Phospholamban, Activator of the Cardiac Sarcoplasmic Reticulum Calcium Pump. Physicochemical Properties and Diagonal Purification†

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ABSTRACT: Phospholamban, the phosphorylatable activator of the cardiac sarcoplasmic reticulum calcium pump, was found to remain strongly associated with the ATPase molecule after solubilization of membrane proteins by either Triton X-100 or deoxycholate as shown by isoelectric focusing or sucrose density gradient ultracentrifugation. Phospholamban appears to be an acidic proteolipid (pI = 3.9) that can be extracted from sarcoplasmic reticulum vesicles by chloroform–methanol–HCl mixtures. As expected, its phosphorylatable sites [Le Peuch, C. J., Haiech, J., & Demaille, J. G. (1979) *Biochemistry 18*, 5150–5157] are exposed at the cytosolic side of the membrane, and the corresponding phosphopeptides are cleaved off by trypsin, chymotrypsin, thermolysin, and subtilisin but not by the staphylococcal protease or by pepsin. Phospholamban was purified to homogeneity by a dodecyl sulfate–polyacrylamide gel electrophoresis diagonal technique. In the first dimension, the phosphoprotein was isolated as the dimeric species (apparent molecular weight 22000) as previously reported [Kirchberger, M. A., & Tada, M. (1976) *J. Biol. Chem. 251*, 725–729]. The protein was then dissociated into the monomeric M_r 11000 species upon treatment with Triton X-100 and dodecyl sulfate and isolated as such. It was found to be phosphorylated only on serine residues and to contain ~45% hydrophobic and 22% acidic residues. Its solubility properties were found to be similar to those exhibited by the membrane-bound protein. Phospholamban could be reinserted into monolamellar phosphatidylcholine liposomes. The liposome-bound protein was dephosphorylated by potato acid phosphatase and by canine heart phosphoprotein phosphatase and then partially rephosphorylated by the catalytic subunit of cyclic adenosine 3′,5′-monophosphate dependent protein kinase. The liposome-bound purified phospholamban will provide the possibility of reconstituting a modulated calcium-transport system.

The Ca^{2+}-dependent ATPase (EC 3.6.1.3, ATP phosphohydrolase) of canine cardiac sarcoplasmic reticulum and its calcium uptake are activated upon phosphorylation of an integral membrane protein, phospholamban. Such a covalent modification is brought about either by cAMP[1] and cAMP-dependent protein kinases (Wray et al., 1973; Kirchberger et al., 1974; Kirchberger & Tada, 1976; Schwartz et al., 1976; Will et al., 1976; Wray & Gray, 1977; Tada et al., 1979) or by a calcium–calmodulin-dependent protein kinase. The latter enzyme is membrane bound and exhibits the same substrate specificity as that of skeletal muscle glycogen phosphorylase *b* kinase (Le Peuch et al., 1979). Their major difference lies in the absolute requirement of the sarcoplasmic reticulum kinase for exogenous calmodulin.

Therefore, calcium uptake is stimulated in response to either catecholamines or increase in cytosolic-free Ca^{2+}. The properties of phospholamban, either in the lipid bilayer or in a purified state, were studied as a first step toward the understanding of the mechanism of the calcium pump regulation. Phospholamban appears to be an acidic proteolipid, strongly associated with the pump. We also report here for the first time the purification of phospholamban to homogeneity through a diagonal technique.

The protein is first isolated under its classical dimeric form (M_w 22000), then dissociated into monomers (M_r 11000), and purified as such. It can be reinserted into a liposome bilayer, dephosphorylated, and rephosphorylated under this form.

Materials and Methods

Materials

Soybean L-α-phosphatidylcholine type IIS, PMSF, *o*-phthaldialdehyde, and cAMP were from Sigma; fluorescamine was from Hoffmann-La Roche. TPCK–trypsin (228 units/mg), pepsin (2700 units/mg), and chymotrypsin (50.9 units/mg) were from Worthington; subtilisin (5 units/mg) and potato acid phosphatase (60 units/mg) were from Boehringer; *Staphylococcus aureus* protease (467 units/mg) was from Miles; thermolysin (17 units/mg) was from Serva. [γ-^{32}P]-ATP (400 Ci/mol) was prepared according to Glynn & Chappell (1964). [^{32}P]Orthophosphoric acid was obtained from NEN. Polyacrylamide gel electrophoresis reagents were from Bio-Rad.

