ml increments) was generated from 1 liter each of successively increasing concentrations (10%) of mobile phase (solvent A, 60% formic acid/H₂O; solvent B, 20% formic acid/isopropanol alcohol). A flow rate of 80 ml/min was used to elute the product. A distinct component that eluted at 70% B contained the desired product. Fractions containing purified PLB were pooled, concentrated, and lyophilized to yield 27 mg of PLB.

Preparation of Monoclonal Antibody, the Immunoaffinity Column, and Polyclonal Antisera—PLB peptide 1-25 synthesized as above was coupled to keyhole limpet hemocyanin using a carbodiimide cross-linking reagent. Mouse monoclonal antibody was prepared by procedures described in standard protocols. Purified mAb 1D11 was obtained from ascites fluid using an Immobilized Recomb® Protein A kit (Pierce) and subtyped to IgG2a. Coupling of 1D11 to Affi-Gel 10 was performed following the Bio-Rad instructions. Polyclonal antibody 94339 was produced in New Zealand white rabbits using the same antigen, purified from serum using Protein A affinity resin, and used for Western blot analyses.

Enzyme-linked Immunosorbent Assay Measurement—Cardiac SR (200 μl/well of 2 μg protein/ml in 10 mM sodium carbonate, pH 9.6) was incubated overnight at 4°C in a 96-well microtiter plate (Costar). The plate was rinsed with phosphate-buffered saline and blocked with 2% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Monoclonal antibody 1D11 (1 μg/ml) was added to each well, and 1 μg/ml of goat anti-mouse IgG conjugated to alkaline phosphatase (Pierce) for 1 h. Antibody binding was visualized by the addition of 2.5 μl p-nitrophenyl phosphate in 10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.5, and the absorbance was read at 405 nm.

2 Cys-to-Ser PLB refers to a PLB analog in which the three natural cysteine residues (36, 41, and 46) are replaced with serine residues. Single amino acid replacements are denoted as the natural residue, position, and replacement residues (i.e., C41F represents replacement of cysteine 41 by phenylalanine).
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determined using the Bradford method (Bio-Rad dye) with bovine serum albumin employed as a standard. Cardiac SR membranes were stored frozen at -70°C.

PLB was purified from cardiac SR using previously described extraction and solubilization procedures (24). The solubilized protein in Zwittergent 3-14 was applied to a mAb 1D11 immunoaffinity column with the Ca\(^{2+}\)X mAb bed volume pre-equilibrated with column buffer (10 mM MOPS/KOH, pH 7.0, 0.2% Zwittergent 3-14). Following extensive washing including a high salt wash, PLB was eluted with 50 mM citric acid, pH 2, 0.2% Zwittergent 3-14, and the elutants were neutralized in tubes containing 1/20th volume of 2 M Tris/HCl, pH 9.5. The peak PLB fractions, as assessed by silver staining and Western blot analysis, were precipitated in 2 volumes of ice-cold acetone (1:1). The precipitate was dried under vacuum and subsequently redissolved and applied to the reverse-phase HPLC column as described for s-PLB.

Characterization of n- and s-PLB—Synthetic PLB was solubilized in 0.2% Zwittergent 3-14, 20 mM dithiothreitol, and 10 mM MOPS, pH 7.0. PLB was incubated 1:1 in sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 100 mM dithiothreitol, 24% glycerol, and 0.002% bromphenol blue), boiled for 2–5 min immediately before loading, and electrophoresed on a 10–20% precast acrylamide gel (Integrated Separation Systems) using a Tricine buffer system. Protein was visualized with Coomassie Blue R250 or silver stain (Rapid-Ag-Stain, ICN) or transferred electrophoretically to 0.2 μm nitrocellulose membrane. The nitrocellulose was blocked with 3% nonfat dry milk in phosphate-buffered saline and reacted with a 1:1000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Cappel). Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for the alkaline phosphatase reaction to visualize the immunoreactive protein bands.

