The human 31-amino acid integral membrane protein sarcolipin (SLN), which regulates the sarcoplasmic reticulum Ca-ATPase in fast-twitch skeletal muscle, was chemically synthesized. Appropriate synthesis and purification strategies were used to achieve high purity and satisfactory yields of this hydrophobic and poorly soluble protein. Structural and functional properties of SLN were analyzed and compared with the homologous region of human phospholamban (PLB) comprising residues Ala^{24}–Leu^{52} (PLB-{24–52}), the regulatory protein of the cardiac sarcoplasmic reticulum Ca-ATPase. Circular dichroism spectroscopy showed that SLN is a predominantly α-helical protein and that the secondary structure is highly resistant to SDS and thermal denaturation. In this respect SLN is remarkably similar to PLB-{24–52}. However, SLN is monomeric in SDS gels, whereas PLB-{24–52} shows a monomer-pentamer equilibrium typical for native PLB. Analytical ultracentrifugation experiments revealed that SLN oligomerizes in the presence of the nonionic detergents octylpolyoxyethylene and octyl glucoside in a concentration-dependent manner. No plateau was observed, and a pentameric state was only reached at much higher protein concentrations compared with PLB-{24–52}. Chemical cross-linking showed that also in liposomes SLN has the ability to self-associate to oligomers. PLB-{24–52} specifically oligomerized to pentamers in the presence of octylpolyoxyethylene as well as in liposomes at low protein concentrations. In the presence of octylpolyoxyethylene pentamers were the main oligomeric species, whereas in liposomes monomers and dimers were predominant. Increasing the protein concentration led to self-association of PLB-{24–52} pentamers in the presence of octylpolyoxyethylene. Functional reconstitution of Ca-ATPase with PLB-{24–52} and SLN in liposomes showed that both proteins regulate the Ca-ATPase in a similar manner.

Phospholamban (PLB)\(^1\) and sarcolipin (SLN) are integral membrane proteins involved in the regulation of the sarcoplasmic reticulum Ca-ATPase. The transmembrane domain of the two proteins is highly conserved, but SLN lacks an extended cytoplasmic domain. Structural similarities between the sln and the plb gene, as well as the homology between the protein sequences of their products, led to the proposal that the two genes are members of a family (1).

The 52-residue protein PLB is located in the sarcoplasmic reticulum of cardiac and slow-twitch skeletal muscle (2, 3). PLB is a critical regulator of the cardiac sarcoplasmic reticulum Ca-ATPase (SERCA2a) and myocardial contractility (4). Recently it has been shown for a mouse model of dilated cardiomyopathy that PLB is a key element in the progression of the disease (5). PLB binds to and inhibits SERCA2a in its unphosphorylated form, and phosphorylation of PLB removes the inhibitory effect. PLB consists of a hydrophilic N-terminal domain, located in the cytosol, and a hydrophobic C-terminal domain traversing the sarcoplasmic reticulum membrane. The latter has an α-helical conformation and is located within residues 26–52 (6).

The transmembrane α-helical domain is responsible for protein oligomerization, and SDS-PAGE suggests that the oligomeric structure of PLB is a homopentamer (7). Purified PLB and the transmembrane domain PLB-{26–52} show conductance properties similar to channels, suggesting that PLB can form a pentameric pore structure in membranes (8).

Based on mutagenesis data, Simmerman et al. (9) proposed a model for the PLB pentamer as a left-handed coiled coil stabilized by a leucine-isoleucine zipper. Although other models for the PLB pentamer were presented (10) the leucine-isoleucine zipper model was supported by investigation of the cysteine reactivity of PLB and its mutants (11) and by infrared spectroscopic analysis of PLB and molecular modeling (12).

The 31-amino acid proteolipid SLN is located in the sarcoplasmic reticulum of fast-twitch skeletal muscle (13). The muscle-specific expression of SLN is complementary to PLB expression, mimicking the expression pattern of SERCA1 (1). SLN has been described to be a regulator of SERCA1, as PLB inhibits Ca^{2+} uptake at low Ca^{2+} concentrations. In addition...
and, in contrast to most reports on PLB, it enhances Ca-ATPase activity by increasing $V_{\text{max}}$ at high Ca$^{2+}$ concentrations (14).

In contrast to PLB, no structural data are available about the SLN protein. In order to get high amounts as well as a high purity of SLN, we chemically synthesized the full-length protein. Modifications to standard protocols were required for synthesis and purification of this extremely insoluble intrinsic membrane protein. We then analyzed the structural and functional properties of human SLN and compared it with the homologous region of human PLB comprising residues Ala$^{24}$-Leu$^{52}$, PLB-(24–52), which we also have synthesized.

The second structure of SLN was found to be very similar to the one of PLB-(24–52). Both proteins are predominantly α-helical, and their secondary structures showed resistance to SDS denaturation and to thermal perturbation. However, the oligomeric state of both proteins proved to be different. PLB-(24–52) forms pentamers in 1% n-octylpolyoxyethylene (octyl-POE) and in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes, and these oligomers are stable to SDS denaturation and to thermal perturbation. However, the pentamers are not specifically formed, and the oligomers are not SDS-stable. Both synthetic proteins regulate the Ca-ATPase in a similar manner.