The catalytic subunit of bovine heart cAMP-dependent protein kinase (type II) [specific activity 1.16 μmol of ^{32}P incorporated/(min mg)] was prepared according to Peters et al. (1977). Calmodulin was purified from brain according to Teo et al. (1973) as modified by Walsh & Stevens (1978). Glycogen phosphorylase *b* kinase was prepared according to Cohen (1973). Phosphoprotein phosphatase S was partially purified from canine heart through the ethanol and ammonium sulfate precipitation steps as described by Li et al. (1978).

Methods

Miscellaneous Methods. Sarcoplasmic reticulum vesicles were prepared from diastole-arrested canine hearts as previously described (Le Peuch et al., 1979).

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SR, sarcoplasmic reticulum.

0006-2960/80/0419-3368$01.00/0

Protein concentrations were determined either by the Lowry procedure (Lowry et al., 1951) or by the Coomassie blue technique (Spector, 1978). Amino acid analyses were performed according to Moore & Stein (1963) after 24-, 48-, and 72-h hydrolyses in 5.7 N HCl under vacuum by using a Beckman Multichrom Model 4255 analyzer. Amino acids were detected in the eluate either by the ninhydrin reaction or by the fluorescent adducts obtained after reaction with *o*-phthaldialdehyde (Benson & Hare, 1975). [^{32}P]Phosphoserine and phosphothreonine were identified after partial acid hydrolysis of the phosphoprotein by 6 N HCl for 4 h at 110 °C (Bylund & Huang, 1976). ^{32}P-Labeled and cold phosphoamino acids were spotted together and separated by high-voltage electrophoresis (1000 V, 2.5 h) at pH 1.9 on cellulose thin-layer plates (20 × 20 cm). Electrophoretograms were stained with fluorescamine as described by Stephens (1978) and autoradiographed with a Kodak X-Omat R film.

Edman degradation was carried out manually on 20 nmol of phospholamban as described by van Eerd & Takahashi (1976). Phenylthiohydantoin (PTH) amino acids were identified by high-performance liquid chromatography on μBondapak C 18 (Waters Associates) by using a 20–48% methanol gradient in 10 mM sodium acetate, pH 4.17. Isocratic elution of water-soluble PTH amino acids was carried out with 27% methanol in the above buffer.

Dodecyl sulfate–polyacrylamide (0.1%:15%) gel electrophoreses were carried out according to Laemmli (1970) with analytical 1.5-mm thick gels and preparative 3-mm thick gels.

Polyacrylamide gel isoelectric focusing was performed according to O'Farrell (1975) by using in the first dimension 2% Ampholytes 3–6 (LKB) supplemented with 3 mg each of lysine hydrochloride, arginine hydrochloride, and glutamic acid. The pH gradient was between 3.8 and 5.6.

Isoelectric focusing was also carried out, with the LKB 8100-1 column (110 mL), in a liquid phase stabilized by a sucrose gradient and containing 1% Ampholines, pH 3–10, and 0.5% Triton X-100, essentially as previously described (Pechère et al., 1971). SR vesicles (12 mg) were dissolved in 1% Triton X-100 and 5 mM potassium phosphate buffer, pH 7.0, and the resulting suspension was clarified by centrifugation at 100000*g* for 90 min. The solution was made 4% Ampholine, and its density was adjusted with sucrose to allow the introduction in the middle of the preestablished pH gradient.

Sarcoplasmic reticulum phosphorylations were carried out at 20 °C in 0.05 mM DTT, 10 mM NaF, and 50 mM sodium phosphate buffer, pH 7.0, containing 10–20 mg of SR protein/mL. Additions were 25 μg/mL catalytic subunit of cAMP-dependent protein kinase and/or 0.5 μM calmodulin and 50 μM $CaCl_2$. Adequate labeling was obtained within 30 min after addition of 10 mM magnesium acetate and 0.3 mM [γ-^{32}P]ATP (400–600 cpm/pmol). The reaction mixture was made 5 mM EDTA and 50 mM NaF and centrifuged at 100000*g* for 90 min. The pellet was resuspended in 1 mM DTT and 50 mM Hepes buffer, pH 7.4, and the resulting suspension (6 mg of protein/mL) was either used immediately or stored frozen under nitrogen.

