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Overexpression, purification, and characterization of recombinant Ca-ATPase regulators for high-resolution solution and solid-state NMR studies☆

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Abstract

Phospholamban (PLB) and Sarcolipin (SLN) are integral membrane proteins that regulate muscle contractility via direct interaction with the Ca-ATPase in cardiac and skeletal muscle, respectively. The molecular details of these protein–protein interactions are as yet undetermined. Solution and solid-state NMR spectroscopies have proven to be effective tools for deciphering such regulatory mechanisms to a high degree of resolution; however, large quantities of pure recombinant protein are required for these studies. Thus, recombinant PLB and SLN production in *Escherichia coli* was optimized for use in NMR experiments. Fusions of PLB and SLN to maltose binding protein (MBP) were constructed and optimal conditions for protein expression and purification were screened. This facilitated the large-scale production of highly pure protein. To confirm their functionality, the biological activities of recombinant PLB and SLN were compared to those of their synthetic counterparts. The regulation of Ca-ATPase activity by recombinant PLB and SLN was indistinguishable from the regulation by synthetic proteins, demonstrating the functional integrity of the recombinant constructs and ensuring the biological relevance of our future structural studies. Finally, NMR spectroscopic conditions were established and optimized for use in investigations of the mechanism of Ca-ATPase regulation by PLB and SLN.

Keywords: Membrane protein overexpression; Maltose binding protein; Recombinant phospholamban; Recombinant sarcolipin; Solution NMR; Solid-state NMR; Micelles

The integral membrane proteins phospholamban (PLB) and sarcolipin (SLN) regulate contraction in cardiac and skeletal muscle, respectively [1]. PLB, a 52 amino acid membrane protein, and SLN, a 31 amino acid membrane protein, interact with the sarcoplasmic reticulum Ca-ATPase to regulate calcium uptake in the sarcoplasmic reticulum. Aberrant regulation of myocyte calcium concentration has been associated with various myopathies [2,3]. However, the mechanistic details of Ca-ATPase interaction with PLB and SLN remain elusive, warranting the need for high-resolution structural studies.

Nuclear magnetic resonance (NMR) protein structural investigations require milligram quantities of highly purified, isotopically ^{13}C- and ^{15}N-labeled proteins. Expression in insect cells has been used to produce milligram quantities of pure and biologically active PLB, and solid-phase peptide synthesis has produced even larger yields of pure and active PLB [4] and SLN [5], but neither of these methods can be used to produce uniform isotopic labeling that is affordable. In addition, it is time-consuming to use either of these methods to produce a variety of mutant proteins or proteins containing selective isotopic labeling. *Escherichia coli* is generally the system of choice for

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recombinant protein production due to the ease of its manipulation in isotopically enriched media; however, various parameters must be empirically optimized for each target protein (i.e., DNA construction, growth conditions for protein expression, and protein purification) [6]. Production of membrane proteins poses additional constraints due to their highly hydrophobic nature, which often causes insoluble protein aggregates that are difficult to purify, low yields of pure protein, and toxicity to the host cell [7].

Various systems have been developed to overcome the difficulties inherent in heterologous membrane protein expression in *E. coli*. Bacterial cells have been derived that enhance membrane protein accumulation and reduce proteolysis [8]. Fusion partners have also been incorporated to facilitate high yields of solubilized membrane protein and to aid in their purification [9]. In addition, the formation of inclusion bodies upon expression of certain fusion proteins has been utilized to protect the target against proteolysis and circumvent its cellular toxicity [6]. Methodologies using Gram-positive bacteria, as an alternative to *E. coli*, have also been established [10].

In this study, the *E. coli* strain BL21(DE3) and a maltose-binding protein (MBP) fusion partner were used to produce the recombinant membrane proteins AFA-PLB and wt-SLN. AFA-PLB is a fully functional mutant of PLB where cysteines 36, 41, and 46 of the PLB sequence have been changed to alanine, phenylalanine, and alanine, respectively, to maintain PLB in its monomeric form [11]. While these mutations prevent the formation of pentameric PLB, they confer the same inhibitory activity of PLB [11]. MBP was chosen because it has been shown to increase the solubility of hydrophobic membrane proteins while increasing their resistance to degradation by host cell proteases [12,13]. Since the expression products are soluble, harsh denaturing steps can be avoided. In addition, MBP-fusion proteins are easily purified in one step under relatively mild conditions using an amylose resin affinity chromatography system with a competitive maltose elution step [14]. In this report, the conditions for uniform isotopic labeling of both AFA-PLB and wt-SLN for solution and solid-state NMR investigations of Ca-ATPase regulation are summarized.

Materials and methods

Synthesis of DNA primers and DNA sequencing were all performed at the University of Minnesota Microchemical Facility.

