Structure and Function of Integral Membrane Protein Domains Resolved by Peptide-Amphiphiles: Application to Phospholamban

Abstract: We have used synthetic lipidated peptides (“peptide-amphiphiles”) to study the structure and function of isolated domains of integral transmembrane proteins. We used 9-fluorenylmethyl-oxycarbonyl (Fmoc) solid-phase peptide synthesis to prepare full-length phospholamban (PLB$_{1–52}$) and its cytoplasmic (PLB$_{1–25K}$: phospholamban residues 1–25 plus a C-terminal lysine), and transmembrane (PLB$_{26–52}$) domains, and a 38-residue model α-helical sequence as a control. We created peptide-amphiphiles by linking the C-terminus of either the isolated cytoplasmic domain or the model peptide to a membrane-anchoring, lipid-like hydrocarbon tail. Circular dichroism measurements showed that the model peptide-amphiphile, either in aqueous suspension or in lipid bilayers, had a higher degree of α-helical secondary structure than the unlipidated model peptide. We hypothesized that the peptide-amphiphile system would allow us to study the function and
INTRODUCTION

Peptide-amphiphiles consist of a hydrophilic peptide covalently bonded through the N- or C-terminus to a synthetic mono- or dialkyl lipid-like tail.1 The number and length of tails, tail functional groups, and peptide sequence are all variable due to the modular design of peptide-amphiphiles. Lipidated peptides under the names of “peptide-amphiphiles,” “lipopeptide conjugates,” or “lipopeptides” have been developed for biomaterials functionalization,2,3 for drug delivery systems,4 for cell receptor mimetics,3,5 for biosensors,6 as a tool for probing signal transduction,7 and for enhancement antimicrobial peptide activity.8

Peptide-amphiphiles self-assemble into micelles and bilayer aggregates in aqueous solution,9 placing the peptide headgroup into an environment similar to that experienced by the cytoplasmic or extracellular portion of a transmembrane protein. Previous work has shown that tethering a peptide to an aggregate interface in this manner stabilizes secondary structure even in short peptide head groups that show no structure on their own.5,10,11 Peptide-amphiphiles also preserve the native function of peptide sequences. Surfaces coated with peptide-amphiphiles containing the short cell-adhesion sequence RGD show increased cell adhesion compared to untreated surfaces.2 Peptide-amphiphiles containing longer RGD-based peptides also interact with cells and bind specific integrin receptors.12 Monolayers of amphiphiles containing a peptide based on the 15-residue melanoma cell adhesion region of type IV collagen increases specific cell adhesion compared to untreated surfaces.5

Self-assembly, preservation of structure and function, and the lipophilic nature of peptide-amphiphiles make them good candidates for studying isolated domains of transmembrane proteins. This would be particularly useful for proteins in which the function of the cytoplasmic or extracellular domain is not clear. This is the case for phospholamban, a 52-residue integral membrane protein that regulates the calcium transport activity of the cardiac sarcoplasmic reticulum calcium pump (Ca-ATPase).13

The 52 residues of phospholamban (PLB) are organized into two major domains.14–16 The cytoplasmic domain (domain Ia, residues 1–25) contains the phosphorylation site. Phosphorylation at serine 16 by cAMP-dependent protein kinase or at threonine 17 by Ca/calmodulin-dependent protein kinase or both causes PLB dissociation from the Ca-ATPase, relieving its inhibitory effect.15,17,18 It has been proposed that a region of the cytoplasmic domain, PLB1–31, is essential for functional association with Ca-ATPase.19–21 The hydrophobic transmembrane domain (domain II, residues 26–52) contains a leucine–isoleucine zipper region that stabilizes the pentameric structure of wild-type PLB.15,22,23

Several investigators have studied the interaction of isolated PLB domains with the Ca-ATPase. It was reported that the reconstitution of purified Ca-ATPase with an excess of PLB1–31 inhibited Vmax without affecting Ca2+ affinity of Ca-ATPase, while in vitro reconstitution of Ca-ATPase with 100–300 molar ratios of PLB28–47 to Ca-ATPase lowered the Ca2+ affinity of Ca-ATPase.19 In a subsequent study, using a well-characterized reconstitution system, no significant effect of PLB1–25 was found on Ca-ATPase.24 Site-directed mutagenesis and functional studies have shown that the inhibitory association of PLB with the Ca-ATPase involves interactions with PLB not only in the cytoplasmic domain but also within the transmembrane region.25

