Lipid−Protein Interactions with Cardiac Phospholamban Studied by Spin-Label Electron Spin Resonance†

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ABSTRACT: Phospholamban is a cardiac regulatory protein that, in its monomeric form, inhibits the Ca^{2+}-ATPase. Lipid−protein interactions with a synthetic variant of phospholamban, in which all cysteine residues are replaced with alanine, have been studied by spin-label electron spin resonance (ESR) in different lipid host membranes. Both the stoichiometry and selectivity of lipid interactions were determined from the two-component ESR spectra of phospholipid species spin-labeled on the 14 C atom of the *sn*-2 chain. The lipid stoichiometry is determined by the oligomeric state of the protein and the selectivity by the membrane disposition of the positively charged residues in the N-terminal section of the protein. In dimyristoylphosphatidylcholine (DMPC) membranes, the stoichiometry (N_b) is 7 lipids/monomer for the full-length protein and 4 for the transmembrane section (residues 26−52). These stoichiometries correspond to the dimeric and pentameric forms, respectively. In palmitoyloleoylphosphatidylcholine, $N_b = 4$ for both the whole protein and the transmembrane peptide. In negatively charged membranes of dimyristoylphosphatidylglycerol (DMPG), the lipid stoichiometry is $N_b = 10-11$ per monomer for both the full-length protein and the transmembrane peptide. This stoichiometry corresponds to monomeric dispersion of the protein in the negatively charged lipid. The sequence of lipid selectivity is as follows: stearic acid > phosphatidic acid > phosphatidylserine = phosphatidylglycerol = phosphatidylcholine > phosphatidylethanolamine for both the full-length protein and the transmembrane peptide in DMPC. Absolute selectivities are, however, lower for the transmembrane peptide. A similar pattern of lipid selectivity is obtained in DMPG, but the absolute selectivities are reduced considerably. The results are discussed in terms of the integration of the regulatory species in the lipid membrane.

Phospholamban (PLB)[1] is a 52-residue integral membrane protein that exerts a phosphorylation-dependent regulatory effect on the cardiac calcium pump (Ca^{2+}-ATPase) (*1*, *2*). The protein is bitopic, and is characterized by a single, C-terminal, transmembrane domain and a charged N-terminal section that contains a preponderance of positively charged residues. Figure 1 gives the amino acid sequence of rabbit heart phospholamban. Three functional "domains" are indicated. Domain IA contains an amphipathic helix and the regulatory phosphorylation sites. Domain IB follows the hinge region and contains a high proportion of amidated residues. Domain II is hydrophobic and constitutes a transmembrane helix. The secondary structure indicated in Figure 1 is that determined for the C41F mutant of pig phospholamban by high-resolution NMR in a chloroform/methanol solution (*3*). A similar helix−turn−helix motif was determined earlier for residues 1−36 of human phospholamban in aqueous trifluoroethanol (*4*). In solution, the helix axes are tilted relative to one another by ca. 100−110 ± 20°. Recent solid state NMR with oriented membranes indicates

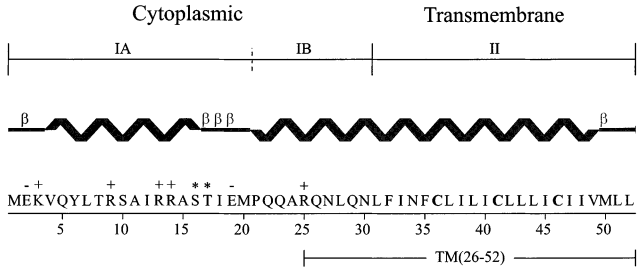

FIGURE 1: Amino acid sequence of rabbit heart phospholamban. Cysteine residues that are replaced with alanine are given in bold. Charged residues are denoted with + and − and phosphorylation sites with asterisks. The secondary structure cartoon corresponds to C41F pig heart phospholamban in chloroform-methanol (*3*). The region marked *βββ* is a type III turn.

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; 14-PCSL, 14-PESL, 14-PGSL, 14-PSSL, and 14-PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxy)]stearoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoglycerol, -phosphoserine, and -phosphoric acid, respectively; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxy)stearic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PLB, rabbit heart phospholamban; TM^{26-52}, transmembrane peptide of PLB (residues 26−52); ESR, electron spin resonance; NMR, nuclear magnetic resonance.

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that the amphipathic helix is oriented closer to the membrane plane than to the bilayer normal, with a tilt relative to the transmembrane helix that is in the range of 60−100° (*5*). The latter is in approximate accord with a tilt for domain IA of 60 ± 15° determined by polarized infrared spectroscopy (*6*). Nevertheless, other infrared spectroscopic measurements and solution NMR studies have depicted full-length phospholamban as a continuous helix (*7*, *8*).