Part of the data was published previously in abstract form (15).

**EXPERIMENTAL PROCEDURES**

**Protein Synthesis**—Synthesis of human PLB-(24–52) was performed as described (16). Synthesis of human SLN was performed on solid support using Fmoc-tert-Bu chemistry on an ABI 431A fully automated synthesizer (ABI System Inc., Forster City, CA). The C-terminal amino acid was loaded onto a Tentagel S PHB resin (Rapp Polymers, Tübingen, Germany) using 4-dimethylaminopyridine-catalyzed esterification with preformed Fmoc-Tyr(tert-Bu)-OH symmetrical anhydride. Loading was 0.2 mmol/g resin as determined spectrophotometrically. Double coupling procedures with a total of 8-fold Fmoc-amino acid excess in the presence of 2-1HF-benzenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-1-hydroxybenzotriazol-N,N,N',N'-diisopropylamine (1:1:2 equivalents) in NMP(dimethylformamide/CH$_{2}$Cl$_{2}$, 1:1:1, v/v) were used. Fmoc protection was performed in two steps of 4 and 12 min, with 2% piperidine, 2% 1,8-diazabicyclo[5.4.0]undec-7-ene in NMP/CHCl$_{3}$, (1:1, v/v). All the washing steps were performed with NMP(dimethylformamide/CHCl$_{3}$, 2:1, v/v). The final protein was cleaved from the resin and desalted with a mixture of trifluoroacetic acid/water/thioanisole/ethanedithiol (90:5.5:3:1.5, v/v). The slurry was shaken for 100 min at room temperature and then filtered. The crude product was precipitated with methyl tert-butyl ether, filtered, and dried (0.31 g).

**Purification of SLN**—Preparative HPLC was performed with UV monitoring at 210 nm using C8 columns (220 × 12.5 mm; Machery & Nagel, Düren, Germany) with 0.1% aqueous trifluoroacetic acid (eluent A) and CH$_{3}$CN/2-propanol (1:1, eluent B) at flow rates of 8 ml/min. The crude product was divided into 50-mg fractions, dissolved in trifluoroacetic acid, and quickly injected into the preparative HPLC. The protein was eluted with a step gradient starting with 2% isocratic elution of 10% B followed by an 18-min gradient from 40% B to 60% B and finally from 60% B to 80% B in 42 min. The fractions from 41 to 47 min were collected, pooled, and lyophilized. A further purification step of the lyophilized product was performed, using the same gradient as before. Fractions from 42 to 45.5 min were collected and lyophilized; yield, 7 mg of pure product (16%). For electrospray ionization-mass spectroscopy: $m/z$ [M + 2H]$^{2+}$ = 1881.4 Da; calculated $m/z$ [M + 2H]$^{2+}$ = 1882.0 Da. For amino acid analysis: Asp 2.0 (2); Thr 2.59 (3); Ser 1.29 (1); Gly 2.21 (2); Val 2.11 (3); Met 1.83 (2); Ile 2.58 (4); Leu 5.19 (6); Tyr 1.91 (2); Phe 1.47 (2); Trp 0.89 (1); Arg 2.56 (2).

**Fluorescence Spectroscopy**—Spectra were recorded on a Jasco FP-777 spectrophluorimeter with 2-mm quartz cuvettes thermostated at 20 °C. The excitation wavelength was set to 280 or 295 nm. The excitation slit width was 1.5 nm, and the emission slit width was 3 nm. The background intensity of the buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl) including detergent or lipid vesicles was subtracted from the fluorescence spectra.

**Circular Dichroism Spectroscopy**—An Aviv 62DS circular dichroism spectropolarimeter was used with thermostated 1-mm quartz cuvettes. Each spectrum was the average of four scans and was corrected for the buffer contribution including detergent or lipid vesicles. Buffers used were 10 mM Tris-HCl, pH 7.5, or 50 mM sodium phosphate, pH 7.5.

**Analytical Ultracentrifugation**—A Beckman model XLA analytical ultracentrifuge was employed. Sedimentation velocity runs were performed in 12-mm double sector cells at 56,000 rpm at 20 °C. Sedimentation equilibrium runs were performed using the same cells also at 20 °C but at a filling height of 3 mm only; in case of OG, the filling height was decreased to 1.5–2.5 mm. The wavelength and double sector cell thickness were varied according to the protein concentration used. The molecular masses were calculated from sedimentation equilibrium runs using a floating base-line computer program that adjusts the base-line absorbance to obtain the best linear fit of In $A$ versus $r$ ($A$ is the absorbance and $r$ is the distance from the rotor axis). A partial specific volume of the proteins of 0.73 cm$^{3}$/g in octyl-POE or of 0.757 cm$^{3}$/g in OG (17) was used for the calculations. The buffer density including octyl-POE is close to 1.0 g/cm$^{3}$, and therefore runs were performed as in aqueous solutions (18). By using OG the runs were performed at densities of gravitational transparency by adding sucrose as described (17).