Construction of wt-SLN and AFA-PLB expression plasmids

The wt-SLN gene insert was constructed by polymerase chain reaction (PCR) using two overlapping oligonucleotides in which all codons were optimized for usage in *E. coli*. The 5′-oligonucleotide incorporated an extended *Eco*RI site and a thrombin protease cleavage site, while the 3′-oligonucleotide included two stop codons and an extended *Hin*dIII. The sequence of the forward primer was: 5′-CCG GAA TTC CTG GTT CCG CGT GGA TCC ATG GGC ATT AAC ACC CGC GAA CTG TTT CTG AAC TTT ACC ATT GTG C-3′. The sequence of the reverse primer was: 5′-CCC AAG CTT TTA TTA ATA CTG ATA GCT GCG CAC CAG CAG CCA CAT CAG AAT CAC GGT AAT GAG CAC AAT GGT AAA GTT CAG AAA CAG TTC CGC GG-3′. PCR was carried out in a total volume of 100 μl with a final concentration of 0.5 μM of each oligonucleotide, 200 μM dNTP mix (Promega), 10× buffer (10 μl), and 5.0 U *Pfu Turbo* DNA polymerase (Stratagene). DNA was amplified using 25 cycles with annealing at 60 °C and elongation at 72 °C in a Perkin–Elmer GeneAmp PCR system 2400.

The AFA-PLB insert also had all codon usage optimized for *E. coli* expression. The nucleic acid sequence was partitioned into four overlapping primers. Three PCRs were performed as described above. Reaction 1 consisted of the two overlapping primers: 5′-ATG GAA AAA GTG CAG TAT CTG ACC CGC AGC GCG ATT CGC CGC GCG AGC ACC ATT GAA ATG-3′ and 5′-AAT AAA CAG GTT CTG CAG GTT CTG GCG CGC CTG CTG CGG CAT TTC AAT GGT GCT CGC GCG-3′. Reaction 2 included primers: 5′-AAC CTG CAG AAC CTG TTT ATT AAC TTT GCG CTG ATT CTG ATT TTT CTG CTG CTG ATT GCG-3′ and 5′-CGC AAG CTT TTA TTA CAG CAG CAT CAC AAT AAT CGC AAT CAG CAG CAG AAA AAT CAG-3′. Reaction 3 amplified the full AFA-PLB gene sequence from overlapping PCR products of the first two reactions. Two more rounds of PCR incorporated a TEV protease cleavage site and an *Eco*RI restriction site at the 5′end, and a *Hin*dIII site at its 3′ end, using the following primers: 5′-G TAT TTT CAG GCC ATG GAA AAA GTG-3′ and 5′-CGC GAA TTC GAA AAC CTG TAT TTT CAG-3′.

Wt-SLN and AFA-PLB PCR products were ligated into *Eco*RI and *Hin*dIII sites on a maltose binding protein (MBP) vector (New England Biolabs) using T4 DNA ligase (Promega). Ligated products were used to transform XL1-Blue competent cells (Stratagene) and transformants were selected by growth on Luria–Bertani (LB)/ampicillin plates. All sequences were confirmed by DNA sequencing.

wt-SLN and AFA-PLB expression

E. coli BL21(DE3) (Novagen) cells were transformed with wt-SLN or AFA-PLB fusion constructs. A 100 ml starter culture containing LB growth media supplemented with 50 μg/ml ampicillin was inoculated with a

single wt-SLN or AFA-PLB colony, and agitated for ~16 h at 250 RPM at room temperature (RT) to OD_{600} of ~1.0. For wt-SLN expression, bacteria were diluted (1:50) into standard M9 minimal media containing [^{15}N]ammonium chloride and grown at 37 °C until $OD_{600} \sim 0.6$ and at RT to $OD_{600} \sim 0.8$. Protein expression was induced with IPTG (1 mM) and cells were harvested after 16 h of induction. AFA-PLB was diluted (1:50) into M9 media containing mineral and vitamin supplements (Minerals: 6 mg/L $CaCl_2$, 6 mg/L $FeSO_4$, 1 mg/L $MnCl_2$, 0.8 mg/L $CoCl_2$, 0.7 mg/L $ZnSO_4$, 0.3 mg/L $CuCl_2$, 0.02 mg/L H_3BO_3, 0.25 mg/L $(NH_4)_6MO_7O_{24}$, and 5 mg/L EDTA; Vitamins: 1 mg/L calcium pentothenate, 1 mg/L biotin, and 1 mg/L folic acid, 1 mg/L niacinamide, and 1 mg/L pyridoxal phosphate) and grown at 37 °C to an $OD_{600} \sim 1.0$. Protein expression was induced with IPTG (1 mM) for 6 h at 37 °C. All cells were harvested by centrifugation at 6370*g* for 20 min at 4 °C. Pellets were stored at −20 °C.