Phospholamban samples (400 ng) were incubated with 40 units of catalytic subunit of PKA in a reaction buffer containing 10 mM MOPS, 0.1% Zwittergent 3-14, and 5 mM MgCl\(_2\) for 10 min at 30°C in a total volume of 80 μl. The phosphorylation reaction was initiated by the addition of 20 μl of ATP (final concentration, 100 μM) containing 2 μCi of [γ\(^{32}\)P]ATP. After 10 min, the phosphorylation reaction was stopped by the addition of an equal volume of SDS sample buffer. The gel was dried after staining and subjected to autoradiography.

Amino Acid Compositional and Sequence Analysis—Samples were hydrolyzed with 6 N constant boiling HCl in sealed, evacuated vials at 110°C for 20 h. Amino acids were analyzed and quantified on a Beckman 6300 analyzer. NH\(_2\)-terminal sequence analyses were performed with an Applied Biosystems model 470A gas-phase sequenator equipped with a model 120A phenylthiohydantoin analyzer.

Mass Spectrometry—Mass analysis was performed with a Kratos Analytical MALDI-II laser desorption time-of-flight mass spectrometer. PLB (0.3 μl of ~10 μM) was applied to a sample slide with α-cyano-4-hydroxycinnamic acid (Aldrich; 1 μl of ~50 μM). Bovine insulin (Sigma; 0.4 μl of 10 μM) was added to the s-PLB sample slide as an internal standard for mass calibration. The sample was air dried prior to introduction into the instrument. Spectra were collected in the linear mode.

Circular Dichroism Spectra—CD spectra were collected with a jasco j-720 spectropolarimeter calibrated with camphorsulfonic acid. Three spectra were recorded at a 10 nm/min scan rate, baseline corrected, and averaged using a 1-mm path length quartz cuvette thermostated at 20°C. Spectra were collected using PLB concentrations of approximately 10 μM in 10 mM sodium phosphate, pH 7.0, with 0.3% C\(_2\)E\(_5\) and are expressed as molar ellipticity. Protein concentrations were verified by amino acid analysis.

Analytical Ultracentrifugation—Velocity sedimentation experiments were performed with a Beckman XL-A analytical ultracentrifuge equipped with an optical scanning detector. Data were collected in a Beckman An-60Ti rotor at 47,000 rpm and 20°C. The sedimentation profiles were monitored by the absorbance at 235 nm. PLB samples were solubilized in 0.1% C\(_2\)E\(_5\) and 10 mM MOPS, pH 7.1.

Reconstitution of PLB and Skeletal Muscle Ca\(^{2+}\) ATPase—Skeletal muscle Ca\(^{2+}\) ATPase was purified and reconstituted alone or with either native or synthetic PLB into proteoliposomes, and 45Ca\(^{2+}\) uptake in the absence and the presence of mAb 1D11 was measured as described (34).

RESULTS

Characterization of mAb 1D11 and Preparation of the 1D11 Affinity Column—Production of the PLB-specific monoclonal antibody 1D11 was required for construction of a PLB immunoaffinity column as well as a diagnostic probe for PLB reactivity.
SR (Fig. 2B) but has no effect on fast twitch skeletal muscle SR (data not shown). Maximum stimulation occurred at unitary antibody to PLB molar ratios. Treatment with 1D11 primarily increased the Ca$^{2+}$ sensitivity of the Ca$^{2+}$ ATPase, although sometimes a modest increase in $V_{\text{max}}$ was observed. The magnitude of the stimulation by 1D11 in the Ca$^{2+}$ ATPase and $^{45}$Ca uptake assay at each free calcium concentration ranged from about 10-fold at low free calcium to 0.2-fold at 1 $\mu$M free calcium. At very low free calcium concentrations (<31 nM) where large stimulation has been reported (7), calcium uptake in antibody-free cardiac SR was not measurable, but activity was discernible in the presence of 1D11. The biological effects of mononclonal antibody 1D11 were also similar to those reported with other stimulatory anti-PLB monoclonal antibodies such as A1 (4, 5), IIC2 (35), and 2D12 (6, 7, 36). Antibody effects are remarkably similar to those produced by PLB phosphorylation, although they tend to be somewhat greater in magnitude. Their ease of use and marked biological effects make these antibodies ideal tools for verifying the presence of PLB inhibition of Ca$^{2+}$ ATPase and studying its mechanism.