**Protein Insertion into Lipid Vesicles**—Lyophilized PLB-(24–52) or SLN was solubilized in trifluoroethanol and adsorbed to POPC liposomes that was dissolved in chloroform. The mixture was dried, dissolved in CH$_{3}$CN, and again dried. Buffers were added to get a final POPC concentration of 6.6 mM. The dispersion was vortexed extensively. For preparation of small unilamellar vesicles, the dispersion was sonified (in ice water, under nitrogen) using a titanium tip ultrasonicator until the solution became transparent (about 20 min). Titanium debris and nonsolubilized protein were removed by centrifugation, and the protein concentration was measured.

**Chemical Cross-linking**—Chemical cross-linking of PLB-(24–52) was performed using bis(sulfosuccinimidyl)suberate (BS$^3$), and ethylene glycol bis(sulfosuccinimidylsuccinate) (Sulfo-EGS) (Pierce). Lyophilized PLB-(24–52) was solubilized in 20 mM sodium phosphate, pH 7.5, 1% octyl-POE (v/v), or PLB-(24–52) was incorporated into liposomes (see above) using 20 mM sodium phosphate, pH 7.2 as buffer. The protein (40 µM) was incubated at a 50–fold molar excess of cross-linker for 30 min at 25 °C. The reaction was stopped by adding 100 mM Tris-HCl, pH 6.8.

Sulfo-EGS is a hydroxylamine-cleavable cross-linker and was cleaved in control experiments by incubating the cross-linked protein with 1 M hydroxylamine for 6 h at 37 °C at pH 8.5.

**Chemical cross-linking of SLN** was performed using p-azidophenyl glyoxal monohydrate (APG) and 4-p-azidosacilylamidobutyramide (ASBA) (Pierce). Buffers used were 20 mM sodium phosphate, pH 7.5, in case of cross-linking experiments with APG, or 20 mM MES, pH 4.5, in case of experiments using ASBA. SLN was incorporated into liposomes (see above), and the protein (60 µM) was incubated at different concentrations of APG for 4 h at 25 °C in the dark. The following photocleavage procedure was performed with a 300 nm UV light source for 15 min at 25 °C. Cross-linking with ASBA was performed according to the manufacturer's instructions.

**SR Vesicles**—SR vesicles were prepared from the fast-twitch skeletal muscle of New Zealand White rabbits (19). The SR vesicles were purified on a sucrose gradient, and the Ca-ATPase was purified using a reative-red affinity column (20).

**Ca-ATPase Activity Measurements**—Ca-ATPase activity was measured by an enzyme-linked assay performed in microtiter plates as described (21).

**Protein Quantitation**—Concentrations of SLN were determined by measurement of $A_{280}$, using a calculated extinction coefficient of 8480 $10^{-3}$ for a folded Protein according to Pace et al. (22) (SLN is not soluble in guanidine HCl, thus using the Edelhoch method unsuitable for the calculation of the extinction coefficient). Concentrations of PLB-(24–52) or SLN were determined with the BCA Protein Assay (Pierce) using BSA as standard protein; the values were increased by a factor of 1.37 because of underestimation by this assay compared with amino acid analysis. In case of protein incorporated into lipid vesicles, 1% SDS was included into the BCA Protein Assay Reagent for protein solubilization.
PLB$_{24-52}$ $^{24}$ARQLQNLFVINCLILICLICITIVML$^{52}$
SLN  $^{52}$MGIINRELPLFTTLVTVILMVLVRSYQQ

FIG. 1. Amino acid sequences and alignment of synthetic human PLB-(24–52) and SLN. The position of the first and last amino acid of PLB-(24–52) in full-length PLB is indicated. Identical amino acids are shown by solid vertical lines and conserved amino acids by dashed vertical lines. The putative transmembrane domains are shaded.

(24). Proteins were analyzed by Tricine/SDS-PAGE (25), using 4–5% SDS and 1% 2-mercaptoethanol final concentrations in the sample buffer. Proteins in SDS gels were silver-stained as described (26).

RESULTS

Synthesis of Sarcolipin and the Transmembrane Domain of Phospholamban—Human SLN and the homologous region of human PLB, PLB-(24–52) (Fig. 1; (1)), were chemically synthesized on solid phase using Fmoc/t-Bu chemistry. PLB-(24–52) was obtained in good yields and purity applying standard protocols with double coupling and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole activation (16).

For synthesis of SLN several modifications were applied to the standard protocols to improve the yield of the highly hydrophobic protein. The thiazolidine building blocks procedure proposed by Mutter and co-workers (27) did not produce the expected results, and the major product showed a mass correspondent to the des-Ile$^{17}$-Thr$^{18}$ protein. Similarly unsuccessful were attempts of fragment coupling on solid support. Best results were obtained adding chloroform to the solvent mixture in the coupling, washing, and deprotection steps (see “Experimental Procedures”). Difficulties were also encountered in the purification of the crude SLN product using HPLC. Insolubility and high affinity to the stationary reverse phase caused irreversible adsorption to a C18 column. Satisfactory results in terms of resolution and recovery were obtained by using C8 columns and a gradient elution by 0.1% trifluoroacetic acid as eluent A and a mixture of acetonitrile/2-propanol (1:1) as eluent B. However, multiple preparative runs were required to isolate homogenous material with an experimental yield of 16% based on resin loading and quantitative amino acid analysis.