Cell lysis

Wt-SLN pellets were resuspended on ice in 200 ml lysis buffer (20 mM PBS, pH 7.3, 120 mM NaCl, 8 mM EDTA, 0.1 mM DTT, 52.6 mM glycerol, 0.5 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 2.5 μM lysozyme, and 4.5 mM Tween 20). The suspension was homogenized with a cell grinder and sonicated using a Branson sonifier 450 (output of 4, duty cycle 45%) for 10 min. Cell debris was pelleted by centrifugation at 45,700*g* for 20 min at 4 °C and the supernatant was collected for purification. AFA-PLB pellets were lysed in the same manner as wt-SLN using an alternative lysis buffer (20 mM PBS, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 52.6 mM glycerol, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 2.5 μM lysozyme, 0.5 mM PMSF, and 8 mM Triton X-100).

Protein purification

After lysis, pooled supernatant containing MBP fusion protein (either wt-SLN or AFA-PLB) was applied to an amylose resin affinity chromatography column at 4 °C (New England Biolabs; 166 nmol protein/mol resin). The column was washed (12 column volumes) with buffer (20 mM PBS, pH 7.3,120 mM NaCl, 1 mM EDTA, and 3.1 mM NaN_3). Fusion protein was eluted with 250 ml maltose buffer (46 mM maltose in 20 mM PBS, pH 7.3, 120 mM NaCl, 1 mM EDTA, and 3.1 mM NaN_3). For wt-SLN, cleavage was performed using 30 U/mg thrombin (Calbiochem) at 28 °C for 14 h. The cleavage reaction was stopped (1.6 mM PMSF, 16 mM DTT, 4 μg/ml pepstatin A, and 4 μg/ml leupeptin) and wt-SLN was isolated via an S-100 Sephacryl gel filtration column using an AKTA*prime* liquid chromatography system (Amersham Pharmacia Biotech; 20 mM PBS, pH 7.3, 120 mM NaCl, 3 mM DTT, 0.05 mM PMSF, 4 mM SDS, and 3.1 mM NaN_3). For AFA-PLB, cleavage was accomplished with TEV protease (Invitrogen) at 100 U/mg at 30 °C for ~10 h. SDS present in the FPLC column buffer was utilized to stop the TEV cleavage reaction. AFA-PLB was isolated from MBP similar to wt-SLN, except that 1 mM EDTA was also included in the buffer. SDS–PAGE and Coomassie/silver staining identified fractions containing pure wt-SLN or AFA-PLB. Pure fractions were pooled and dialyzed against 4 L of water to remove the detergent. The protein was lyophilized and stored at −20 °C.

Quantification and identification

Total protein concentration at each purification step was assessed via protein absorbance at 280 nm, or assayed with densitometry measurements of Coomassie- or silver-stained gels. Densitometry data were collected on a Bio-Rad Molecular Imager FX using Bio-Rad Quantity One quantitation software. The final protein concentrations of AFA-PLB were determined by comparison to a standard curve of 1, 2, and 3 μg synthetic AFA-PLB standard previously quantified by amino acid analysis. MBP/wt-SLN fusion protein concentration, based on absorption at 280, was used as a reference to assess the final wt-SLN concentration from SDS–PAGE by comparative densitometric volumes. Pure AFA-PLB was confirmed with MALDI-MS and amino acid sequence analysis, while pure wt-SLN was confirmed by MALDI-MS.

Peptide synthesis

Synthesis of AFA-PLB was performed as described [15]. Synthesis of wt-SLN was performed by following stepwise Fmoc solid-phase peptide synthesis and HPLC purification protocols similar to those described previously for PLB [4]. Starting with Fmoc-Tyr(tBu)-PAC-PEG-PS resin (0.75 g, loading 0.16 mmol/g), the 31 residue SLN was assembled on PE Biosystems Pioneer automated peptide synthesizer. Acidolyzable side-chain protecting groups were Pmc for Arg, Trt for Asn and Gln, O*t*Bu for Glu, and *t*Bu for Ser, Thr, and Tyr. Fmoc removal was achieved with 20% piperidine and 2% DBU in NMP. The activation of the coupling was performed with HBTU/HOBt/DIEA. Final deprotection was performed by treatment of 200 mg peptide resin with 2 ml of freshly prepared reagent R: 90% TFA, 5% thioanisole, 3% of 1,2-ethanedithiol, and 2% anisole, for 4 h at 25 °C. The cleavage mixture was filtered and 30 ml 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 60% aqueous TFA and purified by HPLC on a Diphenyl column (Vydac, 219TP152022; 15–20 μm, 300 Å; 22 × 250 mm) with a linear AB gradient. The

mobile phase A was 0.1% aqueous trifluoroacetic acid in water and B was 0.1% TFA in acetonitrile (eluent B). The peptides were characterized by mass spectrometry (MALDI-TOF) and amino acid analysis (Hewlett Packard AminoQuant II System). MALDI (*m/z*): wt-SLN $[M + H]^+$ *m/z* 3763.9 Da, calculated 3761.6 Da; AFA-PLB $[M + H]^+$ *m/z* 6060.8 Da, calculated 6060.5 Da.