Purified 1D11 antibody was coupled to an Affi-Gel support resin (Bio-Rad). Multiple applications of protein to the column have been made with very little loss in performance. Results of a representative PLB purification are summarized in Table I. Cardiac SR was treated with sodium carbonate to extract calsequestrin with the loss of only trace amounts of PLB as detected by Western blot analysis. The extracted membranes were solubilized with deoxycholate, which was exchanged with Zwittergent 3-14 before being applied to the immunoaffinity column. The flowthrough fraction contained the majority of the protein with limited loss of PLB. A high salt wash removed several contaminants and only small amounts of PLB. The eluted fraction contained 1.4 mg of protein of which >80% was PLB. PLB readily precipitated upon addition of an equal volume of acetone/ethanol (1:1). This fraction was further purified using reverse-phase HPLC to yield a homogeneous PLB preparation.

| Fraction                  | Protein | Protein recovery | PLB content<sup>a</sup> |
|---------------------------|---------|------------------|-------------------------|
| Cardiac SR                | 383     | 100              | <1                      |
| Na$_2$CO$_3$-extracted SR | 210     | 55               | <1                      |
| DOC-solubilized protein   | 80      | 21               | <2                      |
| Zwittergent 3–14 solubilized | 71     | 19               | <2                      |
| mAb column eluate         | 1.4     | 0.4              | 80                      |
| Reverse-phase HPLC        | 0.9     | 0.2              | 100                     |

<sup>a</sup> PLB content estimated from silver-stained polyacrylamide gels.

Silver Stain  

Western Blot  

Phosphorylation  

Fig. 3. Characterization of native and synthetic phospholamban. Samples of purified PLB were electrophoresed without boiling or after boiling. The gel was silver-stained (left two panels) or electrophoresed onto nitrocellulose for Western blot analysis (middle two panels). Alternatively, the samples were prephosphorylated using [$\gamma$-32P]ATP and the catalytic subunit of PKA, then were visualized by autoradiography (right two panels). The positions of molecular mass standards are indicated on the far left side, and the positions of the monomer, pentamer, and catalytic subunit (PKA) are indicated on the right.

Comparison of Purified Native PLB with Chemically Synthesized PLB—Fig. 3 shows a side-by-side comparison of the two PLB proteins following SDS-PAGE. In all cases, the results for n- and s-PLB are indistinguishable. The first panels show a silver-stained gel before and after boiling. Both pentamer (28,000 Da) and monomer (6,000 Da) forms are present as well as a small amount of dimer (12,000 Da) and a higher order aggregate (>60,000 Da) in the nonboiled samples. After boiling, only the monomer and some dimer are present. The middle panels illustrate the results of Western blot analysis. The immunoblot confirms that all the silver-stained bands were derived from PLB. The results from autoradiography (final panels) show that PLB is phosphorylated using [$\gamma$-32P]ATP and exhibits the same electrophoretic behavior. In addition, auto-phosphorylation of the catalytic subunit of PKA is detected.

The purified proteins were subjected to amino acid analysis (Table II). The results were then compared with the predicted amino acid composition derived from the canine PLB cDNA. Determinations of tryptophan and cysteine were not made. The amino acid yields were normalized to threonine and corrected to an internal standard. A comparison of n- and s-PLB finds virtually identical compositions with only small deviations from predicted values. These deviations can be attributable to incomplete hydrolysis of these sterically hindered amino acid residues (Val, Ile, Leu, and Phe), which diminishes their recovery. In addition, methionine is partially destroyed during acid hydrolysis. The amino acid compositions also agree closely with reported values (22).