When perphosphorylation was required for subsequent evaluation of the extent of dephosphorylation and rephosphorylation, phosphorylation conditions were as previously described (Le Peuch et al., 1979).

Proteolytic removal of [^{32}*P*]*phosphopeptides* was followed on ^{32}P-phosphorylated SR vesicles. The protease (E/S = 0.01) was added to the SR vesicles suspension (6 mg/mL) in 1 mM DTT and 50 mM Hepes buffer, pH 7.4, except for pepsin that was added to a suspension of SR vesicles (6 mg/mL) in formic acid–acetic acid–water (1:4:45 v/v/v), pH 2.0.

Aliquots (10 μL) were withdrawn and filtered through GSWP Millipore filters (0.22 μm) saturated with bovine serum albumin. Filters were washed twice with 1 mL of 2 mM ATP and 50 mM potassium phosphate, pH 7.5, and counted in 3 mL of dioxane–naphthalene scintillant.

Solubilization of [^{32}*P*]*phospholamban* was carried out on SR vesicles (100 000 cpm = 200 pmol of phosphate) after lyophilization. The powder was stirred for 30 min on a Vortex mixer at room temperature in the presence of 1 mL of the following solvent mixtures: 98% formic acid, acetone–3 mM HCl, chloroform–methanol (2:1 v/v), chloroform–methanol (2:1 v/v)–10 mM HCl, chloroform–methanol–water (65:25:4 v/v/v)–20 mM HCl, 95% ethanol, and diethyl ether.

The suspension was centrifuged in a clinical centrifuge for 10 min, and 10-μL aliquots of the supernatants were counted in 10 mL of dioxane–naphthalene scintillant.

Purification of phospholamban was carried out by two successive preparative electrophoreses in 0.1% dodecyl sulfate–polyacrylamide (3-mm thick) slab gels (Laemmli, 1970). Phosphorylated SR vesicles (80 mg of protein) were dissolved in 16 mL of sample buffer, incubated at 80 °C for 5 min, and subjected to a first 15% gel electrophoresis. The radioactive band (M_r 22000, R_f ~0.36) was detected by autoradiography, after a 30-min exposure of a Kodak X-Omat R film, and cut out. The gel was extruded through the outlet of a 10-mL syringe into the minimal volume of sample buffer containing 1% Triton X-100. The resulting slurry was loaded onto a 5–20% gradient polyacrylamide gel containing 0.1% dodecyl sulfate. After electrophoresis, the 2-mm wide radioactive band (M_r 11 000, R_f 0.67) was detected by autoradiography as described above and cut out. Phospholamban was eluted from the gel by using an electrophoresis-concentration apparatus as described by Allington et al. (1978), with a 40 mM Tris–borate buffer, pH 8.3. The concentrated protein solution was dialyzed overnight against 1 mM ammonium bicarbonate and lyophilized. The powder was extracted twice with 5 mL of acetone containing 2.5 mM HCl (Lazarides, 1976). The insoluble residue was spun down with a clinical centrifuge at 3000 rpm for 5 min, the supernatant was discarded, and the pellet was lyophilized.

Purification of the phospholamban–ATPase complex was carried out according to van Winkle et al. (1978). ^{32}P-Phosphorylated SR vesicles (50 mg of protein) were spun down by centrifugation at 100000*g* for 90 min, solubilized, and fractionated on a sucrose gradient (van Winkle et al., 1978). Fractions (0.5 mL) were collected after puncture of the bottom of the tube, dialyzed against 1 mM ammonium bicarbonate, lyophilized, and analyzed by 0.1% dodecyl sulfate–5–20% polyacrylamide gradient gel electrophoresis and autoradiography.