Phospholamban shows a strong tendency to oligomerize, forming stable pentamers in sodium dodecyl sulfate (*2*, *9*). The pentamer has been modeled as a left-handed helical bundle with knobs-into-holes packing, with two different models for the possible orientations of the helices in the bundles (*7*, *10*, *11*). In the model of Simmerman et al. (*10*), the pentamer is stabilized by a Leu-Ile zipper, consistent with studies that show that mutagenesis of contact residues in the zipper sequence leads to disruption of the pentamer (*12*). Mutation of the three Cys residues in PLB to Ala also leads to loss of pentamer formation in SDS (*13*, *14*) for steric reasons (*15*). The pentameric form of phospholamban is in equilibrium with monomeric phospholamban, and it has been suggested that the phospholamban pentamer is a storage form whereas the functionally active form is the monomer (*16*, *17*). Reddy et al. (*18*) used a fluorescence energy transfer method to show that in SDS ∼50% of the PLB was present as a monomer. In contrast, in DOPC ∼13% of the PLB is monomeric, the rest being present as an oligomer of 9−11 monomers, which could be due to association of pentamers into larger aggregates (*18*).

Electron spin resonance spectroscopy (ESR) of spin-labeled lipids affords a ready means of investigating the stoichiometry and selectivity of lipid interaction with transmembrane proteins (see ref *19*). The stoichiometry is a measure of the intramembranous perimeter of the protein that is exposed to the lipid chains. This is dependent directly on the oligomer state of the protein (*20*). A selectivity for negatively charged lipids is an indicator for the positioning of positively charged amino acid residues in the vicinity of the lipid headgroups (*21*, *22*). These lipid affinities may be used to investigate the disposition of the extramembranous parts of the protein. In previous work, the lipid stoichiometry of association with native PLB and a single-residue mutant has been determined by spin-label ESR (*23*). The native protein was suggested to be a mixture of the monomer and pentamer in DOPC (*23*). Mutation of cysteine to alanine increases the stoichiometry of the lipid interaction, i.e., reduces the degree of oligomerization, of phospholamban (*24*).

In the work presented here, we investigate lipid−protein interactions with a cysteine-less variant of phospholamban. The mutant protein is used to eliminate the possibility of cysteine cross-linking and to concentrate more on the active monomeric form of phospholamban. Results for the full-length protein (residues 1−52) are compared with those for the transmembrane section (residues 26−52), as done already in studies on the inhibition of the calcium pump (*14*). Potentially, both cytoplasmic and transmembrane domains may affect lipid−protein interactions and protein oligomerization. Comparison of full-length and transmembrane phospholamban dissects out the contributions from the two domains. Different lipid species spin-labeled on the 14 C atom of the *sn*-2 chain are used to establish both the stoichiometry and selectivity of lipid−protein interaction. This is done for the protein in membranes of both zwitterionic and anionic lipids, and in membranes of both saturated and unsaturated-chain lipids. The oligomeric state of phospholamban is found to depend not only on the N-terminal section of the protein but also on the host lipid. Differential lipid selectivities displayed by the N-terminal section of phospholamban are relatively modest, suggesting that several positively charged residues are free to interact with the extramembranous domain of the Ca^{2+}-ATPase.

MATERIALS AND METHODS

Materials. Full-length phospholamban (residues 1−52) and the transmembrane peptide (residues 26−52) were produced by solid-phase synthesis using Fmoc chemistry for coupling, in the case of phospholamban, and t-Boc chemistry for the transmembrane peptide, as described previously (*14*). All cysteine residues of the native rabbit sequence were substituted with alanine, i.e., phospholamban (C36A, C41A, and C46A), and the peptide was N-acetylated. Purification and characterization by mass spectrometry of both peptides were carried out as previously described (*14*). The inhibitory activity of the peptides was assayed with sarcoplasmic reticulum Ca^{2+}-ATPase reconstituted in dioleoylphosphatidylcholine, at a lipid:protein molar ratio of 2000:1, as described in the same reference. The increase in the pCa value required for 50% maximal activity was approximately +0.5 at a peptide:ATPase molar ratio of 20:1, for both peptides. The amount of peptide required for inhibition decreased with decreasing lipid:ATPase ratio.

Dimyristoylphosphatidylcholine (DMPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Spin-labeled stearic acid, 14-SASL, was synthesized according to the method of Hubbell and McConnell (*25*). Spin-labeled phosphatidylcholine, 14-PCSL, was synthesized by acylation of lysophosphatidylcholine with 14-SASL, as described by Marsh and Watts (*26*). Other spin-labeled phospholipids, 14-PSSL, 14-PGSL, 14-PESL, and 14-PASL, were prepared from 14-PCSL by headgroup exchange mediated by phospholipase D (*26*).

Sample Preparation. Phospholamban or the transmembrane peptide was reconstituted in lipid membranes at the required lipid:protein ratio as described previously (*14*). The required amounts of peptide and lipid (ca. 1 mg of lipid) were codissolved in a chloroform/methanol solution (2:1, v/v), together with the desired lipid spin-label at 1 mol % (with respect to total lipid). The organic solvent was evaporated off with a stream of dry nitrogen gas, and the sample was incubated overnight under vacuum. The dry samples were hydrated with ca. 100 μL of 10 mM Hepes buffer (pH 7.4), by incubation at 50 °C for 5 min. The suspension was vortexed and sonicated on a bath-type sonicator (Branson) for 2 min at 35 °C. The sample was further subjected to five freeze−thaw cycles, and membranes were pelleted by centrifugation at 200000*g* on a Beckman TL 100 ultracentrifuge (TLA 100.3 rotor). The centrifugation was carried out at 20 °C for 30 min. The resulting pellet was resuspended in Hepes buffer, and the centrifugation step was repeated. Finally, the membrane pellet was homogenized with a small volume of buffer (∼100 μL), then transferred to a 1-mm diameter 100-μL glass capillary, and pelleted on