Secondary Structure in the Presence of Detergents and Liposomes—The CD spectra of both synthetic PLB-(24–52) and SLN in 1% (v/v) octyl-POE were characteristic of an α-helical structure with dichroic minima at 209 and 222 nm and a maximum centered at 193 nm (Fig. 2). Nearly identical spectra were obtained for the two proteins in 1.5% (w/v) OG and 4% (w/v) SDS (data not shown). The spectrum of PLB-(24–52) is reminiscent of those recorded for full-length PLB and the related tryptic fragment PLB-(26–52) (6, 28, 29).

The two proteins were incorporated into POPC liposomes at a protein concentration of 30–50 μM and a POPC concentration of 6.6 mM, and the CD spectra showed mean molar ellipticities that were in the same range as those obtained in detergents (data not shown).

To determine the thermal stability of both proteins, scans in the temperature range from 4 to 96 °C were carried out by recording the ellipticity at 221 nm. Both PLB-(24–52) and SLN did not melt in any of the detergents tested (4% SDS, 1.5% OG; octyl-POE could not be used because it is not heat-stable), and only a linear signal decrease of about 25% during heating could be observed (data not shown). This loss of helicity was completely reversible. High heat stability of the α-helices was also obtained with both proteins incorporated into POPC liposomes (data not shown). These results fully agree with previous observations about the high thermal stability of full-length PLB and the related tryptic fragment PLB-(26–52) (6, 29).

Oligomerization in SDS and Nonionic Detergents—In Tricine/SDS-polyacrylamide gels according to Schagger and von Jagow (25) (Fig. 3) and in SDS-polyacrylamide gels according to Laemmli (30) (data not shown), PLB-(24–52) showed the typical monomer-pentamer pattern, which also has been observed for native PLB (31). Complete dissociation of pentameric PLB-(24–52) on SDS gels required boiling in the presence of high concentrations of SDS (4–5%) at low protein concentrations, as is the case for native PLB (32). In contrast, and in agreement with published data (14) synthetic SLN was completely in the monomeric state, whether or not the protein was boiled prior to electrophoresis (Fig. 3). Even at very low SDS concentration in the sample buffer (0.1%), no oligomeric structures could be detected (data not shown). The capacity of SLN to be silver-stained was clearly reduced compared with the PLB fragment (Fig. 3). The presence of SLN did not change the monomer-pentamer pattern of PLB-(24–52), even at a 10-fold molar excess (data not shown). This suggests that SLN is not interacting with PLB-(24–52) in SDS solutions.

For investigation of the oligomeric state of both proteins in nonionic detergents ultracentrifugal techniques were used, which are suitable for the determination of the molecular mass of proteins in these detergents (17, 18). The ultracentrifugation studies were preferentially performed in 1% octyl-POE because its density is close to 1.0 g/cm$^3$. However, another nonionic detergent, OG, was also used as described (17). Sedimentation equilibrium experiments in both 1% octyl-POE and 1.5% OG showed that at the lowest protein concentrations used (20–100 μM) SLN was monomeric to dimeric (calculated molecular mass of the monomer 3.8 kDa) (Fig. 4). By increasing the SLN concentration up to 1.06 mM (4 mg/ml), the obtained molecular mass estimates increased up to 22.4 kDa, which is a value in the range of hexamers, and the ability of SLN to self-associate is seen (Fig. 4). No plateau was reached within the concentration range used. By increasing the rotor speed we showed also for the high protein concentrations that a mixture of several oligomeric species was present (data not shown). Different pH (6.8–7.5) and ionic strength (up to 100 mM NaCl) had no significant effect on the measured molecular masses (data not shown).
Characterization of Sarcolipin Structure

In contrast to SLN, analysis of the oligomeric state of PLB-(24–52) by analytical ultracentrifugation clearly showed pentamer formation already at the lowest protein concentration used. In sedimentation velocity runs in 10 mM Tris-HCl, pH 7.5, 1% octyl-POE at 30 μM a main component with a sedimentation coefficient of 2.0 was observed. According to sedimentation equilibrium runs at the same protein concentration and at 28,000 rpm, the molecular mass of this species was estimated to be 17.5 kDa, consistent with a pentamer state (calculated molecular mass of the monomer 3.4 kDa). For this component (assumed partial specific volume of 0.73 cm$^3$/g) an $f/f_0$ value of 1.22 was calculated. This indicates that the pentameric species has an elongated shape, as expected. The experiment was repeated at different protein concentrations in Tris-buffer, 1% octyl-POE (Fig. 4). Sedimentation equilibrium yielded for the lowest protein concentration (35 μM) a main species with an estimated molecular mass of 16.9 kDa, again consistent with a pentameric state. However, higher protein concentrations resulted in significantly higher molecular mass estimates (Fig. 4), which probably reflects association of pentamers. Putative pentamer self-association reached a maximum at around 600 μM. The estimated molecular mass of the main species (99 kDa) at this protein concentration indicates the association of six pentamers (calculated molecular mass of 102 kDa). Increasing the rotor speed at the higher protein concentrations led to the appearance of species with molecular mass estimates close to smaller multiples of pentamers and the one of pentamers themselves (data not shown), probably due to a concentration distribution in the rotor cells during centrifugation.