Because the amino termini are N-acetylated, the samples were deaved using cyanogen bromide (CNBr) to obtain microsequencing data. Fig. 1 indicates the position of the three methionine residues (1, 20, and 50) that constitute the CNBr cleavage sites. The cleaved samples were directly subjected to Edman microsequencing without purification. Two major internal PLB fragments (Table III), recovered in roughly equimolar amounts, were discernible with some minor contaminants recovered in the first few cycles. The recovery yields of the first eight cycles are listed and separated according to the predicted peptide sequence. Recoveries in cycles 19 and 20 are listed showing a small amount of methionine 20 (cycle 19) followed by additional PLB sequence, due to incomplete CNBr digestion.

Laser desorption mass spectral analysis of the two PLB samples yielded molecular mass values of 6123 ± 6 and 6126 ± 6 Da for n- and s-PLB, respectively (Fig. 4). These values agree with the predicted value of 6123 Da for acetylated PLB.

A synthetic full-length PLB molecule in which the three cysteine residues were replaced with serine was also chem-
cally synthesized using the same methods. Unlike s-PLB, Cys-to-Ser PLB does not oligomerize into pentamers, and only monomers are observed on SDS-polyacrylamide gels (Fig. 5). This PLB analog is still detectable by Western blot analysis and can be phosphorylated using the catalytic subunit of PKA. Its amino composition was confirmed as described above.

Structural Characterization of PLB and Cys-to-Ser PLB—CD spectra of PLB were recorded in the presence of various detergents. Spectra cannot be obtained in the absence of detergents because of the low solubility of PLB in aqueous solutions. Surfactants were chosen based on their unique charge characteristics: C12E8 (neutral), dodecylphosphocholine and Zwittergent 3-14 (dipolar), and SDS (negative). Independent of the surfactant used, the CD spectra possessed similar features with positive ellipticity centered at 193 nm and negative peaks at 209 and 222 nm characteristic of α-helical structure. CD spectra of native, synthetic, and Cys-to-Ser PLB in C12E8 are shown in Fig. 6. The spectra were analyzed for secondary structure content by a constrained least squares fitting procedure (37) and a self-consistent algorithm (38). The data are summarized and compared with other values in Table IV. Both methods show that PLB is predominantly α-helical (60–70%), with the balance of the protein containing a mixture of β- and less ordered structure. Overall, little major difference in secondary structure can be resolved between n- and s-PLB because these values are within the uncertainty of the computational methods. In contrast, Cys-to-Ser PLB possesses a CD spectrum that is significantly different from that of n- or s-PLB (Fig. 6). A minimum is observed at 210 nm, and a single positive peak at 194 nm. Analysis of this spectrum for secondary structure content by either method yielded the same results and suggests a substantial decrease in helical content with increases in β- and disordered structures.

PLB samples were also examined by analytical ultracentrifugation to determine their association state under conditions that more closely mimic a membrane environment than those used for SDS-PAGE. In a sedimentation velocity experiment, PLB diffusely migrated with a mean sedimentation coefficient of 4.8 S suggestive of an oligomeric species. Under the same conditions, the Cys-to-Ser PLB did not sediment, as predicted.

### Table II

| Amino acid | Theoretical* | Native | Synthetic |
|------------|--------------|--------|-----------|
| Asx        | 4            | 3.92   | 3.86      |
| Thr        | 2            | 2.00   | 2.00      |
| Ser        | 2            | 2.00   | 2.02      |
| Gix        | 6            | 6.05   | 6.00      |
| Ala        | 3            | 2.99   | 2.98      |
| Val        | 2            | 1.30   | 1.26      |
| Met        | 3            | 1.60   | 1.29      |
| Ile        | 8            | 3.39   | 3.29      |
| Leu        | 10           | 5.76   | 5.53      |
| Tyr        | 1            | 0.92   | 0.83      |
| Phe        | 2            | 1.52   | 1.44      |
| Lys        | 1            | 0.96   | 0.92      |
| Arg        | 4            | 3.92   | 3.79      |
| Pro        | 1            | 0.98   | 1.10      |
| His        | 0            | n.d.   | n.d.      |
| Gly        | 0            | n.d.   | n.d.      |
| Cys        | 3            | 0.83   | 0.83      |
| Trp        | 0            |        |           |

*Theoretical values are derived from the cDNA sequence.