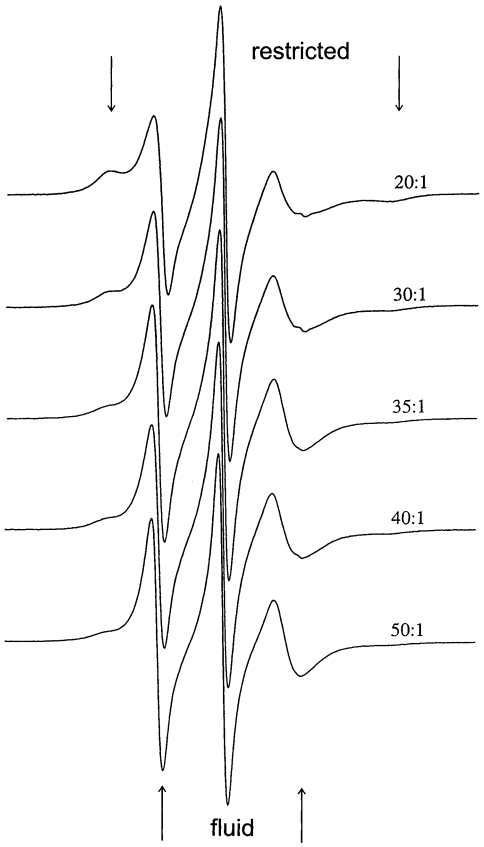

FIGURE 2: ESR spectra of the 14-SASL stearic acid spin-label in complexes of phospholamban with DMPC at the indicated lipid:protein molar ratios. T = 30 °C; total scan width = 100 G. The two spectral components are denoted with arrows above and below the spectra, respectively.

a bench centrifuge. Excess supernatant was removed, and the capillary was flame-sealed.

ESR Spectroscopy. ESR spectra were recorded on a Varian Century Line 9-GHz spectrometer with a rectangular cavity and nitrogen gas flow-temperature regulation. Sample capillaries were accommodated in a standard quartz ESR tube that contained light silicone oil for thermal stability. The temperature was measured with a fine-wire thermocouple positioned in the silicone oil at the top of the microwave cavity. Spectral subtraction and quantitation were performed as described by Marsh (*27*). A library of spectra from 14-PCSL in sonicated vesicles of DMPC at different temperatures in the gel and fluid phases was used to match the motionally restricted and fluid single-component spectra, respectively.

RESULTS

Lipid Spin-Label ESR Spectra. Figure 2 gives ESR spectra of the 14-SASL stearic acid spin-label in complexes of phospholamban with DMPC at different lipid:protein ratios. The spectra were recorded at 30 °C which is above the chain melting transition temperature of DMPC bilayer membranes. Figure 3 gives transition curves registered by the amplitude of the central peak in the ESR spectra. These confirm that the membrane is in the fluid phase at 30 °C, even for samples with the highest protein:lipid ratio.

The spectra of the spin-labeled lipid in Figure 2 all consist of two components that are indicated by the two sets of

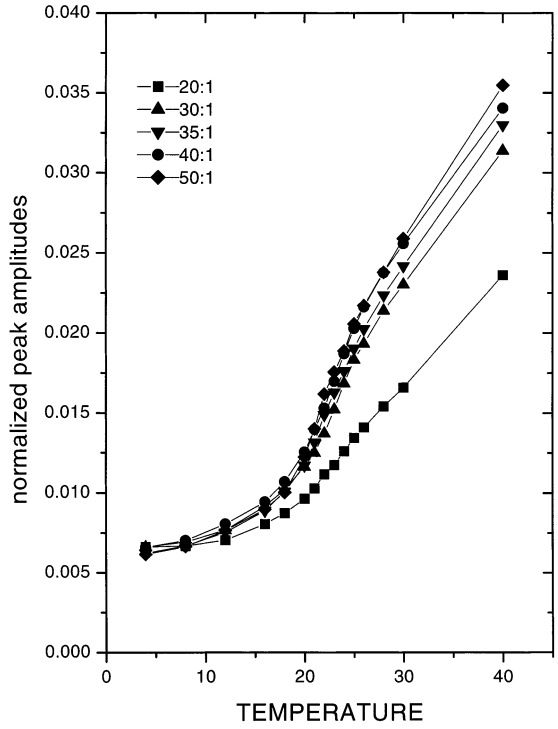

FIGURE 3: Temperature dependence of the central line height in the ESR spectra of the 14-SASL spin-label in complexes of phospholamban with DMPC at the indicated lipid:protein molar ratios.

arrows at the top and bottom of the figure. The relatively sharp three-line spectrum corresponds to lipids that are in the fluid bilayer regions of the membrane. The component with the larger hyperfine splitting that is visible in the outer wings of the spectra increases in intensity with increasing protein content in the membrane. This component arises from lipids where chain motion is restricted, relative to the fluid bilayer lipids, by direct interaction with the intramembranous section of the protein (see ref *28*).