Both the transmembrane and cytoplasmic domains of full-length PLB are strongly α-helical.26 Solution NMR studies in organic mixtures showed that PLB adopts an “L”-shaped structure with the region linking the transmembrane and cytoplasmic domains in either a short flexible turn or a type III β-turn conformation.27–29 Solid-state NMR of full-length PLB shows that the cytoplasmic domain is also α-helical in lipid bilayers.27 Circular dichroism and Fourier transform infrared spectroscopy (FTIR) of the isolated transmembrane domain in lipid bilayers and aqueous sodium dodecyl sulfate (SDS) solution indicate a high degree of α-helical content.26,30 However, the isolated
cytoplasmic domain of PLB is primarily unstructured in aqueous solution, and only forms an α-helical conformation when prepared in SDS or trifluoroethanol (TFE) solutions. The lack of functional effect observed in the isolated cytoplasmic domain of PLB could be due to a lack of structure in the peptide, the low affinity of the free cytoplasmic peptide for the Ca-ATPase, or incorrect positioning of the peptide. In order for the cytoplasmic domain of PLB to have a functional effect on the Ca-ATPase, it is likely than the peptide must be anchored to the membrane surface in a configuration that optimizes the potential for interaction with the Ca-ATPase. Tethering the peptide to the membrane may also impart a more native structure to the peptide.

In the present study we address these issues by extending the use of peptide-amphiphile architecture to the study of the function and structure of monomeric AFA–PLB (replacement of Cys 36, 41, and 46 in phospholamban with alanine, phenylalanine, and alanine). Using monomeric AFA–PLB allowed us to focus on the behavior of isolated AFA–PLB domains independent of oligomeric interactions. We have demonstrated the feasibility of the approach with a model peptide and applied the system to the study of the isolated AFA–PLB domains. We replaced the hydrophobic transmembrane domain of AFA–PLB with a lipid-like tail, creating a nonspecific hydrophobic transmembrane domain of AFA–PLB (isolated transmembrane domain of PLB with Cys 36, 41, 46 changed to alanine, phenylalanine, and alanine). This is a “model” peptide due to its preference for α-helical secondary structure; it is used here only to demonstrate structural behavior of peptide-amphiphiles in aqueous and lipid solutions.

Phospholamban-based sequences included the monomeric AFA–PLB mutant of full-length PLB and the corresponding transmembrane domain (residues 26–52). The cytoplasmic domain peptide (residues 1–25) of PLB included a C-terminal lysine residue to facilitate amphiphile tail linkage.

**MATERIALS AND METHODS**

**Peptide Sequences**

Peptide sequences were derived from a model α-helical sequence and from the AFA mutant of phospholamban (Figure 1). The 38-residue model peptide was composed of a sequence derived from the α-helical coiled-coil region of the DNA-binding protein GCN4 and a short C-terminal portion based on the stereotypical coiled-coil heptad sequence. This is a “model” peptide due to its preference for α-helical secondary structure; it is used here only to demonstrate structural behavior of peptide-amphiphiles in aqueous and lipid solutions.

**Synthesis of the Model Peptide**

The model α-helical peptide (Figure 1) was synthesized by the Microchemical Facility at the University of Minnesota using a Milligen Biosearch 9600 automated peptide synthesizer following standard solid-phase Fmoc procedures. The C-terminus carboxylate group was synthesized in the amide form. The peptide support and side-chain protecting groups were cleaved with a mixture of 95% trifluoroacetic acid (TFA) and 5% water following synthesis. Crude peptide was dissolved in 3:2 water/isopropanol and purified by high performance liquid chromatography (HPLC) on a C4 column using a B phase of isopropanol/0.1% TFA.