### Table III

| Cycle No. | Native PLB Amino acid (nmol) | Synthetic PLB Amino acid (nmol) |
|-----------|------------------------------|---------------------------------|
| 1*        | D (1.41)                     | P (1.51)                        |
| 2         | K (1.26)                     | Q (1.14)                        |
| 3*        | V (2.88)                     | Q (1.23)                        |
| 4*        | Q (1.47)                     | A (1.46)                        |
| 5         | Y (1.54)                     | R (0.45)                        |
| 6         | L (2.54)                     | Q (0.63)                        |
| 7         | T (1.32)                     | L (1.21)                        |
| 8         | R (0.39)                     | L (1.42)                        |
| 9         | S                            | Q                               |
| 10        | A                            | N                               |
| 11        | I                            | L                               |
| 12        | R                            | F                               |
| 13        | R                            | I                               |
| 14        | A                            | N                               |
| 15        | S                            | F                               |
| 16        | T                            | I                               |
| 17        | I                            | L                               |
| 18        | E                            | I                               |
| 19        | M (0.20)                     | L (0.60)                        |
| 20        | P (0.12)                     | I (0.62)                        |
| 21        | Q                            | Q                               |
| 22        | Q                            | Q                               |
| 23        | A                            | A                               |
| 24        | L                            | L                               |
| 25        | Q                            | Q                               |
| 26        | N                            | N                               |
| 27        | L (trace)                    | I (trace)                       |
| 28        | Q (trace)                    | Q (trace)                       |
| 29        | N                            | V                               |
| 30        | L (trace)                    | I (trace)                       |
| 31        | F                            | I                               |
| 33        | N                            | N                               |
| 34        | F                            | F                               |
| 35        |                              |                                 |

*Small amounts of contaminants were detected in these cycles as follows: native PLB, cycle 1, T (0.18) and Y (0.02) and synthetic PLB, cycle 1, T (0.26) and Y (0.10); cycle 3, I (0.05); cycle 4, R (0.07) and Y (0.03). Nondigested protein samples yielded no sequence due to the acetylated N-methionine.
for monomeric PLB, implying little or no self-association.

Functional Characterization of PLB—The ability of n-PLB and s-PLB to regulate Ca\(^{2+}\) ATPase was assessed by co-reconstitution with purified skeletal muscle Ca\(^{2+}\) ATPase. Reconstitution into phosphatidylcholine/phosphatic acid (weight ratio, 10:1) vesicles yielded the best results (34). Even though this system leads only to partial co-reconstitution as compared with cardiac SR, PLB decreased the calcium sensitivity without altering the \(V_{\text{max}}\) of the Ca\(^{2+}\) ATPase as occurs in cardiac SR. Moreover, these effects were reversed by treatment with the anti-PLB monoclonal antibody 1D11. The effects of the PLB samples and the reversibility by treatment with anti-PLB antibody 1D11 are summarized in Table V and compared with the results with recombinant-PLB (34). The rates of spare \(^{45}\text{Ca}^{2+}\) uptake and the activity relative to Ca\(^{2+}\) ATPase without PLB at pCa 6.8 and pCa 5.5 are also shown. The rates of spare \(^{45}\text{Ca}^{2+}\) uptake at a saturating calcium concentration (pCa 5.5) were determined to verify that similar amounts of Ca\(^{2+}\) ATPase were incorporated and retained activity in the proteoliposomes. In all cases, high levels of activity were present and as expected anti-PLB antibody 1D11 had no effect on the maximal activity. Inclusion of PLB did not reduce the activity at pCa 5.5, indicating minimal nonspecific inhibition like that observed with large amounts (molar excess, >100:1) of PLB 1-25 (39). \(^{45}\text{Ca}^{2+}\) uptake at a sub saturating free calcium concentration (pCa 6.8) was diminished by 19 and 35% in the presence of either n-PLB or s-PLB, respectively. Most importantly, treatment of these proteoliposomes with anti-PLB antibody 1D11 reversed the PLB-induced inhibition of Ca\(^{2+}\) ATPase activity to near control levels.