Lipid/Protein Titration in DMPC. The relative proportions of the motionally restricted and fluid spin-label populations, n_f^*/n_b^*, were determined by spectral subtraction, using sonicated DMPC vesicles as a reference library. Double integration yields the fractional contribution, f, of the motionally restricted lipid component to the total intensity where $n_f^*/n_b^* = (1 - f)/f$. The results are given in Figure 4 for the 14-PCSL, 14-PASL, and 14-SASL spin-labels in complexes of phospholamban and the transmembrane peptide with DMPC at various lipid:protein ratios, n_t. The data are plotted according to the equation for equilibrium exchange association of spin-labeled lipids with the protein (*28*, *29*):

$$n_f^*/n_b^* = (n_t/N_b - 1)/K_r \quad (1)$$

where N_b is the number of first-shell lipid association sites per protein and K_r is the mean association constant of the spin-labeled lipid, relative to the background host lipid. The data for spin-labeled phosphatidylcholine, 14-PCSL, correspond reasonably well with the expectation that $K_r \approx 1$ relative to the phosphatidylcholine host lipid. This then yields a first-shell stoichiometry (N_b) of 7 lipids/PLB$^{1-52}$ monomer for phospholamban, but a lower value of N_b = 4 lipids/TM$^{26-52}$ monomer for the transmembrane peptide. Both the phosphatidic acid (14-PASL) and stearic acid (14-SASL)

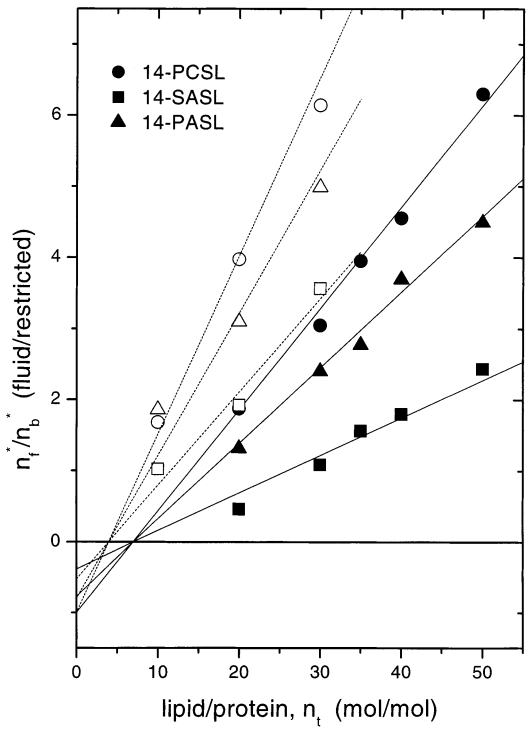

FIGURE 4: Dependence on the lipid:protein molar ratio, n_t, of the ratio of the fluid to motionally restricted populations, n_f^*/n_b^*, of spin-labeled lipids in complexes of phospholamban$^{1-52}$ (filled symbols) or the transmembrane peptide$^{26-52}$ (empty symbols) with dimyristoylphosphatidylcholine: (● and ○) 14-PCSL, (▲ and △) 14-PASL, and (■ and □) 14-SASL. Values of n_f^*/n_b^* [$=(1 - f)/f$] were obtained by spectral subtractions. Lines (solid and dashed) are predictions according to eq 1, with the values of N_b and K_r that are given in the text.

spin-labeled lipids display a selectivity relative to phosphatidylcholine (14-PCSL) for interaction with phospholamban and with the transmembrane peptide. The degree of selectivity differs, however, between these two negatively charged lipids. The lines drawn through the data that are given in Figure 4 are consistent with the stoichiometries determined with 14-PCSL and correspond to relative selectivities K_r/K_r^{PC} of 2.6 (1.3) for 14-SASL (14-PASL) interacting with phospholamban and $K_r/K_r^{PC} = 1.9$ (1.2) for 14-SASL (14-PASL) interacting with the transmembrane peptide.

Lipid Selectivity in DMPC. The selectivity with a wider range of spin-labeled lipids has been investigated in DMPC complexes at a fixed lipid:protein ratio. The spectra of spin-labeled phosphatidylethanolamine (14-PESL), phosphatidylglycerol (14-PGSL), and phosphatidylserine (14-PSSL) are given, along with those for 14-PCSL, 14-PASL, and 14-SASL, in Figure 5, for 1:30 PLB−DMPC complexes of identical lipid:protein molar ratios. The motionally restricted spectral component is clearly greater, relative to the fluid spectral component, for 14-SASL than for the other spin-labeled lipids. Quantitatively, the pattern of selectivity can be established by spectral subtraction for the different spin-labeled lipids. The association constants, K_r, relative to that for phosphatidylcholine, K_r^{PC}, can be determined from eq 1:

$$K_r/K_r^{PC} = (n_f^*/n_b^*)^{PC}/(n_f^*/n_b^*) \qquad (2)$$

where values of n_f^*/n_b^* for the lipid of interest are determined for a complex with a lipid:protein ratio identical to