**Synthesis of Full-Length PLB and TM–PLB Domain**

Materials, solvents, instrumentation, and general methods of solid-phase peptide synthesis were essentially as described previously with minor modifications. Starting with Fmoc–Leu–PAC–PEG–PS resin (PEG-PS, polyethylene glycol–polystyrene [graft support]) (initial loading 0.18 mmol/g), the full-length AFA–PLB and TM–AFA–PLB (isolated transmembrane domain of PLB with Cys 36, 41, 46 changed to alanine, phenylalanine, and alanine) (Figure 1) were assembled by automated Fmoc solid-phase synthesis on a Pioneer automated peptide synthesizer. Acidolyzable side-chain protecting groups were 2,2,5,7,8-pentamethylychroman-6-sulfonyl (Pmc) for Arg; triphenylmethyl (trityl) (Trt) for Asn and Glu; tert-butyl ester (OtBu) for Asp and Glu; tert-butylxycarbonyl (tBoc) for Lys; and tert-butyl ether (tBu) for Ser, Thr, and Tyr. Fmoc removal was achieved with 20% piperidine and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in N-methyl-2-pyrrolidone (NMP). The activation of the double coupling was performed with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBT)/N,N-diisopropylethylamine (DIEA). Final deprotection was performed by treatment of 200 mg peptide resin with 2 mL of freshly prepared reagent K: 82.5% TFA, 5% phenol, 5% thioanisole, 2.5% 1,2-ethanediol, 5% water. The cleavage mixture was filtered and the resin washed with 2 mL of the same cocktail. The combined filtrates were concentrated under N2, and precipitated in 30 mL of diethyl ether 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 1 mL of cold diethyl ether and lyophilized.

**FIGURE 1** Sequence of the model and PLB-based peptides. The stereotypical coiled-coil sequence of the model peptide and the transmembrane domain of PLB are highlighted in gray. C-terminal lysine residues on the model peptide and the PLB cytoplasmic domain were added to facilitate peptide-amphiphile synthesis.
in 5 mL of TFA and purified by HPLC on a C-18 column (Vydac, 218TP54) 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% isopropanol. Fractions containing peptides were lyophilized to yield full-length PLB (15.4% yield based on starting resin) and TM–AFA–PLB (22% yield based on starting resin).

**Synthesis of PLB<sub>1–25K</sub>**

Starting with Fmoc–PAL–PEG–PS resin (initial loading 0.2 mmol/g), the PLB<sub>1–25K</sub> (phospholamban residues 1–25 plus a C-terminal lysine) (Figure 1) was synthesized under similar conditions as described for the full-length PLB. The peptide sequences for the model peptide and the cytoplasmic domain of PLB<sub>1–25K</sub> included a Dde-protected [Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl] C-terminal lysine residue to facilitate coupling to the lipid tail. In order to prevent N-terminal coupling of the tails, the final residue in the peptide sequence included an acid-labile Boc-protecting group (Boc: tert-butylxycarbonyl). Lys–Dde was cleaved with 2% hydrazine in N,N-dimethylformamide (DMF). The final deprotection was performed in a mixture of 90% TFA, 5% water, and 5% tris(propylsilylamin (TIS).

**Synthesis of Peptide-Amphiphiles**

Dialkyl 1',3'-dioctadecyl-N-succinyl-L-glutamate (lipid tails) were synthesized as described previously and linked to the model or PLB<sub>1–25K</sub> peptide through the Lys side chain amino group (Figure 2). The lipid tails were double coupled to the resin-bound peptide for 4 h with N-[dimethylamino]-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-N-methylmethaniminium hexafluorophosphate N-oxide (HATU) (0.45 mmol), DIEA (1.25 mmol), and a 5-fold excess of lipid tails. Peptide-amphiphiles were cleaved from the resin using either 95% TFA, 5% water (model peptide-amphiphile) or 90% TFA, 5% water, 5% TIS (PLB<sub>1–25K</sub> peptide-amphiphile).

**Peptide Analysis by Mass Spectroscopy**

Mass spectral data were acquired with a Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) system equipped with a N2 laser (337 nm, 3-ns pulse length) and a microchannel plate (MCP) detector. Data was collected in linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum is the accumulation of 100–400 laser shots. The samples were cocryostallized with the matrix 3,5-dimethoxy-4-hydroxycinna- mide (3,5-Dimethoxy-4-hydroxycinna- mide) and 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid). Results: model peptide-amphiphile: [M + H]<sup>+</sup> 5127.0 Da, calculated 5124.8 Da; AFA–PLB: [M + H]<sup>+</sup> 6060.8 Da, calculated 6060.5 Da; TM–AFA–PLB: [M + Na]<sup>+</sup> 3153.4 Da, calculated 3151.4 Da; PLB<sub>1–25</sub> amphiphile [M + H]<sup>+</sup> 3813.0 Da, calculated 3813.6 Da.