Increasing the rotor speed showed the presence of species with lower molecular mass than pentamers at the lowest protein concentration of 35 μM. For example, at 44,000 rpm a small fraction of the material showed a molecular mass of about 9.6 kDa, which points to the presence of a mixture of monomers and small oligomers, in addition to the pentamers.

Increasing the ionic strength of the PLB-(24–52) solution at low protein concentrations by adding up to 100 mM NaCl led to a partial shift of the molecular masses of the observed species to higher values. This indicates the formation of higher mass aggregates dependent on the ionic strength, which was not observed in case of SLN.

SLN contains a Trp residue, in contrast to PLB-(24–52). We therefore investigated whether the concentration-dependent oligomerization of SLN can be analyzed by a blue shift of the Trp fluorescence emission maximum upon complex formation (33). The fluorescence spectra of different concentrations of SLN, ranging from 1.5 μM to 0.5 mM, in 1% octyl-POE were recorded (data not shown), but no shift of the fluorescence emission maximum at 332 nm was observed. According to our analytical ultracentrifugation experiments, monomers convert to tetramers and larger oligomers in this concentration range. The absence of a blue shift of the fluorescence emission maximum upon oligomerization can be due to two reasons as follows: (i) the Trp residue of SLN might not be located at the subunit interface, or (ii) the Trp residue is located in the transmembrane domain and thus already in a hydrophobic environment in the monomer.

Demonstration of Oligomerization by Chemical Cross-linking—To confirm the results that PLB-(24–52) forms pentamers in the presence of octyl-POE and to determine its oligomeric state in liposomes, which cannot be investigated in the analytical ultracentrifuge, we performed cross-linking studies. Because PLB-(24–52) contains two suitable amino groups (one lysine and the N terminus of the protein) in its hydrophilic N-terminal cytoplasmic domain, we selected the water-soluble cross-linkers BS$^3$ and Sulfo-EGS. Both are homobifunctional amine-reactive cross-linkers, which differ in their spacer arm length. PLB-(24–52) (30 μM) in 1% octyl-POE was cross-linked; the octyl-POE micelles were then dissolved with 5% SDS, and the reaction products were analyzed by Tricine/SDS-PAGE (Fig. 5A). In the control incubation without cross-linker, the pentamers were completely converted to monomers after boiling. When a 5-fold molar excess of Sulfo-EGS was added, approximately half of the pentamers observed by SDS-PAGE became stable to boiling, indicating cross-linking. Sulfo-EGS is a hydroxylamine-cleavable cross-linker; therefore, we incubated the cross-linked protein with 1 M hydroxylamine in a control experiment. Despite the finding that the high hydroxylamine concentration that remained in the sample buffer (0.2–0.5 M) and/or the remaining parts of the cross-linker obviously destabilized the non-cross-linked pentamers, it can clearly be seen that the pentamers were no more stable to boiling (Fig. 5A), i.e. the cross-links were cleaved and thus cross-linking is reversible.
PLB-(24–52), which contains three cysteines in its transmembrane domain, was cross-linked by oxidation via interchain disulfide bridges (16). This cross-linking was performed in 4% SDS, in which non-cross-linked PLB-(24–52) occurs in the typical monomer-pentamer pattern (see Fig. 3). However, like the cross-linking by BS<sup>3</sup>, cross-linking by oxidation led to the occurrence of a ladder of cross-linked oligomers in SDS gels (16). This suggests that cross-linking by formation of interchain disulfide bridges destabilizes the pentamers in a similar way as cross-linking by BS<sup>3</sup>. Higher amounts of both cross-linkers or lengthening of the incubation time led to similar results as the one shown in Fig. 5A.

PLB-(24–52) (40 μM) was incorporated into POPC liposomes (6.6 mM POPC) and treated with a 50-fold molar excess of Sulfo-EGS and BS<sup>3</sup>. The POPC liposomes were dissolved with 4% SDS, and the reaction products were analyzed by Tricine/SDS-PAGE (Fig. 5B). A similar cross-linking pattern compared with the one in the presence of octyl-POE was observed, although less of the protein formed pentamers in the liposomes compared with detergent micelles. Again, cross-linking was reversible as shown by hydroxylamine treatment of the Sulfo-EGS cross-linked protein (Fig. 5B). Lower amounts of both cross-linkers resulted in a reduced cross-linking yield (data not shown). With this experiment we identified pentamers of PLB-(24–52) to be a specific oligomeric species also in a lipid environment.