Insertion of Purified Phospholamban into Liposome Lipid Bilayer. Soybean lecithin (30 mg) was dissolved in 1 mL of heptane in a thick-walled Pyrex tube. While the tube was rotating, heptane was evaporated under a nitrogen stream, and a phospholipid film was formed on the tube wall. After addition of 2 mL of 1 mM DDT and 50 mM Hepes buffer, pH 7.5, or 1 mM DTT and 50 mM ammonium acetate buffer, pH 5.5, the contents of the tube were sonicated at 0 °C for three periods of 5 min by using an MSE 100 W ultrasonic disintegrator. The opalescent solution was filtered through a Sepharose 4B column (1.2 × 50 cm) equilibrated with one of the buffers used above. Monolamellar liposomes were eluted after the multilamellar liposomes that emerged with the void volume.

Insertion of phospholamban into the bilayer of monolamellar liposomes was carried out under nitrogen by addition of liposomes (0.5 mL) to a conical vial on the walls of which [^{32}P]phospholamban (100 000 cpm) had been dried as a thin layer. The extent of phospholamban insertion was determined after filtration on GSWP Millipore filters (0.22 μm). The filters were washed and counted as described above.

Dephosphorylation and Rephosphorylation of Liposome-Bound Phospholamban. Liposome-bound [^{32}P]phospholamban (100 000 cpm, 500 pmol of ^{32}P) was incubated at 20 °C either in a 1 mM DTT and 0.1 M ammonium acetate buffer, pH 5.5, in the presence of 10 μg of potato acid phosphatase or in a 1 mM DTT and 50 mM Hepes buffer, pH 7.4, in the presence of an amount of phosphoprotein phosphatase S that catalyzes the hydrolysis of 0.5 nmol of inorganic phosphate (P_i) from a phosphohistone mixture per min. Aliquots (10 μL) were withdrawn and filtered through GSWP Millipore filters (0.22 μm). Filters were washed twice with 0.5 mL of 50 mM phosphate buffer, pH 7.5, and counted as described above. Dephosphorylated phospholamban-containing liposomes were freed from phosphatase by gel filtration through a Sepharose 4B column (1.2 × 50 cm) equilibrated with a 1 mM DTT–50 mM potassium phosphate buffer, pH 7.0. Liposome peak fractions were pooled and incubated in the presence of 10 mM magnesium acetate, 0.3 mM [γ-^{32}P]ATP (400–600 cpm/pmol), and 35 μg of catalytic subunit of cAMP-dependent protein kinase per mL. Liposomes were also incubated in the presence of 94 μg of glycogen phosphorylase *b* kinase per mL, in 50 mM Tris-HCl buffer, pH 8.2, containing MgATP as described above. In both cases, aliquots (10 μL) were withdrawn, and phosphate incorporation was measured after filtration. Blank experiments were carried out in parallel in the absence of enzyme.

Results

Phospholamban Is Strongly Associated with the Calcium Pump. After isoelectric focusing of phosphorylated cardiac SR vesicles solubilized by Triton X-100, most of the protein (Ca^{2+},Mg^{2+}-ATPase), initially soluble, precipitated at its isoelectric point of 6.2. This material exhibited most of the radioactivity whereas free phospholamban of M_r 11 000 (see below) was found at pH ~4.[2] This experiment was indicative of an association between phospholamban and the Ca^{2+} pump, even in the presence of nonionic detergents. Such a strong association was confirmed by copurification of the calcium pump and phospholamban after solubilization of SR vesicles by deoxycholate and fractionation by sucrose density gradient centrifugation (van Winkle et al., 1978). As shown in Figure 1, the dodecyl sulfate gel electrophoretogram of individual fractions exhibits the main Coomassie blue stained band of the Ca^{2+},Mg^{2+}-ATPase (M_r 105 000) whereas a proportional amount of ^{32}P-labeled protein of M_r 11 000 in each of the fractions was only found by autoradiography. Therefore, the published purification procedure of the cardiac Ca^{2+},-Mg^{2+}-ATPase yields in fact a pump–phospholamban complex, the latter protein being undetected after Coomassie blue staining.