**Liposome Preparation**

1,2-Dimyristoyl-sn-glycero-3-phosphocholine; (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) phospholipids were obtained from Avanti Polar Lipids, Inc. Chloroform solutions of pure phospholipid were mixed in a glass tube with the appropriate amount of methanol solutions of peptides or peptide-amphiphiles and dried under a low flow of N2 to form a thin film. Residual solvent was removed under vacuum for several hours. The resulting lipid film was hydrated with an appropriate volume of 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7) to yield a final lipid concentration of 8–10 mM and vortexed at temperatures well above the lipid transition temperature. Solutions for CD experiments were sonicated 20 min or more in a bath sonicator at temperatures above the lipid transition temperature. This process produced clear solutions that scattered light only minimally in the 190–250 nm wavelength range used for CD.

**Circular Dichroism**

CD spectra were recorded on a Jasco J-710 spectrophotometer at 25°C using a 0.01 cm path-length quartz cuvette. Acquisition was performed using a 50 nm/min scan rate, 1 nm bandwidth, and 2 s response. The corresponding baseline (buffer or lipid/buffer solution) was subtracted from each spectrum. Reported spectra are averages of six scans and are expressed as mean residue ellipticity, [θ]. Aqueous solutions for CD were prepared by drying a volume of peptide or peptide-amphiphile stock solution (~3 mg/mL in chloroform or methanol) under a low flow of N2 and adding the appropriate amount of 10 mM Tris (pH 7) to give final peptide concentrations of 0.1–0.5 mM. CD basis spectra were measured with polylysine and polyglutamic acid (Sigma) using conditions and parameters reported by others. Linear combinations of α-helix and random coil basis spectra were used to estimate secondary structure contributions from fits to experimental CD spectra.

**Ca-ATPase/PLB Co-Reconstitution**

Sarcoplasmic reticulum (SR) vesicles for PLB/Ca-ATPase interaction studies were prepared from the fast-twitch skel-
etal muscle of New Zealand white rabbits. The Ca-ATPase from the SR vesicles was purified using a reactive red affinity column. The method used for the functional reconstitution of Ca-ATPase with PLB has been described. In short, 33 μg PLB or its analogues was dried and solubilized in 240 μL chloroform containing 2.4 mg lipids (DOPC/DOPE, 4:1). The solvent was evaporated to dryness and the dried film of lipid and PLB was hydrated with 120 μL of 25 mM imidazole, pH 7.0, by vortexing followed by brief sonication. The resulting vesicles were diluted to 20 mM imidazole, pH 7.0, 0.1M KCl, 5 mM MgCl₂, 10% glycerol. 4.8 mg of octyl-glucoside (-OG) was added, followed by 60 μg of purified Ca-ATPase. The final volume was adjusted to 300 μL with buffer. The detergent was removed by incubation with 120 mg of hydrated Biobeads for 3 h at room temperature. The Ca-ATPase/PLB lipid vesicles were separated from Biobeads (BioRad, Inc), and assayed immediately. All Ca-ATPase/PLB co-reconstitution used a fixed molar ratio of 10 PLB/Ca-ATPase.

Ca-ATPase Activity Measurements

Ca-ATPase activity was measured by an enzyme-linked assay performed in microtiter plates (200 μL total volume in each well) as described previously. Each well contained 0.2–0.6 μg of Ca-ATPase (1–3 μL of vesicles) in 50 mM imidazole (pH 7.0), 0.1M KCl, 5 mM MgCl₂, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM phosphoenol pyruvate, 2.5 mM ATP, 0.2 mM β-nicotinamide adenine dinucleotide (NADH), 2 IU of pyruvate kinase, 2 IU of lactate dehydrogenase, and 1–2 μg calcium ionophore (A23187). The assay was performed in triplicate at 12 free calcium concentration levels. The absorbance of NADH was monitored at 340 nm to determine the rate of ATP hydrolysis. The assays were performed at 15 and 25°C in a Thermomax microplate reader (Molecular Devices). Each data point represents average ± SEM (n ≥ 6). A Student’s t test was used to determine the statistical significance of the differences between peptides and the effects of temperature.