Ca-ATPase/wt-SLN or AFA-PLB co-reconstitution

The method used for the functional reconstitution of Ca-ATPase with PLB and SLN has been described [5,16,17]. AFA-PLB or wt-SLN (33 μg) was dried and solubilized in 240 μl chloroform containing 2.4 mg lipids in a 4:1 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids). After eliminating the organic solvent by N_2 flux, the dried film of lipid and AFA-PLB or wt-SLN 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 AFA-PLB or wt-SLN, were made to 20 mM imidazole (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, and 10% glycerol. Then, 4.8 mg 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/AFA-PLB or wt-SLN lipid vesicles were separated from Biobeads and assayed immediately.

ATPase activity measurements

Ca-ATPase activity was assayed by an enzyme-linked assay [18], performed in microtiter plate wells. Each assay was done in triplicate at different free calcium concentrations in a volume of 175 μl. Between 1 and 3 μg Ca-ATPase (5–15 μl of vesicles) was added to a buffer containing 0.5 mM phosphoenolpyruvate, 2.5 mM ATP, 0.2 mM NADH, 2 IU pyruvate kinase, 2 IU lactate dehydrogenase, and 1–2 μg 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 37 °C in a Thermomax Microplate Reader (Molecular Devices).

NMR spectroscopy

NMR samples for wt-SLN were prepared as ~0.5 mg lyophilized protein in 20 mM PBS (pH 4.0), 600 mM sodium dodecyl sulfate (SDS), and 10% D_2O. AFA-PLB NMR samples were prepared by dissolving ~1 mg lyophilized protein in 20 mM PBS (pH 4.0), 600 mM dodecyl phosphocholine (DPC), and 10% D_2O. $^1H/^{15}N$ heteronuclear single quantum coherence (HSQC) spectra were collected at a probe temperature of 323 K on a Varian Inova 800 spectrometer operating at 800.235 MHz, equipped with a standard triple resonance, triple axis probe for wt-SLN, and a similarly equipped Inova 600 spectrometer operating at 600.478 MHz for AFA-PLB. All NMR spectra were processed using NMRPipe [19].

Results and discussion

AFA-PLB and wt-SLN expression in E. coli and purification

Our objective is to characterize further the regulation of cardiac and muscle Ca-ATPase by PLB and SLN at a molecular level employing solution and solid-state

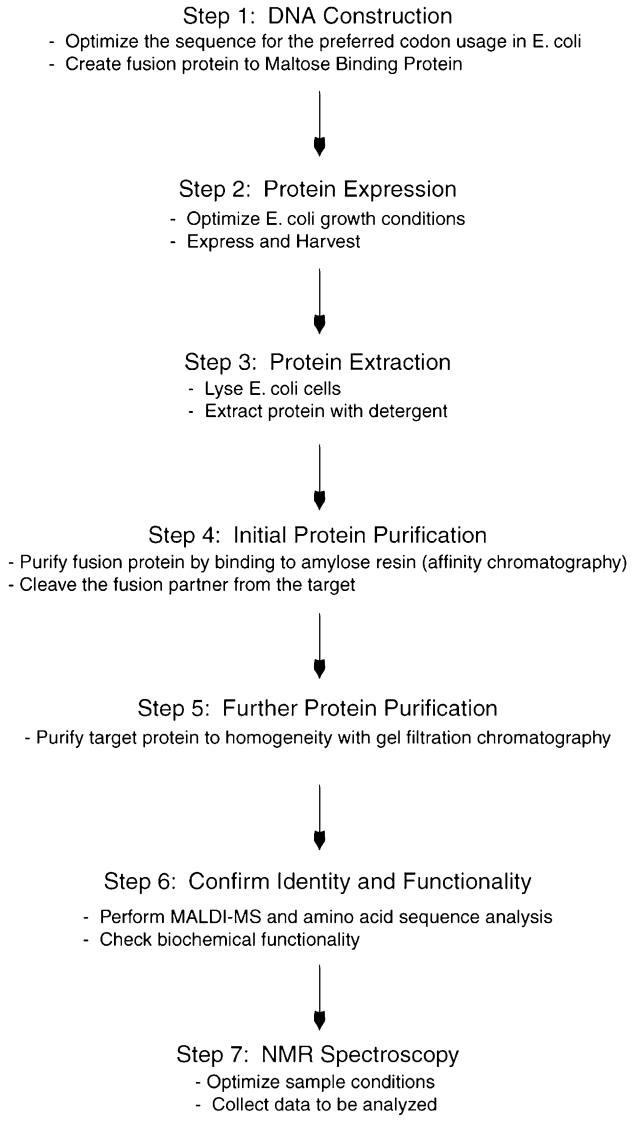

Fig. 1. Flowchart illustrating the sequential protein purification steps for the integral membrane proteins AFA-PLB and wt-SLN.

NMR spectroscopy. Due to the inherent need for large quantities of isotopically labeled PLB and SLN for these studies, we sought a purification method that was more cost-efficient than currently published methods. We chose recombinant expression utilizing *E. coli*, as it yields the high levels of pure uniformly labeled protein necessary for solution and solid-state NMR studies (Fig. 1).