Phospholamban Is Partly Exposed at the Cytosolic Side of SR Vesicles. Proteolytic removal of [^{32}P]phosphopeptides from phospholamban was easily achieved through incubation of SR vesicles with chymotrypsin, thermolysin, trypsin, and subtilisin (Figure 2). Subtilisin and chymotrypsin proved to be the most efficient with a $t_{1/2}$ of 24 min. However, 90% of the membrane-bound ^{32}P was finally removed by subtilisin while only 72% was removed by chymotrypsin. Trypsin attacked phospholamban somewhat more slowly ($t_{1/2}$ = 36 min) and removed 85% of the bound radioactivity. Thermolysin was less active ($t_{1/2} \simeq$ 60 min), and pepsin exhibited a low activity (30% removal of phosphopeptides at 24 h). [^{32}P]-Phosphopeptides are likely to be short since they were not precipitated by 15% trichloroacetic acid in the presence of bovine serum albumin as carrier. The staphylococcal enzyme was essentially ineffective in removing phosphopeptide, excluding the possible presence of more than one glutamoyl residue in the stretch of the polypeptide chain that is exposed to kinases and proteases at the cytosolic side of the membrane.

Solubilization of [^{32}P]Phospholamban from SR Vesicles by Organic Solvents. Acetone containing 2.5 mM HCl failed to extract more than 1% of labeled phospholamban from SR vesicles and could thus be used to extract detergents from purified phospholamban. Similarly, ethanol or diethyl ether or chloroform–methanol (2:1) were ineffective in extracting phospholamban. The best results were obtained under conditions in which carboxylic side chains are protonated and the

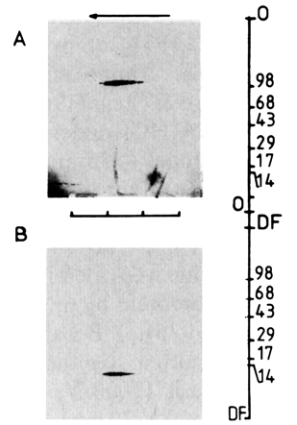

FIGURE 1: Copurification of the calcium pump and phospholamban. The Ca^{2+},Mg^{2+}-ATPase of phosphorylated cardiac SR vesicles (50 mg of protein) was purified as described under Methods. The 20 fractions were analyzed by 0.1% dodecyl sulfate–0.1% Triton X-100–5–20% polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue (A) and autoradiographed (B). The arrow is directed from the top (20% sucrose) to the bottom (50% sucrose) of the gradient. Most of the M_r 105 000 ATPase and the M_r 11 000 [^{32}P]phospholamban are present from 30 to 35% sucrose (fractions 8–13). O = top of the separating gel and DF = dye front. The figures refer to molecular weight markers ($\times 10^{-3}$): phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme. Every fourth fraction is indicated on the horizontal line, from 4 to 16.

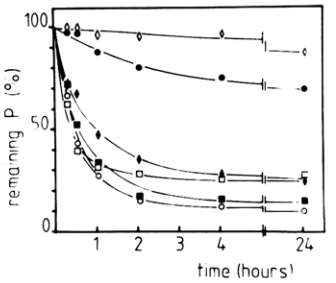

FIGURE 2: Proteolysis of [^{32}P]phospholamban in cardiac SR vesicles. ^{32}P-Phosphorylated SR vesicles (6 mg/mL) were incubated in the presence of 1% protease as described under Methods, and the remaining membrane-bound radioactivity was measured after Millipore filtration. (◊) Staphylococcal protease; (●) pepsin; (□) chymotrypsin; (♦) thermolysin; (■) trypsin; (○) subtilisin.

[2] The material is not shown but submitted to reviewers for examination. The material will be sent upon request to interested readers.

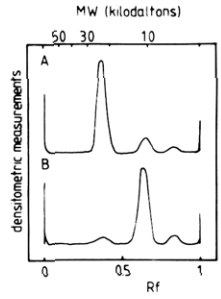

FIGURE 3: Dodecyl sulfate–polyacrylamide (0.1%–15%) gel electrophoresis of ^{32}P-labeled cardiac SR proteins (A) in the absence of Triton X-100 and (B) after addition of 1% Triton X-100 in the sample buffer. The autoradiograms were scanned with a Joyce–Loebl MK III CS microdensitometer.

bilayer disrupted, i.e., by formic acid (90–95% solubilization) and by either chloroform–methanol (2:1) containing 10 mM HCl or chloroform–methanol–water (65:25:4 v/v/v) containing 20 mM HCl. The latter conditions allowed ~75 and 80% extraction of phospholamban, respectively. Phospholamban is therefore expected to be an acidic molecule. Indeed, the isoelectric point of [^{32}P]phospholamban was found to be 3.9 upon isoelectric focusing of labeled SR vesicles. When protonated, phospholamban thus behaves as a proteolipid, in agreement with similar results reported by Bidlack & Shamoo (1979).