RESULTS

Secondary Structures of the Model Peptide and Amphiphile

We hypothesized that a peptide tethered to a lipid bilayer would assume its native secondary structure. We carried out CD studies of a model peptide and amphiphile in buffer and lipid bilayers to test this hypothesis and form a basis for our work with phospholamban. In buffer solution, the model amphiphile showed an enhancement of peptide -helical secondary structure as compared to the unmodified peptide (Figure 3A). A linear combination of basis spectra fit to the measured spectra showed that the peptide was 37% α-helix in its isolated form and 50% α-helix in the amphiphile. Nearly identical spectra and structure distributions were obtained for the two peptides in DMPC lipids (Figure 3B), 38% helix for the model peptide, 49% helix for the model amphiphile. These results showed that the stabilization of peptide secondary structure operates for peptide-amphiphiles both in aqueous solution and in lipid bilayer environments. The results also encouraged us to apply peptide-amphiphiles to the PLB system.
Activity of PLB Derivatives

Full-length AFA–PLB showed inhibition of the Ca-ATPase, shifting the pKCa = 0.35 units (Figure 4A). This level of inhibition is in good agreement to that observed for wild-type PLB for this co-reconstitution system. The isolated transmembrane domains (TM–AFA–PLB) showed a similar inhibitory effect (Figure 4A). Under the same method of preparation, no inhibitory effect was observed for the isolated cytoplasmic domain (PLB1–25K) at 10-fold excess of PLB1–25K (Figure 4B). The PLB1–25K amphiphile showed behavior similar to that of the peptide, yielding no inhibition of the Ca-ATPase at 10-fold excess of amphiphile (Figure 4C). The results were similar for experiments run at 50-fold excess PLB1–25K peptide and amphiphile.

These results show that the isolated TM–AFA–PLB is sufficient for Ca-ATPase inhibition, while the isolated cytoplasmic domain has no inhibitory effect, even when anchored to the membrane by a lipid tail. The results thus resolve the functional properties of the isolated domains of PLB.

Secondary Structures of PLB Domains in Lipids

In order to more fully characterize the PLB system, we used CD spectroscopy to measure the secondary structure of AFA–PLB and its isolated domains. CD spectra of AFA–PLB and TM–AFA–PLB mixed with DOPC/DOPE (4:1) lipids showed characteristic α-helical minima at 207 and 222 nm and maximum at 193 nm (Figure 5A). In contrast, the CD spectrum of PLB1–25K peptide indicates very little helical structure. The PLB1–25K amphiphile also showed a spectrum consistent with an unstructured state. We observed no significant difference between the PLB1–25K peptide and amphiphile spectra.

Secondary Structures of PLB1–25K Peptide and Amphiphile in Buffer

CD spectra of PLB1–25K peptide in 10 mM Tris buffer showed essentially disordered structure, while PLB1–25K amphiphile showed a significant α-helical conformation (Figure 6). Linear fits of the spectra yielded 16% helix for PLB1–25K peptide and 57%...
DISCUSSION

Structural Enhancement in the Model Peptide-Amphiphile System

We hypothesized that peptide-amphiphile architecture would be a useful method for studying the structure and function of isolated transmembrane peptide domains. Our hypothesis was based on the known properties of peptide-amphiphiles: self-assembly in solution to form micelles and vesicles, secondary structure stabilization in the peptide headgroup, and preservation of peptide biological function.9,10,12 These properties of peptide-amphiphiles were established primarily in single-component systems; for studying membrane peptides, the same properties must exist when peptide-amphiphiles are prepared in lipid bilayers. We used a model peptide to examine the structural behavior of peptide-amphiphiles mixed with bilayer-forming lipids.

The model amphiphile increased the level of α-helical secondary structure of the peptide headgroup in buffer solutions, in the absence of added lipid (Figure 3). The α-helix content as measured by CD increased from 37% in the model peptide to 50% in the model peptide-amphiphile. More importantly, the same level of structural enhancement occurred when the amphiphile was mixed with DMPC lipids, presumably forming bilayers. The level of α-helicity increased from 38% in the model peptide to 49% in the model peptide-amphiphile. In these experiments, the lipids showed no independent effect on peptide structure; secondary structure stabilization occurred was the same for pure peptide-amphiphiles as for peptide-amphiphiles mixed with lipid bilayers.