Fusion proteins were created between the soluble MBP (MW of ~42 kDa) and AFA-PLB (MW of ~6 kDa) or wt-SLN (MW of ~4 kDa) (Fig. 2). This was accomplished by first optimizing the DNA sequence of the target for preferred protein translation codon usage in *E. coli* (Step 1 of Fig. 1). Since wild-type PLB is primarily pentameric, we generated monomeric PLB by mutating its three transmembrane cysteines at positions 36, 41, and 46 to alanine, phenylalanine, and alanine, respectively [11]. This monomeric AFA-PLB mutant has previously been shown to function with enhanced activity over the pentameric wt-PLB, implying that the monomer is the active species for Ca-ATPase inhibition [20]. Thus, the AFA-PLB construct is appropriate for use in NMR studies of PLB–Ca-ATPase interactions. The synthetic oligonucleotides were then introduced into a pMal expression vector for growth in bacterial cells (Step 2 of Fig. 1). Once extracted from the cells (Step 3 of Fig. 1), pure AFA-PLB or wt-SLN protein was isolated by the application of two basic protein purification techniques: (1) affinity chromatography of the fusion protein and (2) gel filtration chromatography to separate cleavage products (Steps 4 and 5 of Fig. 1).

The MBP fusion proteins were isolated from cell lysates using amylose resin. Elution with maltose buffer yielded over 100 mg/L of culture of MBP/AFA-PLB fusion protein at >90% purity (Fig. 3 and Table 1). The MBP/wt-SLN fusion yield was much less, at 34 mg/L of growth at >90% purity (Table 1). Fusion proteins were then cleaved from their MBP fusion partners by enzymatic digestion with Tobacco Etch Virus (TEV) protease or thrombin protease for AFA-PLB and wt-SLN, respectively.

Our first attempts to use a thrombin cleavage site for the MBP/AFA-PLB construct failed. In fact, we detected a substantial amount of secondary cleavage between R9 and S10 (data not shown), which increased as the cleavage reaction proceeded and became almost complete for cleavage reactions longer than 30 h. We determined that this proteolysis occurred even in the absence of thrombin by monitoring the loss of the cytoplasmic PLB antibody epitope during the course of incubation (data not shown). Attempts to prevent proteolysis using other protease-deficient *E. coli* strains, or additional protease inhibitors such as EDTA or aprotinin, only marginally improved the outcome. Alteration of the protease recognition site for other proteases (Factor Xa, Genenase (Genecore International), and enterokinase) was similarly unsuccessful.

Much better results, however, were obtained by using a TEV protease recognition site. The TEV cleavage allowed us to obtain a selective, quantitative reaction in a shorter amount of time. The efficiency of cleavage for AFA-PLB was approximately 95% using 100 U of TEV/mg of fusion protein at 30 °C for approximately 10 h (Fig. 3).

The wt-SLN construct did not exhibit the secondary cleavage observed for AFA-PLB, so a thrombin protease recognition site was used to obtain selective cleavage

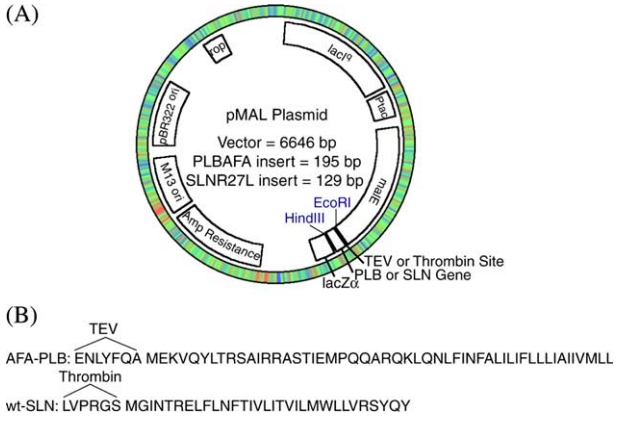

Fig. 2. (A) The pMal plasmid used to create maltose binding fusion proteins for expression and purification of AFA-PLB and wt-SLN. The protease cleavage site following the mal*E* was modified from the original vector (pMal-c2X, NEB) to remove the Xa factor cleavage site. In its place, either the thrombin site (for SLN) or the Tobacco Etch Virus (TEV) recognition sequence (for PLB) was introduced via PCR. The restriction enzymes used to introduce the insert are highlighted. (B) Amino acid sequences of TEV–AFA-PLB and Thrombin-wt-SLN.

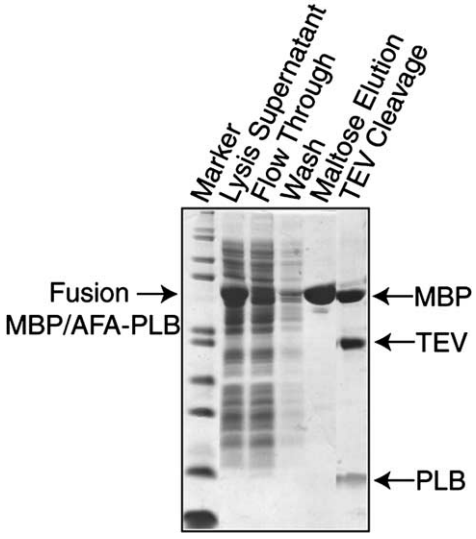

Fig. 3. Twelve percent SDS–PAGE gel showing fractions from cell lysis, affinity chromatography, and cleavage of AFA-PLB by TEV. Similar results were obtained for wt-SLN, which was cleaved with thrombin protease rather than TEV (data not shown).