Next we investigated the oligomeric state of SLN in liposomes. Amine-reactive cross-linkers could not be used for cross-linking experiments with SLN, because SLN contains only one suitable amino group. However, it contains two carboxyl groups and two arginines, which are suitable for cross-linking. Both of these functional groups are located within the hydrophilic N-terminal as well as the C-terminal part of SLN and are separated from each other by the transmembrane domain. We therefore selected the membrane-permeable heterobifunctional cross-linkers APG and ASBA. APG is arginine-specific, and ASBA is carboxyl-reactive on one end, and both are nonspecific (photoreactive) on the other end. SLN (60 μM) was incorporated into POPC liposomes (6.6 mM POPC) and treated with different amounts of APG and ASBA, ranging from an equimolar ratio to a 50-fold molar excess of the cross-linkers. Convincing results were only obtained with APG (Fig. 5C) since ASBA was less efficient, and only at high concentrations (20–50-fold molar excess) could a cross-linking activity be observed (data not shown). In the control incubation without cross-linker SLN was monomeric in the SDS gels. Increasing amounts of APG led to the appearance of protein ladders in the SDS gels consisting of monomers and oligomers of increasing molecular mass. The oligomers were stable to boiling, indicating that they were cross-linked. The intensity of the protein bands in the SDS gels decreased with increasing molecular mass of the oligomers. This kind of protein pattern in SDS gels probably arises from the low cross-linking efficiency of APG after photoactivation, which is due to the characteristics of the highly reactive aryl nitrene. At a molar ratio of APG to SLN of 10:1 maximum cross-linking was achieved. Distinct protein bands ranging from monomers to tetramers and a faint pentameric band are visible (Fig. 5C). Increasing the development time of the silver-staining procedure showed the pentameric band better and a faint hexameric band became visible (data not shown). However, under these conditions some of the protein bands became over-stained, because the low capacity of SLN to be silver-stained (see Fig. 3) obviously was increased by the attachment of the cross-linker (Fig. 5C). The higher amounts of APG (20–50-fold molar excess) did not result in the detection of higher molecular mass oligomers than at the 10-fold molar excess (Fig.

Interestingly, using BS<sup>3</sup> for cross-linking under the same conditions as Sulfo-EGS, beside pentamers of PLB-(24–52) as the main cross-linked species, a ladder of higher molecular mass oligomers appeared at the expense of monomers (Fig. 5A). BS<sup>3</sup> possesses a significant shorter spacer arm (1.14 nm) than Sulfo-EGS (1.61 nm) and may thus meet the spatial requirement in pentamers less well. In a former cross-linking experiment we have made a similar observation. In that case
CHARACTERIZATION OF SARCO-LIPIN STRUCTURE

FIG. 6. Regulation of Ca-ATPase activity by synthetic SLN (top) and PLB-(24–52) (bottom) after reconstitution in lipid bilayers. Circles, control (Ca-ATPase only). Squares, Ca-ATPase plus SLN. Triangles, Ca-ATPase plus PLB-(24–52). Left, specific activity normalized to \( V_{\text{max}} \) of the control, illustrating the change in \( V_{\text{max}} \). Right, each curve normalized to its own \( V_{\text{max}} \) illustrating the shift in \( pK_{\text{Ca}} \). The curves are fits to experimental data (symbols), means ± S.E. (n ≥ 6).

5C). However, even higher molecular mass oligomers than hexamers may have been present in the liposomes, which might not have been efficiently cross-linked due to the low cross-linking efficiency of APG. These results show that SLN forms oligomers also in a lipid environment.

PLB-(24–52) appears not to interact with SLN to form mixed pentamers in liposomes. PLB-(24–52) was reconstituted with an up to 5-fold molar excess of SLN in POPC liposomes and treated with different amounts of Sulfo-EGS and BS3. The amount of cross-linked PLB-(24–52) pentamers was not reduced by the presence of SLN (data not shown). In case of mixed pentamers the amount of cross-linked pentamers should have been reduced because SLN contains only one suitable amino group for the amine-reactive cross-linkers.

Investigation of an influence of PLB-(24–52) on the cross-linking of SLN with APG was not possible, because after silver-staining of the reaction products in SDS gels the PLB-(24–52) protein bands were much stronger than the SLN protein bands.

Functional Properties of Synthetic SLN and PLB-(24–52)—Fig. 6 shows the effect of synthetic SLN (top) and PLB-(24–52) (bottom) on Ca-ATPase activity as a function of \([\text{Ca}^{2+}]\), measured in reconstituted membranes. Both proteins decrease the Ca-ATPase activity below \( p\text{Ca} \). The shift in \( pK_{\text{Ca}} \) (Fig. 6, right) was \(-0.15 \pm 0.025 \) (from 6.36 to 6.21) due to SLN (top right) and \(-0.19 \pm 0.02 \) due to PLB-(24–52) (bottom right). In addition, at saturating \([\text{Ca}^{2+}]\) both proteins stimulate \( V_{\text{max}} \) (left) by 17.5 ± 2.7% due to SLN (top left) and by 11.2 ± 2.4% due to PLB-(24–52) (bottom left).