DISCUSSION

Co-expression studies (14) have provided some insight into the nature of the interaction between PLB and Ca\(^{2+}\) ATPase, including identification of the regions of both proteins involved in PLB's regulation of Ca\(^{2+}\) ATPase activity. Ultimately, knowledge of the three-dimensional structure of PLB and the Ca\(^{2+}\) ATPase will be required for understanding the reversible inhibition elicited by PLB. Detailed structural information on intact native PLB is lacking, even though its small size makes it an attractive molecule for structural analyses. Major limitations have included the lack of availability of large amounts of highly purified PLB and its poor solubility. The ability to chemically synthesize a native form of PLB should eliminate the first problem. Because it is critical to verify the biochemical integrity of s-PLB by comparison to native protein, sufficient quantities of highly purified n-PLB are necessary to establish its biochemical properties.
The use of a 1D11 immunoaffinity column facilitated the purification of n-PLB by dramatically decreasing isolation time and improving the yield of highly purified protein as compared with procedures using sulfhydryl affinity chromatography (21, 22, 24). The immunoaffinity column proved to be very efficient at purifying solubilized PLB because more than 90% of the PLB bound to the column was recovered. PLB comprises about 0.3% of the total cardiac SR protein (40), which makes it a poor source of material for large scale purification. In addition, solubilization of PLB from the SR membrane is inefficient, with at least a third of the PLB remaining in the insoluble SR pellet. The overall protein recovery from the immunoaffinity column of 0.36% is better than the previously reported values of 0.2 (40) and 0.19% (22), and 10-fold more protein was used. Some minor contaminants were also recovered requiring the use of a reverse-phase HPLC column to obtain a homogeneous PLB sample. The purity of n-PLB was verified by mass spectral analysis, amino acid composition, and microsequencing. Final recovery was 0.2%. The PLB immunoaffinity column will allow rapid purification from richer sources as well as mutant PLBs from expression systems. In a preliminary report, the immunoaffinity column has been successfully used to purify PLB expressed in yeast (26), although solubilization of PLB from the yeast membrane limits the yield.

### Table IV

| PLB Source | α-helix | β-structure | Other | Reference   |
|------------|---------|-------------|-------|-------------|
| Native     | 78      | 22          | 0     | 43          |
|            | 62      | 22          | 16    | This report |
|            | 68      | 11          | 21    | This report |
| Synthetic  | 68      | 14          | 18    | 28          |
|            | 65      | 14          | 21    | This report |
| Cys-to-Ser | 71      | 29          | 0     | This report |
|            | 31      | 37          | 32    | Both methods |

^a Chen and Yang method (37).
^b Self-consistent method (38).