Table 1
Protein yields at various purification steps for AFA-PLB and wt-SLN

Protein	Amount (mg/L)	Purity (%)[a]	Fold enrichment[b]
E. coli soluble fraction (MBP/wt-SLN fusion)	34	4	—
Affinity chromatography (MBP/wt-SLN fusion)	34	>90	22.5
Gel filtration chromatography (isolated wt-SLN)	2	>99	1.04
E. coli soluble fraction (MBP/AFA-PLB fusion)	100	13.5	—
Affinity chromatography (MBP/AFA-PLB fusion)	100	>90	6.7
Gel filtration chromatography (isolated AFA-PLB)	6	>99	1.04

[a] Protein quantities were estimated from densitometric measurements using 12% SDS–PAGE.
[b] Calculated as the fold increase in protein purity (percent purity in purification step divided by percent purity of preceding step).

of MBP from wt-SLN. The efficiency of this reaction was 70% cleavage using 30 U of thrombin/mg of fusion protein at 28 °C for 15–18 h (data not shown). To maintain solubility of the cleaved membrane proteins in solution, small amounts of the mild detergents Tween 20 or Triton X-100 were added to the cleavage reaction of wt-SLN and AFA-PLB, respectively.

For both proteins, the cleavage products (~7 mg/ml) were applied directly to a high-resolution Sephacryl S-100 matrix and separated from MBP using a liquid chromatography system. The elution profiles are shown in Fig. 4. AFA-PLB and wt-SLN peaks (detected by SDS–PAGE of the fractions) were independently pooled, dialyzed, and lyophilized. The final yields of purified protein per liter of culture were ~6 mg for AFA-PLB and ~2 mg for wt-SLN (Table 1). The apparent decrease in protein yield after size exclusion chromatography was due to removal of the fusion partner and not from an actual loss of PLB or SLN. The identity of each protein was confirmed by mass spectrometry (Fig. 5).

Activity assays

To ensure the functionality of recombinant AFA-PLB and wt-SLN proteins, their ability to inhibit the activity of Ca-ATPase was tested and compared to previously published values [5,15]. Both synthetic AFA-PLB and recombinant AFA-PLB decreased the apparent calcium affinity of the Ca-ATPase at pCa below 5.5, resulting in an increase in pK_{Ca} (the calcium concentration, in pCa units, required for 50% Ca-activation). Synthetic AFA-PLB shifted pK_{Ca} by −0.34 (control: 6.51 ± 0.02; AFA-PLB: 6.17 ± 0.03), while the shift by recombinant AFA-PLB was −0.32 (6.19 ± 0.02) for this co-reconstitution system (Fig. 6). Synthetic and re-

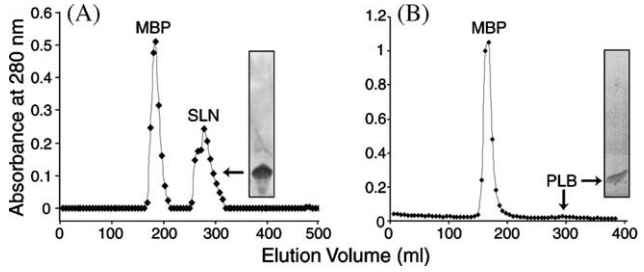

Fig. 4. Separation of SDS-solubilized MBP from wt-SLN (A) or AFA-PLB (B). Gel filtration chromatography was performed on the cleavage products. AFA-PLB showed a broad elution peak around 300 ml indicated by the arrow. Fractions containing AFA-PLB were identified by gel electrophoresis. The left inset panel shows a silver-stained 15% SDS–PAGE gel for the pooled fractions of purified wt-SLN (260–310 ml). The right inset panel is a 12% SDS–PAGE Coomassie-stained gel of pure AFA-PLB pooled fractions (300–384 ml).