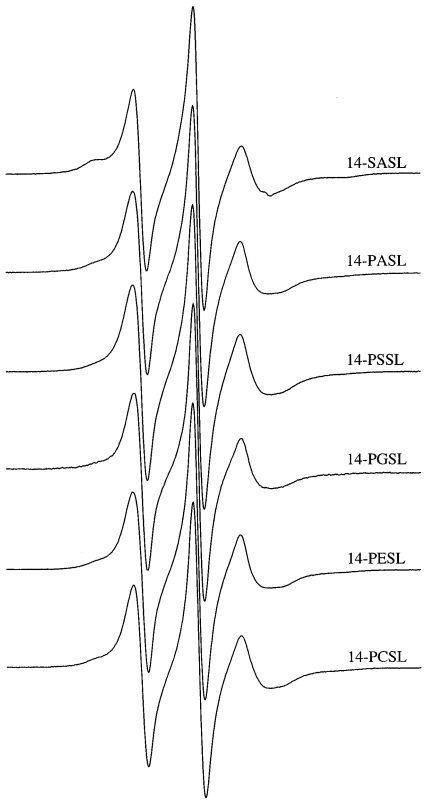

FIGURE 5: ESR spectra of 14 C atom spin-labeled lipids in complexes of phospholamban and DMPC at a 1:30 molar ratio at 30 °C. The stearic acid (14-SASL) and different phospholipid (14-PXSL) spin-labels that were used are indicated. Total scan width = 100 G.

Table 1: Fractions, *f*, of Motionally Restricted Spin-Labeled Lipid and Relative Association Constants, K_r/K_r^{PC}, in 1:30 (Molar Ratio) Phospholamban−DMPC Complexes [PLB(1−52)] and 1:20 (Molar Ratio) Transmembrane Peptide−DMPC Complexes [PLB(26−52)] at 30 °C

	PLB(1−52)		PLB(26−52)		$\Delta\Delta G^a$
lipid	*f*	K_r/K_r^{PC}	*f*	K_r/K_r^{PC}	(kJ/mol)
14-SASL	0.48	2.8	0.34	1.8	1.1
14-PASL	0.29	1.3	0.24	1.1	0.3
14-PSSL	0.25	1.0	0.23	1.0	0.0
14-PGSL	0.24	1.0	0.23	1.0	−0.2
14-PESL	0.23	0.9	0.19	0.8	0.4
14-PCSL	0.25	1.0	0.22	1.0	0.0

[a] $\Delta\Delta G$ is the contribution to the free energy of selectivity in lipid−protein interaction from the peptide truncation.

that for which $(n_f^*/n_b^*)^{PC}$ is determined for 14-PCSL. The resulting values of the relative association constants are given in Table 1 for both phospholamban, PLB (1−52), and for the transmembrane peptide, PLB (26−52).

The free energy associated with the selectivity of lipid−protein interaction is given by

$$\Delta G - \Delta G^{PC} = -RT \ln(K_r/K_r^{PC}) \qquad (3)$$

relative to phosphatidylcholine (PC). Table 1 lists values for the difference ($\Delta\Delta G$) in free energy of interaction with full-length phospholamban and the transmembrane peptide.

Lipid−Protein Interactions in POPC. Comparable experiments have also been performed with reconstitution of the protein in a host phospholipid of different chain composition,

Table 2: Fractions, *f*, of Motionally Restricted Spin-Labeled Lipid and Relative Association Constants, K_r/K_r^{PG}, in 1:50 (Molar Ratio) Phospholamban−DMPG Complexes [PLB(1−52)] and 1:20 (Molar Ratio) Transmembrane Peptide−DMPG Complexes [PLB(26−52)] at 30 °C

	PLB(1−52)		PLB(26−52)		$\Delta\Delta G^a$
lipid	*f*	K_r/K_r^{PG}	*f*	K_r/K_r^{PG}	(kJ/mol)
14-SASL	0.28	1.3	0.61	2.0	−1.0
14-PASL	0.25	1.1	0.37	0.7	1.0
14-PCSL	0.22	1.0	0.38	0.8	0.5
14-PGSL	0.23	1.0	0.44	1.0	0.0

[a] $\Delta\Delta G$ is the contribution to the free energy of selectivity in lipid−protein interaction from the peptide truncation.