The migration of phospholamban upon 15% polyacrylamide gel electrophoresis was studied under different conditions on ^{32}P-phosphorylated SR vesicles (Figure 3). In the presence of 0.1% dodecyl sulfate, the usual pattern of phosphorylation is obtained (Figure 3A); the major [^{32}P]protein migrates with an R_f of 0.36, corresponding to a molecular weight of 22000 (Kirchberger & Tada, 1976). Two additional minor bands were observed with M_r 11000 and ~7000, as already described (Kirchberger & Tada, 1976; Jones et al., 1979; Bidlack & Shamoo, 1979). Inclusion of 1% Triton X-100 in the sample buffer prior to electrophoresis resulted in a dramatic change of the pattern. The M_r 22000 band almost disappeared (Figure 3B) with a concomitant proportional increase in the intensity of the M_r 11000 band. The fastest band (M_r 7000) was left unchanged. This experiment indicates phospholamban to be a polypeptide chain of ~96 residues, assuming that dodecyl sulfate–polyacrylamide gel electrophoresis is a reliable technique for the determination of membrane protein molecular weight. As observed in the absence of nonionic detergents, phospholamban behaves as a dimer, only partly dissociated by dodecyl sulfate.

Diagonal Purification Procedure of Phospholamban. The above observations of different migration rates of phospholamban during electrophoresis under denaturing conditions according to the absence or presence of Triton X-100 prompted the establishment of a diagonal purification scheme (Figure 4). Losses of ^{32}P-labeled protein were encountered during the first electrophoretic step both through partial dissociation (see Figure 3A) to the M_r 11000 monomer and through incomplete dissociation from the ATPase, as shown by the radioactive band at the top of the gel. A further loss of 50% of the material cut out as the 22000 band is encountered during the electrophoretic elution from the second dimension gel. Finally, the overall yield amounts to ~400 μg (i.e., 37 nmol of peptide, M_r 11000) from 80 mg of SR protein. If as suggested earlier (Le Peuch et al., 1979) phospholamban were associated with the pump in a 1:1 stoichiometry, the overall yield would be ~6%. No precise estimate of the yield could, however, be performed due to the limitations in the

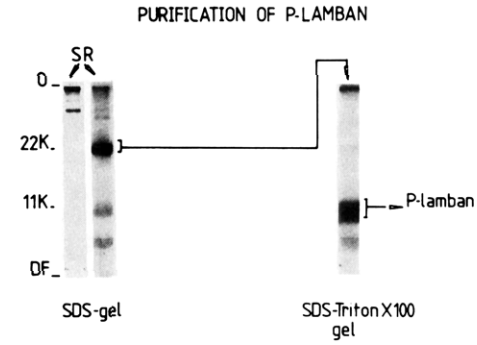

FIGURE 4: A scheme of the diagonal purification procedure for phospholamban (P-lamban). (Left) Coomassie blue pattern and autoradiogram after 0.1% dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis. The phosphoprotein bands are only visible on the autoradiogram. (Right) Autoradiogram after electrophoresis under similar conditions of the M_r 22000 band cut out from the gel on the left side and treated with 1% Triton X-100 in the sample buffer.

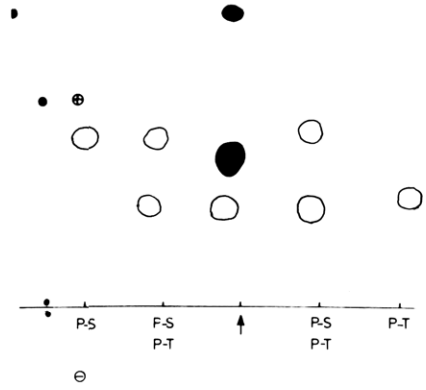

FIGURE 5: High-voltage electrophoretic separation of phospho amino acids obtained after partial acid hydrolysis of 1 nmol of [^{32}P]-phospholamban (see Methods). The arrow indicates the starting line on which a mixture of the hydrolysate and 10 nmol each of unlabeled phosphoserine (P-S) and phosphothreonine (P-T) were loaded. The same amount of unlabeled phosphoamino acids was loaded on each side as indicated. The fluorescamine-positive spots were circled, and the thin-layer plate was autoradiographed. The fast-migrating spot is [^{32}P]phosphate.

determination of the original content of phospholamban and of its molecular weight.