### Table V

| Condition | 45Ca2⁺ Uptake (nmol/mg/min) at 25°C | pCa 6.8 Uptake (nmol/mg/min) | Control |
|-----------|-----------------------------------|-----------------------------|---------|
| Ca²⁺ ATPase alone | 160 100 3017 100 | 166 104 3226 107 |
| + mAb 1D11 | 148 93 3342 111 | 130 81 3242 107 |
| + Synthetic PLB | 173 108 3388 112 | 148 93 3342 111 |
| + Native PLB | 270 100 4870 100 | 250 93 4600 94 |
| + Cys-to-Ser PLB | 250 93 4730 97 | 130 48 4730 97 |

^a These data were adapted from Reddy et al. (34).
uptake at pCa 6.8 without affecting the maximal activity at pCa 5.5 and, importantly, the addition of anti-PLB mononal antibody abolished the inhibition. Complete pCa curves for calcium-stimulated ATP hydrolysis in the absence and the presence of anti-PLB monoclonal antibody were constructed for Ca\textsuperscript{2+} ATPase co-reconstituted with s-PLB (data not shown). Whereas with cardiac SR, monoclonal antibody produces a 0.2–0.3-pCa unit left-ward shift in the pCa curve (see Fig. 2, A and B), in the reconstituted system with s-PLB only a 0.05-pCa unit shift was observed as was reported with recombinant-PLB (34). Thus, optimal reconstitution conditions have not been found, but in our hands, these were the best yet obtained.

Secondary Structure of PLB—PLB has very poor aqueous solubility but dissolves readily in Me\textsubscript{2}SO, chloroform, trifluoroethanol, 50% acetic acid, or formic acid. Limited solubility can be achieved using mild detergents such as 0.1% Zwittergent 3-14, 0.1% octyl \(\beta\)-D-glucopyranoside, or 0.1% C\textsubscript{12}E\textsubscript{8}. Because these amphiphiles more closely mimic natural membrane environments, they are often used for analyzing the structural properties of membrane proteins like PLB. A hydrophathy plot of the primary structure of PLB suggests that it is an amphipathic protein and should adopt a highly helical structure. CD spectra suggest that roughly 70% of PLB is in an \(\alpha\)-helical conformation, whereas the presence of other types of secondary structure is less well defined. Similar secondary structural compositions (Table IV) have been derived from CD spectra of n-PLB (43) and s-PLB (28). All of these results, including those in this work, generally agree. Small differences in structural content appear to arise from the methods used to analyze the CD spectra rather than any real differences in PLB structure. Many of the \(\alpha\)-helical residues probably reside in the COOH-terminal hydrophobic region (amino acid residues 31–52) that is thought to form a transmembrane helix (19). Much of the NH\textsubscript{2} terminus, however, must also be \(\alpha\)-helical in order to account for the high total \(\alpha\)-helical content.

The structure of the NH\textsubscript{2} terminus has not been clearly defined. Secondary structural models (1, 13, 14) predict helices of varying lengths. Based upon data from homonuclear NMR spectroscopy, chemical shift assignments have recently been obtained for PLB\textsubscript{1–25} in 30% trifluoroethanol/H\textsubscript{2}O (44). Using sequential and medium range nuclear Overhauser effect connectivities and secondary C\textsubscript{\beta} shifts as criteria, residues 1–17 appear to form a regular \(\alpha\)-helix. Other work has also shown that the NH\textsubscript{2} terminus is capable of forming an \(\alpha\)-helical structure in trifluoroethanol (PLB\textsubscript{1–25}) and PLB\textsubscript{1–32} (46) and in charged detergents (PLB\textsubscript{1–25}). The NH\textsubscript{2}-terminal PLB peptide, PLB\textsubscript{1–25}, possesses little structure in aqueous buffer solution (40, 44). Likewise, PLB\textsubscript{1–31} (45) showed predominantly disordered structure. Nevertheless, it has been reported that PLB\textsubscript{1–25} (9, 46) and PLB\textsubscript{1–31} (47) can regulate purified and reconstituted Ca\textsuperscript{2+} ATPase. Presumably, interaction with itself, membranes, or the Ca\textsuperscript{2+} ATPase may stabilize the secondary structure of the NH\textsubscript{2}-terminal region facilitating functional association.