Table 3: Ionic Strength Dependence of the Fraction, *f*, of Motionally Restricted 14-SASL and Relative Association Constants, K_r/K_r^{PG}, Referred to 14-PGSL, in 1:50 (Molar Ratio) Phospholamban−DMPG Complexes at 30 °C

lipid	[NaCl] (M)	*f*	K_r/K_r^{PG}	$\Delta G - \Delta G^{PG}$ [a] (kJ/mol)
14-SASL	0.0	0.28	1.3	−0.7
14-SASL	0.05	0.23	1.0	−0.1
14-SASL	0.2	0.23	1.0	0.0
14-SASL	0.5	0.21	0.9	0.2
14-PGSL	0.0	0.23	1.0	0.0

[a] $\Delta G - \Delta G^{PG}$ is the free energy of lipid−protein interaction, relative to 14-PGSL without NaCl.

palmitoyloleoylphosphatidylcholine (POPC), which has longer chains, one of which is unsaturated (data not shown). For PLB−POPC complexes with molar ratios of 1:20 and 1:30, the results with the phosphatidylcholine spin-label 14-PCSL at 30 °C are both consistent with a first-shell stoichiometry (N_b) of 4.0−4.1, assuming that $K_r = 1$. This stoichiometry is considerably lower than that found for PLB in DMPC and identical with that found for TM^{26-52} in the latter host. The lipid selectivity for stearic acid and for phosphatidic acid is preserved in the POPC host lipid, giving essentially consistent values of $K_r/K_r^{PC} = 2.3-2.6$ and 1.3 for 14-SASL and 14-PASL, respectively, at both lipid:protein ratios. For the transmembrane peptide TM^{26-52}−POPC complexes at molar ratios of 1:10 and 1:20, spin-labeled phosphatidylcholine again yields a stoichiometry (N_b) of 3.8−4.2 and the selectivity for stearic acid is given by a K_r/K_r^{PC} of 1.7−2.4, where the smaller values in each range correspond to the lower lipid:protein ratio.

Lipid−Protein Interactions in DMPG. Experiments have been performed additionally with reconstitution of the protein in a host lipid of different polar headgroup, dimyristoylphosphatidylglycerol (DMPG) (data not shown). This lipid has the same acyl chain composition as DMPC, but the phosphoglycerol headgroup is negatively charged at neutral pH, unlike the zwitterionic phosphocholine headgroup. For a 1:50 mol/mol PLB−DMPG complex, the fraction of motionally restricted phosphatidylglycerol spin-label 14-PGSL corresponds to a first-shell stoichiometry N_b of 11, assuming that $K_r = 1$ for 14-PGSL relative to DMPG. This stoichiometry is yet higher than that found for PLB in DMPC. For the transmembrane peptide TM^{26-52}−DMPG complexes with molar ratios of 1:20 and 1:30, spin-labeled phosphatidylglycerol again yields a high stoichiometry N_b of 9−11.

The selectivity of full-length PLB and the transmembrane segment TM^{26-52} for different spin-labeled lipids was investigated in complexes with DMPG at a fixed lipid:peptide ratio. Table 2 gives the fractions of the motionally restricted spin-label and the values of the relative association constants, K_r/K_r^{PG}, referred to that of 14-PGSL. Because the DMPG host is a negatively charged lipid, the effect of screening the membrane surface charge was also studied. This was done with 14-SASL, the lipid that displays the greatest selectivity. Table 3 gives values of the fraction of motionally restricted 14-SASL in DMPG complexes of phospholamban with a fixed lipid:protein ratio, at different salt concentrations. Also given in Table 3 are the relative association constants, K_r/K_r^{PG}, referred to 14-PGSL in samples with the same lipid:protein ratio in the absence of salt. In 0.05 M NaCl, the fraction of motionally restricted 14-SASL is reduced to a plateau value that is equal to that for 14-PGSL in the DMPG host.

DISCUSSION

The experiments presented here reveal considerable differences in both the stoichiometry and selectivity of lipid−protein interaction between full-length phospholamban PLB^{1-52} and its transmembrane peptide TM^{26-52}. Further, both stoichiometry and selectivity can be modulated by the host lipid. This is particularly significant in the case of the stoichiometry of lipid interaction.

Stoichiometry and Aggregation State. The number of motionally restricted lipids associated with the intramembranous perimeter directly reflects the oligomerization state of the transmembrane protein (*30*). A single transmembrane helix with a diameter of 1 nm can accommodate $N_b \sim 10$ diacyl lipids around its perimeter in a lipid bilayer, consistent with the observation that a DOPC:phospholamban molar ratio of >10:1 was required for efficient reconstitution of phospholamban (*14*). Dimerization will occlude ~25% of the intramembranous surface from interaction with lipid. This would result in a stoichiometry per monomer of $N_b^{(1)} \sim 7-8$. For a pentameric structure of transmembrane helices, the number of perimeter lipids per helix is given by (*20*)

$$N_b^{(1)} = \frac{\pi}{n_\alpha}\left(\frac{D_\alpha}{d_{ch}} + 1\right) + \frac{D_\alpha}{d_{ch}} \quad (4)$$

where n_α is the number of transmembrane helices and D_α and d_{ch} are the diameters of an α-helix and a lipid chain, respectively. This expression applies equally to a helical sandwich or a regular polygonal arrangement. When $n_\alpha = 5$, it is predicted that $N_b^{(1)} \approx 4$. This corresponds to the oligomerization state that is found for PLB on SDS gels (*9*).