The increase in helicity is likely associated with aggregation of the peptide-amphiphile, by self-assembly in the case of pure peptide-amphiphile, and by incorporation into lipid bilayers in mixed systems.9,47

FIGURE 5 CD spectra of PLB molecules in DOPC/DOPE mixtures (50:1 lipid:peptide) 10 mM Tris, pH 7, 25°C. (A) AFA-PLB and TM-AFA-PLB show spectra characteristic of a dominant α-helical structure. Fits yielded 85–90% helix for each. (B) PLB1–25K peptide and amphiphile spectra indicate a dominantly unstructured conformation. Fits yielded 19% helix for PLB1–25K, 24% helix for PLB1–25K amphiphile.

helix for the PLB1–25K amphiphile. The increase in secondary structure was substantially greater in the PLB1–25K system than in our model peptide system (Figure 3).

FIGURE 6 CD spectra of PLB1–25K peptide and amphiphile in 10 mM Tris, pH 7.0, 25°C. Fits indicate 16% helix in PLB1–25K peptide, 57% helix in PLB1–25K amphiphile.
The level of structural increase we observed may appear somewhat small compared to the enhancement levels of other peptide-amphiphile sequences, but the relatively high level of \( \alpha \)-helical content in the model peptide alone may limit the possible structural enhancement. Given the length of the peptide (38 residues), it is unsurprising that the model peptide shows some preferred conformation even without the lipid tail.

The results from our model peptide study suggest that peptide-amphiphiles are a promising means of studying membrane protein domains. The results encouraged us to use peptide-amphiphile architecture to help isolate the function and structure of the cytoplasmic domain of phospholamban.

**Structure and Inhibitory Function of PLB Domains**

We compared Ca-ATPase inhibition by the isolated cytoplasmic domain PLB\(_{1-25K} \), PLB\(_{1-25K} \) amphiphile, isolated TM–AFA–PLB, and full-length AFA–PLB. When monomeric AFA–PLB or TM–AFA–PLB was reconstituted with Ca-ATPase in lipid bilayers, both PLB derivatives decreased the apparent Ca affinity (increased \( K_{Ca} \)) of the Ca-ATPase (Figure 4A). The effects of AFA–PLB and TM–AFA–PLB were similar to one another, to other null-cysteine mutants, and to wild-type PLB. In the same reconstitution system, neither isolated cytoplasmic PLB\(_{1-25K} \) nor PLB\(_{1-25K} \) amphiphile showed any decrease in the apparent Ca affinity of the Ca-ATPase (Figure 4B,C). Despite the discovery of several loss-of-function point mutations in the cytoplasmic domain of full-length PLB, suggesting an important functional effect in the domain, isolated PLB\(_{1-25K} \) nor closely related peptides show no Ca-ATPase inhibition. The failure of the PLB\(_{1-25K} \) amphiphile to inhibit the Ca-ATPase shows that the lack of inhibitory activity in the cytoplasmic domain is not a direct result of the peptide being free in solution. The large hydrophobic tail of the PLB\(_{1-25K} \) amphiphile anchored the peptide in the liposome membrane, and yet the peptide showed no inhibitory activity. These results strengthen previous evidence that the transmembrane domain is the inhibitory domain of PLB.

Isolated cytoplasmic domains of PLB have been observed to be unstructured in aqueous solution, but structured in SDS micelles and TFE. Our CD spectra showed that the isolated cytoplasmic domain (PLB\(_{1-25K} \)) is also unstructured in the presence of lipid bilayers (Figure 5B). This is, to our knowledge, the first evidence that the isolated cytoplasmic domain of PLB remains unstructured in the presence of lipid bilayers. Lipid bilayers do not appear to influence the conformation of PLB\(_{1-25K} \) in the same manner that SDS micelles do. The PLB\(_{1-25K} \) amphiphile also showed an unstructured conformation in lipid bilayers; the CD spectrum is essentially the same as that recorded for PLB\(_{1-25K} \) peptide. This is contrary to expectation based on the results of our model peptide, which showed nearly identical structural enhancement in aqueous solution and lipid bilayers (Figure 3).