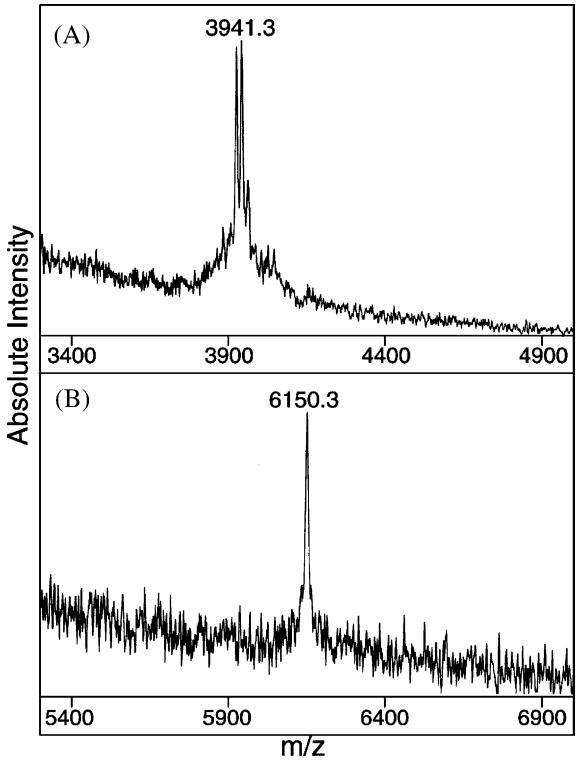

Fig. 5. MALDI-TOF mass spectra of ^{14}N-wt-SLN (A) and ^{14}N-AFA-PLB (B). The calculated mass of wt-SLN was 3905.7 (including two additional Gly and Ser residues left at the N-terminus after thrombin cleavage). The experimental *m/z* of 3941.3 observed for wt-SLN and the shouldering peaks were due to alternative oxygenation states of the protein since it was prepared under non-reducing conditions. TEV-cleaved AFA-PLB had a predicted mass of 6145.6, which was in good agreement with the observed *m/z* of 6150.3. The identity of AFA-PLB was also confirmed by Western blotting (data not shown).

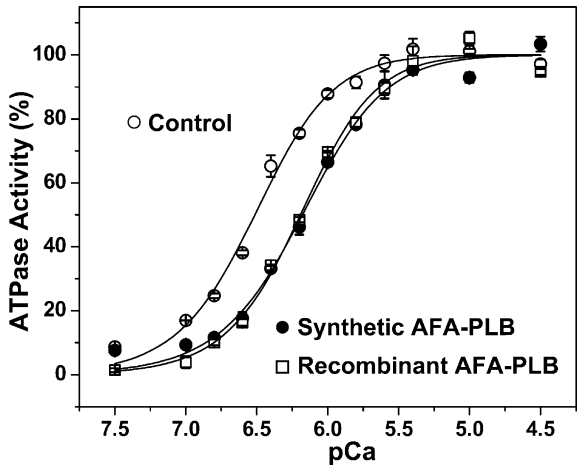

Fig. 6. Regulation of purified skeletal SR Ca-ATPase at 37 °C, after reconstitution in DOPC bilayers for AFA-PLB. (○) Control (Ca-ATPase only). (●) Ca-ATPase plus synthetic AFA-PLB. (□) Ca-ATPase plus recombinant AFA-PLB. The curves were fit to the Hill equation with each data point representing the mean ± SEM ($n \geqslant 6$).

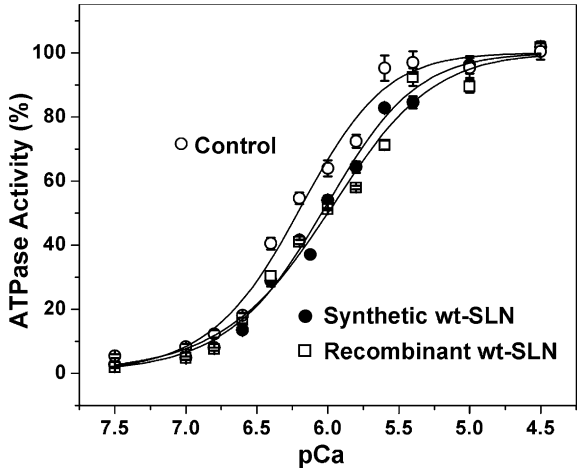

Fig. 7. Regulation of purified skeletal SR Ca-ATPase at 37 °C, after reconstitution in DOPC bilayers for wt-SLN. (○) Control (Ca-ATPase only). (●) Ca-ATPase plus synthetic wt-SLN. (□) Ca-ATPase plus recombinant wt-SLN. The curves were fit to the Hill equation with each data point representing the mean ± SEM ($n \geqslant 6$).

combinant wt-SLN also decreased the activity of the Ca-ATPase at pCa below 5.5, resulting in an increase in pK_{Ca}. Synthetic wt-SLN shifted pK_{Ca} by −0.17 (control: 6.21 ± 0.04; wt-SLN: 6.04 ± 0.03), while the shift by recombinant wt-SLN was −0.21 (6.00 ± 0.05) for this co-reconstitution system (Fig. 7).

Optimal spectroscopy conditions

AFA-PLB and wt-SLN both tend to aggregate at the high protein concentrations required for NMR studies. Therefore, we sought optimized conditions for the spectroscopy of monomeric AFA-PLB and wt-SLN. Several $^{1}H/^{15}N$ HSQC (Heteronuclear Single Quantum Coherence) experiments were conducted in order to improve spectral resolution of the amide backbone region (“protein fingerprint”). A highly resolved HSQC spectrum with dispersion of both proton and nitrogen chemical shift is indicative of a well-folded protein which sets the ground for structure elucidation by NMR spectroscopy. While this task is relatively easy to achieve with soluble proteins, it requires several steps of optimization for membrane proteins [21].