Estimates for the effect of random protein−protein contacts on the stoichiometry of lipid−protein interaction can be made from a simple lattice theory (*31*, *32*):

$$N_b^{(1)} = n_t[1 - \exp(-N_{b,o}^{(1)}/n_t)] \quad (5)$$

where $N_{b,o}^{(1)}$ is the value of N_b per helix in the absence of statistical protein−protein contacts. Equation 5 predicts a 20% reduction in the measured stoichiometry $N_b^{(1)}$ when the lipid:protein ratio, n_t, is $2N_{b,o}^{(1)}$, a 60% reduction for $n_t = N_{b,o}^{(1)}$, and a very steep decrease beyond this. Little dependence on the lipid:protein ratio is found experimentally over

Table 4: Stoichiometries (N_b) of Lipid−Protein Interactions and the Degree of Oligomerization (n_α) for Phospholamban (PLB) and Transmembrane Peptide (TM) Mutants in Different Lipid Hosts

protein	lipid	N_b (mol/mol)	n_α	ref[a]
PLB (C36A/C41A/C46A)	DMPC	7.1 ± 0.2	1.9 ± 0.1	1
	POPC	4.0 ± 0.1	4.9 ± 0.1	1
	DMPG	11.3	1.0	1
	DOPC	7.8 ± 0.3	2.2 ± 0.5	2
TM^{26-52} (C36A/C41A/C46A)	DMPC	4.0 ± 0.2	5.1 ± 0.6	1
	POPC	4.0 ± 0.2	5.0 ± 0.5	1
	DMPG	10.1 ± 1.3	1.2 ± 0.2	1
	DOPC	6.0 ± 0.6	3.3 ± 0.4	2
PLB (WT)	DOPC	5.6 ± 0.5	3.5 ± 0.4	3
PLP (L37A)	DOPC	12 ± 0.5	1.15 ± 0.15	3

[a] For references, 1 represents this work [means and standard deviations (or absolute deviations) for samples with different lipid:protein ratios], 2 ref *24*, and 3 ref *23*.

the ranges that were studied. A systematic decrease in $N_b^{(1)}$ with decreasing n_t is found only for samples with an $N_{b,o}^{(1)}$ of $\approx$4, corresponding to a pentameric assembly. For these samples, the overall decrease in $N_b^{(1)}$ is considerably smaller than that predicted by eq 5, although fractional changes with n_t [for $n_t \geq 2N_{b,o}^{(1)}$] are reproduced well by this equation. From this, it can be concluded that the peptides in their stable oligomeric forms are rather well solubilized by the lipid, and that statistical protein−protein contacts are relatively unimportant.

Comparing the theoretical predictions with the results presented here on the stoichiometry of lipid−protein interaction, it is found that the transmembrane peptide TM^{26-52} is in a pentameric state in both phosphatidylcholine lipid hosts that were tested. This is irrespective of the chain length and degree of unsaturation. In a negatively charged lipid host, however, the transmembrane peptide is predominantly monomeric. On the other hand, the oligomerization state of the full-length protein PLB^{1-52} depends not only on the headgroup charge of the lipid host but also on the lipid chain composition. It varies from monomeric in DMPG to pentameric in POPC. In DMPC, however, the oligomerization state of PLB is on average dimeric. As discussed by Cornea et al. (*23*), a mean degree of oligomerization of 1.9 in DMPC could reflect either a predominantly dimeric state or a mixture of the monomer and pentamer with mostly monomer present.

The difference in the oligomeric state of the peptides in DMPC and DMPG must be attributed to the surface charge, and possibly also different surface hydration, of anionic DMPG bilayers. In terms of chain mobility and chain melting, bilayer membranes of the two lipids are very similar (see ref *33*). Presumably, the monomeric form of phospholamban is better solubilized or stabilized by the negatively charged lipid, at least in part because of favorable electrostatic interactions. In the pentameric state, it is expected that the helices will pack parallel, given the specific interactions between PLB monomers that lead to pentamer formation. However, as in other reconstitution studies, we have no direct evidence on the polarity of the helix packing.

Table 4 summarizes the lipid stoichiometries, N_b, obtained in the different lipid hosts, and the resulting degree of oligomerization, n_α, deduced from eq 4. Values of N_b in DMPC and POPC are deduced from measurements with 14-PCSL and those in DMPG from measurements with 14-PGSL. In each case, it is assumed that $K_r = 1$ for the spin-labeled lipid, relative to the host lipid (cf. Figure 4). The values given for N_b are means and standard deviations for the different lipid:protein ratios that were studied, or the absolute deviation in the case of only two lipid:protein ratios. For PLB in DMPC (five samples), the maximum deviation from the mean is 0.3, and there is no dependence on the lipid:protein ratio. The random error for samples measured at fewer lipid:protein ratios is therefore likely to be in the region of $\pm$0.3.