Chemical Analysis of Phospholamban. Purified [^{32}P]-phospholamban (100000 cpm, 244 pmol) was lyophilized and extracted by different solvents as described above for the SR vesicles. Similar results were obtained. Acetone–3 mM HCl did not dissolve the protein whereas 98% formic acid solubilized 100% of the phosphoprotein and chloroform–methanol (2:1 v/v) dissolved only 58%. Essentially all the radioactive protein was soluble either in chloroform–methanol (2:1) containing 10 mM HCl or in chloroform–methanol–water (65:25:4) containing 20 mM HCl.

Phospholamban contained only phosphoserine, as shown in Figure 5. No trace of phosphothreonine could be detected, at variance with what was previously reported in SR vesicles phosphorylated by cAMP-dependent protein kinases (Kirchberger et al., 1974).

Purified phospholamban was not stained by Coomassie blue (see Figure 4) and was not contaminated by Coomassie blue stained proteins. This is in line with the fact that no Coomassie blue stained band is visible in gradient gel electrophoretograms of SR vesicles that can be superimposed on the autoradiogram band.

A criterion of purity was given by Edman degradation of the purified protein. While the first step could not be inter-

Table I: Amino Acid Composition of Phospholamban[a]

residue		residue	
Asx	11.1	Met	1.7
Thr[b]	5.5	Ile[e]	4.9
Ser[b]	5.5	Leu[e]	9.5
Glx	11.4	Tyr	2.1
Pro	5.5	Phe	3.4
Gly[c]	10.6	His	2.0
Ala	11.0	Lys	4.9
Cys[d]	0.0	Arg	4.4
Val[e]	6.5	Trp	nd[f]

[a] Expressed as residues per 100 residues. [b] Extrapolated to zero time of hydrolysis. [c] Determined after Tris–borate gel electrophoresis purification. [d] After performic acid oxidation. [e] From the 72-h hydrolysis values. [f] Not determined.

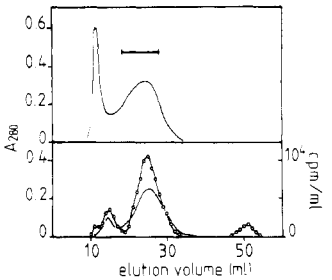

FIGURE 6: Reinsertion of [^{32}P]phospholamban into monolamellar liposomes. (Upper graph) Purification of monolamellar liposomes on Sepharose 4B (see Methods). The multilamellar liposomes eluted with the void volume were discarded, and the fractions under the bar were pooled and incubated overnight with 500 pmol [^{32}P]phospholamban. (Lower graph) Gel filtration of liposome-bound phospholamban on the same Sepharose 4B column as above. (—) A_{280}; (O) radioactivity.

preted satisfactorily due to the presence of contaminating free amino acids, the second step clearly gave a single residue of PTH–alanine with a yield of 37% compared to the starting material.

The amino acid composition of phospholamban is reported in Table I. The protein does not contain cysteinyl residues, and the 22 000-dalton dimer cannot be due to formation of a disulfide bond, as already expected from the use of reducing agents in the gel. Surprisingly enough, it does contain tyrosine, at variance with the noniodination of the protein previously reported (Louis & Katz, 1977) even in the presence of Triton X-100. In fact, the protein may well have migrated under its monomeric form of M_r 11 000 that would have escaped detection in the gel system used by Louis & Katz (1977). As expected from the acidic isoelectric point, there is a large excess of Asx and Glx residues over the basic residues. Hydrophobic residues represent ~45% of the residues.

The results of the amino acid analyses were expressed as residues per 100 residues since molecular weight determination by dodecyl sulfate–polyacrylamide gel electrophoresis cannot be considered as perfectly reliable for membrane proteins.