DISCUSSION

We compared the properties of human SLN with that of the homologous region of human PLB, PLB-(24–52), in which the 23 N-terminally located amino acids of the cytoplasmic domain were omitted. With this approach we wanted to examine whether the homology in the amino acid sequence also leads to similar structural and functional properties of both proteins. SLN and PLB-(24–52) are similar in length (31 and 29 amino acids, respectively) and in theoretical pI (8.3 and 8.7, respectively). Both consist of a hydrophilic N-terminal domain of 7 amino acids, which faces the cytosol in the native proteins, followed by the hydrophobic transmembrane domain. In case of PLB-(24–52) the putative transmembrane domain consists of 22 amino acids, and in case of SLN it is 3 amino acids shorter, but SLN contains an additional hydrophilic C-terminal domain of 5 amino acids, which protrudes into the lumen of the sarcoplasmic reticulum in the native protein (14).

We have chemically synthesized SLN and PLB-(24–52). Appropriate synthesis and purification strategies had to be developed for SLN to achieve high purity and satisfactory yields. In contrast to PLB, the chemical synthesis of SLN has not yet been reported, and the synthesis protocol may also be of future use since large quantities of SLN are difficult to purify from muscle tissue or from recombinant cells.

From the CD results it can be concluded that SLN in a hydrophobic environment is a highly stable, predominantly helical protein, similar to PLB-(24–52). Quantitation of the secondary structure content of membrane proteins based on CD spectral intensities is ambiguous (34), but the high ellipticity values of PLB-(24–52) indicate a helical content between 80 and 100%, if calculated for soluble proteins (35). This is expected from the structure prediction (3), according to which ~75% of PLB-(24–52) consists of an \( \alpha \)-helix proposed to span the lipid bilayer (Fig. 1). The molar ellipticity values of SLN were clearly lower than that of PLB-(24–52), which is expected from the sequence (1) as only ~60% of the SLN protein should represent the predicted transmembrane helix (Fig. 1). The secondary structure of SLN, like the one of PLB-(24–52), is stable in the anionic detergent SDS, which denatures most protein structures. Furthermore, both structures are highly resistant to thermal denaturation. These results not only demonstrate the high stability of SLN, but also indicate that the helical content of SLN is a basic feature of the monomer rather than a consequence of oligomeric interactions. Resistance to SDS denaturation and high thermal stability are known to occur in the transmembrane domains of other membrane-spanning proteins.

By analytical ultracentrifugation and chemical cross-linking, we have shown that PLB-(24–52) forms pentamers in 1% octyl-POE. Both methods revealed that pentamers are the main
oligomeric species at low protein concentrations (30–35 μM). Also significant amounts of monomers and/or smaller oligomeric forms of PLB-(24–52) were present. These findings are consistent with the properties of PLB-(24–52) in SDS-PAGE runs. Experiments with the analytical ultracentrifuge showed that at higher protein concentrations PLB-(24–52) self-assembles to larger species than pentamers in 1% octyl-POE. Most likely pentamers associate to larger oligomers, and at a concentration of around 600 μM a maximum of six associated pentamers was observed. Self-associated pentamers were never observed by SDS-PAGE, indicating that these oligomeric structures are less stable than the pentamers themselves, as they are obviously dissolved in the stronger detergent SDS. Whether self-association of PLB pentamers occurs in a lipid environment and plays a physiological role in vivo remains to be established.

In two former studies analytical ultracentrifugation was used for the analysis of the oligomeric state of PLB. Mayer et al. (31) examined full-length wild type PLB and Cys-to-Ser PLB, in which all of the three cysteines were substituted by serines, by sedimentation velocity experiments in 0.1% C12E8. Wild type PLB migrated with a mean sedimentation coefficient of 4.8 S suggestive of an oligomeric species, whereas Cys-to-Ser PLB did not sediment, suggesting that the protein is monomeric, a feature that is expected from SDS-PAGE runs. Molecular masses (and thus the specific oligomeric state) cannot be determined by sedimentation velocity, and a comparison with our sedimentation coefficient of PLB-(24–52) is not possible because C12E8 has another density (1.037 g/cm² (36)) as octyl-POE (1.015 g/cm³ (18)). Vorherr et al. (28) examined full-length wild type PLB in 1% octyl-POE in the presence of 100 mM NaCl by sedimentation equilibrium experiments. These studies resulted in aggregates of 100–180 kDa at pH 7.0, whereas at pH 4.2 three components were observed with estimated molecular masses corresponding to the monomeric, the pentameric to hexameric, and the decameric state (28). A direct comparison with our data is not possible because the protein concentrations were not indicated for the experiments by Vorherr et al. (28).

We clearly showed by chemical cross-linking that PLB-(24–52) forms pentamers also in POPC liposomes. However, monomers and dimers were predominant. These results are qualitatively consistent with electron paramagnetic resonance (EPR) experiments, which showed that PLB exists in an average oligomeric size of 3.5 in DOPC vesicles (37).