The two proteins were reconstituted in several different detergent solutions, with varying protein concentrations, detergent concentration and type, pH, temperature, and ionic strength. Non-optimized spectra showed a typical pattern of aggregated membrane proteins, with multiple resonances for most flexible regions of the protein (i.e., loops and termini) and with very broad lines for the transmembrane domains (data not shown).

For wt-SLN measurements, we chose to use sodium dodecyl sulfate (SDS), and for AFA-PLB measurements, we chose dodecyl phosphocholine (DPC). Both detergents have been utilized to obtain high resolution of NMR spectra of membrane proteins for structural determination [21]. At low DPC or SDS concentrations (<150 mM), both wt-SLN and AFA-PLB show multiple resonances due to aggregation, which has been observed for membrane proteins of similar size [22]. Consequently, the optimized spectrum shown in Fig. 8A for wt-SLN in 600 mM SDS exhibits 29 peaks (two overlapped) for the 31 resonances expected. Similarly, the optimized spectrum shown in Fig. 8B for AFA-PLB in 600 mM DPC exhibits 48 peaks (three peaks containing

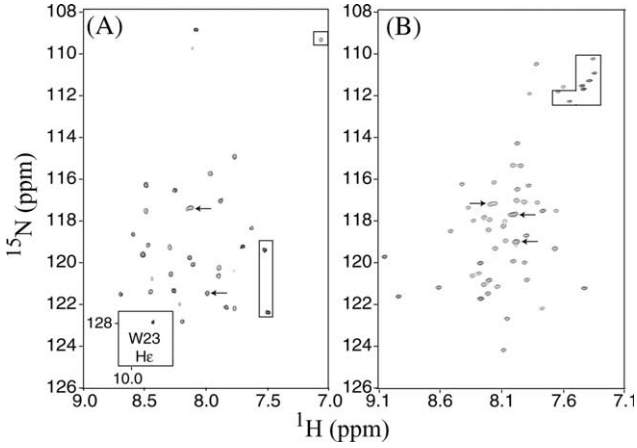

Fig. 8. $^{1}H/^{15}N$ HSQC NMR spectra of wt-SLN (A) and AFA-PLB (B). Optimal sample conditions were established to achieve well-resolved peaks in conventional 2D spectra. For wt-SLN, 600 mM SDS in 20 mM PBS at pH 4.0 gave distinguishable peaks in the HSQC. The resolution of the AFA-PLB spectrum was highest at 600 mM DPC, 20 mM PBS, and pH 4.0. For both (A) and (B), arrows indicate overlapped resonances and boxed peaks represent side chain resonances.

two overlapping resonances) for the 51 resonances expected. In both instances, the number of amide backbone peaks correlates well with the number of residues in each protein and the large dispersion of peaks in the proton and nitrogen dimensions indicates well-defined folding of the two Ca-ATPase inhibitors.

Comparison with previous expression methods

While there are no methods reported for the expression of recombinant wt-SLN, several different methods have been used to express recombinant PLB, but none have proven suitable for our purposes. Reddy et al. [23] reported the first use of a heterologous system to produce large quantities of PLB, using expression in *Spodoptera frugiperda* insect cell culture and purification by PLB monoclonal antibody affinity chromatography. While this method produces milligram quantities of PLB, it is difficult and cost-prohibitive to produce isotopically enriched protein. In addition, large amounts of anti-PLB monoclonal antibody are required for the purification step, which is not yet readily available.

Yao et al. [24] were the first to report successful expression and purification of PLB from *E. coli*, using an N-terminal fusion of PLB with glutathione-*S*-transferase (GST), connected by a linker containing a thrombin cleavage site. This fusion protein, however, was primarily localized to inclusion bodies within the bacteria, requiring strong denaturing conditions to solubilize the protein. This preparation is thus not optimal for conventional purification methods employing glutathione affinity chromatography. Alternatively, Yao et al. [24] achieved purification of PLB using two successive preparative SDS–PAGE purification steps, one to separate the GST-PLB fusion protein from other inclusion body proteins, and a second to separate the thrombin-cleaved PLB product from GST and uncleaved GST-PLB, which negates the advantage of having an affinity tag.

Conclusions

We report a robust method to express, isolate, and purify PLB and SLN, the two regulators of Ca-ATPase in muscle. Due to their high hydrophobic content, these two membrane-bound proteins have proven difficult to express and isolate in sufficient quantities for structural and functional studies. Our approach exploits the favorable effects of MBP, which increases the solubility of hydrophobic PLB and SLN, while increasing their stability toward degradation of the host cell proteases. Our protocol allows the production of 2 mg wt-SLN and 6 mg AFA-PLB per liter of culture making possible the structural studies of these important proteins by solution and solid-state NMR.

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