Secondary Structure of Cys-to-Ser PLB—The three cysteine residues (36, 41, and 46) in the transmembrane domain of PLB are essential for stability of the pentameric structure observed during SDS-PAGE (11, 19). Using site-directed mutagenesis, all of these cysteines have been replaced with alanine, and essentially no pentameric structures are observed with this mutant. Single cysteine replacements lead to intermediate pentameric stabilities with C41A PLB possessing the weakest stability. Single replacements with serine had similar effects with C41S PLB having the poorest stability. Replacement of all three cysteine residues with serine again completely abolished oligomerization. A monomeric C41F PLB mutant retained the ability to inhibit the Ca\textsuperscript{2+} ATPase when the two proteins were co-expressed in mammalian cell systems (14). Scanning alanine mutagenesis (43) and random mutagenesis (19) suggests that other transmembrane amino acids (L37, L40, L44, and I47) are also necessary for pentamer formation. Some of these monomeric PLB mutants (46) functionally altered Ca\textsuperscript{2+} ATPase activity when co-expressed in SF21 cells. Therefore, the Cys-to-Ser PLB should be useful from a functional perspective and can potentially be used to separate the effects of oligomerization from direct interaction of PLB with the Ca\textsuperscript{2+} ATPase.

Cys-to-Ser PLB possesses about half of the \(\alpha\)-helical content of natural PLB in C\textsubscript{12}E\textsubscript{8}. Upon first analysis, this result was somewhat surprising because serine and cysteine are thought to be indifferent helix formers. Although there is as yet no entirely reliable predictive algorithm for determining peptide secondary structure, neither the Chou and Fasman (48) nor Kyte and Doolittle (49) methods predict a major disruption in helix structure for these substitutions. Serine can make shared H bonds within neighboring backbone NH or CO groups or more typically with solvent in exposed locations on turns and loops that can destabilize the \(\alpha\)-helix (50). Assuming that the NH\textsubscript{2}-terminal region is unaffected, the serine replacements appear to essentially abolish formation of the putative transmembrane \(\alpha\)-helix. Thus, the finding that Cys-to-Ser PLB does not oligomerize is easily rationalized.

The relative sedimentation profiles of PLB and Cys-to-Ser PLB indicate that the results from SDS-PAGE experiments accurately reflect actual solution conditions. The sedimentation results are in agreement with those obtained by Vorherr et al. (28), who examined a variety of aggregate complexes under various experimental conditions. Attempts to form monomeric PLB or to disrupt the PLB aggregates by boiling the sample in the presence of 10 mM dithiothreitol prior to velocity ultracentrifugation were unsuccessful. However, Cys-to-Ser PLB, which ran solely as a monomer in SDS-PAGE, failed to sediment under the same conditions used for natural PLB, suggesting that the protein is monomeric.

Closing Comments—High resolution structural information about PLB and the Ca\textsuperscript{2+} ATPase will be essential in defining the mechanism of PLB regulation of calcium uptake and ATP hydrolysis. Elucidation of protein structure requires large amounts of highly purified protein as a starting point. s-PLB behaves identically to n-PLB in the tests described in this report and therefore should be appropriate for further study because it is much easier to obtain large quantities of the synthetic protein. Through a combination of chemical synthesis and high volume expression/m hashmap affinity purification systems, sufficient amounts of PLB should be attainable for higher resolution structural analysis.

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Note Added in Proof—A similar analysis of the secondary structure of PLB\textsubscript{1–25} has been published (Hubbard, J. A., MacLachlan, L. K., Meenan, E., Salter, C. J., Reid, D. G., Lahouratate, P., Humphries, J., Stevens, N., Bell, D., Neville, W. A., Murray, K. J., and Darker, J. G. (1994) Molec. Membr. Biol. 11, 263–269). Full-length PLB in supported bilayers was 64–67% \(\alpha\)-helical which decreased to 54% upon phospholipidation (Tatulian, S. A., Jones, L. R., Reddy, L. G., Stokes, D. L., and Tamm, L. K. (1995) Biochemistry 34, 4448–4456).

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