In contrast to PLB-(24–52), SLN showed no oligomeric structure in SDS gels, suggesting that putative oligomers of SLN are less stable than PLB oligomers. Sedimentation equilibrium experiments revealed that SLN weakly self-associates in 1% octyl-POE. At low protein concentration (20 μM), at which PLB-(24–52) is predominantly pentameric, the protein formed only monomers and dimers. Increasing the protein concentration increased the average molecular mass of the SLN oligomers. Molecular masses corresponding to pentamers were only reached at around 1 mM. Within the concentration range used there was no indication for the reaching of a plateau, and at even higher protein concentrations even higher average molecular mass values are expected. In another detergent, 1.5% OG, a similar tendency of SLN to self-association was observed. This suggests that this property is not dependent on a specific nondenaturing detergent. The weaker oligomerization and weak tendency to form pentamers may be caused by differences between its hypothetical pentameric structure and that of the PLB pentamer. In the left-handed coiled-coil model of PLB a leucine-isoleucine zipper was predicted (9), which would be replaced by an interhelical interface stabilized by differently placed leucines and isoleucines. However, whether SLN oligomers are formed via a coiled-coil structure is not yet clear.

For measurements in a lipid environment, it was necessary to use photoactivatable cross-linkers in the case of SLN. Cross-linking in POPC liposomes with APG yielded beside monomers cross-linked oligomers ranging from dimers to hexamers. Although we cannot establish a specific oligomeric state with this cross-linker due to its low cross-linking efficiency, the data demonstrate that SLN oligomerizes also in a lipid environment. This is the first report about the ability of SLN to form oligomers, and it suggests that the regulatory function of SLN might be influenced by its oligomeric state, as is proposed for PLB.

SLN and PLB-(24–52) appear not to form mixed pentamers, neither in SDS solutions where SLN is monomeric nor in POPC liposomes where SLN is oligomeric. This indicates that despite the high conservation in the amino acid sequences of the transmembrane domains, there is no or only weak association of both proteins.

Functional reconstitution of Ca-ATPase with PLB-(24–52) and SLN in phospholipid liposomes showed that both proteins regulate the Ca-ATPase in a similar way. Both proteins inhibit the enzyme at low [Ca²⁺⁺] (due to an apparent decrease in calcium affinity, i.e. an increase in pKCa) and activate it at high [Ca²⁺⁺] (increase in Vmax). The pKCa shift is smaller for SLN (0.15 ± 0.02) than for PLB-(24–52) (0.19 ± 0.02), and the Vmax increase is greater for SLN (17.5 ± 2.7%) than for PLB-(24–52) (11.2 ± 2.4%). This is the first Ca-ATPase activity study of SLN reconstituted in membranes. The dual regulatory effects of reconstituted synthetic SLN reported here are consistent with the previously reported effects of SLN coexpressed with Ca-ATPase (14). The finding of a significant Vmax increase for SLN is strengthened by this study. The observed functional effects of PLB-(24–52) are qualitatively similar to those reported previously for similar PLB transmembrane fragments and for fulllength PLB in co-expression (38) and co-reconstitution (39–41) experiments except that most of those studies do not report a significant effect of PLB or its transmembrane fragment on Vmax. Further studies will be required to resolve these issues quantitatively.

PLB-(24–52) and SLN were measured in the reconstituted membranes at a protein to lipid molar ratio of 1:320 to 1:350. In the cross-linking experiments in POPC liposomes the protein to lipid molar ratio was in a comparable range of 1:110 to 1:170 (2–3-fold higher). The cross-linking experiments showed that PLB-(24–52) is present partly as pentamers and partly as monomers/small oligomers. Also SLN was shown to be present partly as oligomers. Several lines of evidence indicate that for wild type PLB, monomers or small oligomers are the regulatory species on the Ca-ATPase, whereas oligomerization to pentamers prevents regulatory function. It was suggested for PLB that pentamers are a storage form from which the active monomers binding to Ca-ATPase are released (42).

Phosphorylation of PLB at Ser¹⁶ or Thr¹⁷ in the cytoplasmic domain favors pentamer formation (37) and reduces inhibition of the Ca-ATPase (43). SLN contains in its short cytoplasmic part only a single conserved potential phosphorylation site, Thr, which is not phosphorylated even by purified kinases (14). The regulatory action of SLN and of PLB-(24–52), which lacks most of the cytoplasmic domain, can therefore not be reduced by phosphorylation. Activity of SLN may only be modulated by changes in SLN levels in the membrane (14), which at high concentration may lead to large oligomers and, if analogous to PLB, to a loss of regulatory action.

In conclusion SLN and PLB-(24–52) exhibit similar secondary structures and Ca-ATPase regulatory function. SLN differs from PLB-(24–52) in its much lower oligomerization tendency,
and its putative oligomerized inactive storage state may only be achieved at high protein levels.

Further studies are needed to unravel the relationship between the oligomeric state of SLN and the regulation of the Ca-ATPase, as well as to determine whether the oligomeric state of SLN is regulated by cellular processes, as is proposed for PLB.

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