One possible explanation for the lack of PLB\(_{1-25K} \) amphiphile structure in lipids is that the amphiphile does not stabilize secondary structure at all for this sequence. However, in buffer solution, PLB\(_{1-25K} \) amphiphile showed a strong enhancement in \( \alpha \)-helical secondary structure relative to the PLB\(_{1-25K} \) peptide (Figure 6). This is the same type of structural enhancement observed in our model peptide system (Figure 3) and in systems containing other peptide sequences. The enhancement of \( \alpha \)-helical content is greater in PLB\(_{1-25K} \) than in the model peptide, perhaps due to the more unstructured state of the PLB\(_{1-25K} \) peptide compared to the model peptide. It is clear that the behavior of the PLB\(_{1-25K} \) peptide-amphiphile in aqueous solution matches that of other peptide-amphiphile systems, but the presence of lipid bilayer somehow reduces structural enhancement in PLB\(_{1-25K} \) amphiphiles.

A possible explanation for this controversy is that structural enhancement in the PLB\(_{1-25K} \) amphiphile arises from interpeptide interactions. The aggregation of PLB\(_{1-25K} \) amphiphile molecules into micelles places the peptide portion in crowded conditions compared to a peptide free in solution or incorporated into lipid bilayers. The crowded conditions of peptide-amphiphile aggregates and templated peptide assemblies can drive the stabilization of secondary structure. Diluting the PLB\(_{1-25K} \) amphiphile with lipids (1 peptide-amphiphile for every 50 lipid molecules in our structural experiments) should increase inter-peptide spacing, decrease crowding, and destabilize secondary structure in the PLB\(_{1-25K} \) amphiphile, as we observed. But CD results from our model peptide-amphiphile suggest that crowding is only part of what drives secondary structure enhancement. If crowding alone drove folding, we would not expect the model peptide-amphiphile to enhance secondary structure when diluted in lipids. But we observed no difference between the level of model peptide-amphiphile structural enhancement in buffer or lipids. Others have made similar observations for lipopeptides in lipid bilayers.

The disordered state of the PLB\(_{1-25K} \) amphiphile in lipid bilayers may explain the lack of PLB\(_{1-25K} \) amphiphile inhibitory function. Our CD results of
full-length PLB in lipids support solid-state NMR studies showing that the cytoplasmic domain is primarily helical in lipid bilayers. A phospholamban model in which long-range interactions between the cytoplasmic and transmembrane domains of PLB are part of a regulatory circuit with the Ca-ATPase has been proposed. One part of this regulatory relationship is the control of PLB function through phosphorylation sites in the cytoplasmic domain of PLB. Our results suggest that another part of the regulatory circuit is a long-range interaction between the TM and cytoplasmic domains that stabilizes secondary structure in the cytoplasmic domain. Our results support this model to the extent that PLB1–25K amphiphile, in which lipid tails serve only to anchor PLB1–25K to the lipid membrane and lack any specific long-range interactions with the peptide, showed neither secondary structure nor Ca-ATPase inhibition.

CONCLUSION

We have demonstrated that peptide-amphiphiles can be a useful tool in the study of the structure and function of isolated domains of integral transmembrane proteins. The peptide-amphiphile molecular architecture tethers the peptide to a membrane interface, creating an environment similar to that experienced by a cytoplasmic domain in its native configuration. Our model peptide-amphiphile showed secondary structure stabilization in both aqueous and lipid solutions. The results from the PLB1–25K domain suggest that anchoring a cytoplasmic domain to the membrane does not necessarily induce structure, even if structural enhancement is observed in aqueous solution. Ca-ATPase inhibition experiments indicate that the transmembrane domain of PLB is sufficient for inhibition of the Ca-ATPase and that the cytoplasmic domain PLB1–25K has no effect on Ca-ATPase activity, even when tethered to the membrane in a manner mimicking its native configuration. It is likely that the lack of helical content of the PLB1–25K amphiphile is related to its lack of Ca-ATPase regulation. Our experiments support the hypothesis that a long-range coupling between the transmembrane and cytoplasmic domains of PLB may act to stabilize structure in the cytoplasmic domain.

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