Table 4 also includes data (*23*, *24*) from different variants of phospholamban in dioleoylphosphatidylcholine (DOPC), for comparison. The lipid stoichiometry of cysteine-less PLB in DOPC is rather similar to that found here in DMPC, but not in POPC. Note, however, that the determinations in DOPC were made at 0 °C (*24*), whereas all described here were made at 30 °C. In DOPC, the transmembrane peptide has a lower lipid stoichiometry and a higher degree of oligomerization than the full-length protein, just as found here in DMPC. Wild-type phospholamban in DOPC has an oligomer state comparable to that of the cysteine-to-alanine mutant of the transmembrane peptide, but is monomerized by the L37A mutation (*23*). In the latter case, the lipid stoichiometry is similar to that found here for PLB (C36, 41,46A) in DMPG. The decreased lipid stoichiometry for TM^{26-52} compared to that of intact phospholamban in DMPC suggests that electrostatic repulsion between the charged cytoplasmic domains leads to destabilization of the pentamer for the intact phospholamban and, therefore, that pentamer formation is driven by favorable interactions between the transmembrane domains, as suggested by Karim et al. (*24*). The increased degree of oligomerization for phospholamban in POPC, relative to DMPC, is consistent with the observation that increasing fatty acyl chain length leads to more favorable helix−helix interactions in the membrane (*34*).

The monomeric stoichiometry found for the L37A mutant and for PLB (C36,41,46A) in DMPG is significant. It is far from self-evident that a single transmembrane α-helix would provide a hydrophobic intramembranous surface that is sufficiently large, in molecular terms, to induce the motional restriction of the lipid chains that is produced by large integral proteins. Indeed, simple alternating leucine-alanine transmembrane α-helices that are trytophan- or lysine-flanked do not induce a motionally restricted spin-labeled lipid population (*35*, *36*), nor does the single hydrophobic α-helix of the lung surfactant protein SP-C (*37*). The phospholamban monomer therefore has a particular sequence composition that is capable of supporting motional restriction of adjacent lipids. Possibly, this feature also contributes to the propensity for helix−helix interactions, but is not so susceptible to specific disruption by mutation of single residues as in the latter case. Helix rigidity may be a further factor contributing to motional restriction of the lipid chains.

Although the data for 14-SASL stearic acid are reasonably consistent with the same stoichiometry as that of 14-PCSL but a higher selectivity than that of 14-PCSL, an alternative interpretation of the lipid−protein titration is possible. A linear least-squares fit for 14-SASL in Figure 4 yields the following parameters: $K_r \approx 1.2 \pm 0.1$ and $N_b \approx 13 \pm 2$. This higher stoichiometry suggests that the single-chain lipid, stearic acid, may be able to access more sites at the intra-

membranous surface of the integral protein than can diacyl phospholipids.

Lipid Selectivity. Both phospholamban and its transmembrane peptide display a selectivity for certain negatively charged lipids, although this is not uniform for lipids with the same formal charge (see Tables 1 and 2). This indicates that the observed selectivity is not solely electrostatic in origin, which is a feature shared in common with the lipid interactions of other integral proteins (*22*). In addition, the degree of selectivity depends, as might be expected, on the headgroup charge of the host phospholipid. In phosphatidylcholine, full-length phospholamban has a higher affinity for those lipids that display a selectivity than does the transmembrane peptide (see the last column of Table 1). Evidently, the residues of the N-terminal region that is truncated in the transmembrane peptide contribute to the enhanced lipid selectivity of the full-length protein. Among these are the positively charged residues of the N-terminal section, some of which (probably in domain IB) must be located in the vicinity of the phospholipid headgroups in the membrane-bound protein. Note also, however, that the oligomerization states of PLB^{1-52} and TM^{26-52} differ in DMPC. This additionally may change the accessibility and orientation to the lipid polar groups of the side chains from corresponding residues in the two peptides.

In phosphatidylglycerol, the affinity of negatively charged lipids for phospholamban is significantly reduced (compare Tables 1 and 2). This can be interpreted as a more effective competition of DMPG than of DMPC for lipid sites on the protein. Exceptionally, the selectivity of stearic acid (14-SASL) for the transmembrane segment is greater than that for whole phospholamban, even though both are essentially monomeric in DMPG. This could point to a preferential association of the single-chain lipid for the transmembrane section, whereby the affinity is diminished proportionally less in DMPG than is that for the more highly basic full-length protein. The selectivity of full-length phospholamban in DMPG for 14-SASL is screened out already at 0.05 M NaCl (see Table 3). This indicates that the preferential interaction is of a classical electrostatic nature and presumably originates from a somewhat different location of the stearic acid polar group, relative to that of phosphatidylglycerol.

Despite the preponderance of basic residues in the N-terminal section of phospholamban, the selectivity for negatively charged lipids is relatively modest compared with that for several other integral transmembrane proteins (cf. refs *19* and *22*). In particular, the five basic (and two acidic) residues in the polar domain of phospholamban exert a much smaller effect on lipid selectivity than do the six basic (and two acidic) residues in the segment of the polar loop of the myelin proteolipid that is deleted in the DM-20 isoform (*21*). Quite possibly, the amphipathic helix of domain IA acts as a surrogate peripheral protein and competes for negatively charged lipids at the intramembranous surface of domain II. Such an effect has been observed in double reconstitutions of the myelin basic protein and proteolipid protein (*38*). Alternatively, the amphipathic helix may be tilted away from the membrane surface and/or be oriented azimuthally such that the positively charged side chains are directed away from the lipid headgroups. The latter possibility would allow the basic residues in phospholamban to interact more freely with the inhibitory site in the cytoplasmic sector of the Ca^{2+}-ATPase, most probably the surface of the nucleotide-binding domain (*39*).

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