Reinsertion of Phospholamban into Liposomes. When incubated in the presence of monolamellar liposomes, ~80% of [^{32}P]phospholamban was incorporated into the lipid bilayer. Gel filtration of phospholamban-containing liposomes shows (Figure 6) that most of the radioactivity was incorporated into monolamellar liposomes (76%). The amount of multilamellar liposomes that contain phospholamban and are eluted with the void volume is not negligible and reaches 18%. Since no sonication was performed at this step, there is no reason to believe that the phosphoprotein was trapped into the liposomes. The third peak of radioactivity (elution volume 50 mL) probably represents free phospholamban.

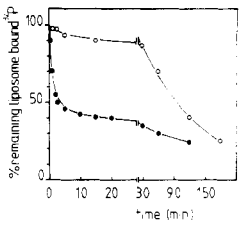

FIGURE 7: Enzymatic dephosphorylation of liposome-bound [^{32}P]-phospholamban (see Methods). (●) Potato acid phosphatase; (O) phosphoprotein phosphatase S.

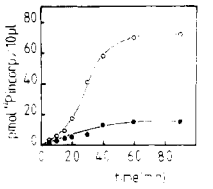

FIGURE 8: Rephosphorylation of dephosphorylated liposome-bound phospholamban (see Methods). Phospholamban-containing liposomes were freed from phosphoprotein phosphatase and incubated with Mg[γ-^{32}P]ATP in the presence (O) or absence (●) of the catalytic subunit of cAMP-dependent protein kinase. The latter curve may be indicative of ATP diffusion into the liposomes.

Enzymatic dephosphorylation was carried out as shown in Figure 7 to further check that phospholamban was inserted into the bilayer in a manner resembling the native protein, i.e., the phosphorylated site at the outer surface. Whereas the first half of bound phosphate was rapidly removed by potato acid phosphatase within 2.5 min, dephosphorylation proceeded sluggishly afterward to a residual 25% bound phosphate after 2 h. The behavior in the presence of phosphoprotein phosphatase S was strikingly different, with a lag phase of ~30 min followed by a faster dephosphorylation rate down to 25% residual bound phosphate after 3 h. At this stage, liposomes were freed from phosphatase S by gel filtration on Sepharose 4B (1.2 × 50 cm) equilibrated with 1 mM DTT–50 mM potassium phosphate, pH 7.0, and incubated in the presence of 0.3 mM [γ-^{32}P]ATP, 10 mM magnesium acetate, and 25 µg/mL catalytic subunit of cAMP-dependent protein kinase. As shown in Figure 8, this resulted in incorporation of phosphate into liposome-bound material, even though the rate of phosphorylation was slow. The extent of phosphorylation could not be determined accurately and was found to be 15% of the amount of [^{32}P]phosphate present before dephosphorylation, if losses that occur at the gel filtration step are not taken into account. Preliminary experiments indicate that liposome-bound phospholamban is also a substrate of glycogen phosphorylase *b* kinase.

Discussion

The criterion of solubility of phospholamban, in the intact SR vesicles as well as in a purified state, qualifies this protein as an acidic proteolipid that becomes fairly soluble in chloroform–methanol mixtures when its carboxylic side chains are protonated. These observations are in agreement with those of Bidlack & Shamoo (1979).

Phospholamban is capable of interacting quite strongly with the Ca^{2+}-stimulated Mg^{2+}-ATPase since most of the [^{32}P]-phosphoprotein is still found associated with the pump after either solubilization and isoelectric focusing of SR proteins in the presence of Triton X-100 or solubilization by deoxycholate and sucrose density gradient centrifugation. It is tempting to speculate that the pump–activator complex is mainly held together by hydrophobic forces since activation of the pump upon phospholamban phosphorylation was sug-

gested to be brought about by a change in the hydrophobic microenvironment of the pump (Chiesi, 1979). Such strong hydrophobic interactions would not be disrupted by "mild" detergents such as Triton X-100 or deoxycholate. Once dissociated by dodecyl sulfate, phospholamban tends to exist as a dimer of 22 000 molecular weight, together with a minor and variable proportion of the M_r 11 000 monomer.

Persistence of a noncovalent dimer in the presence of dodecyl sulfate is against the dogma of full dissociation of individual peptide chains, but similar observations of a noncovalent trimer of a major outer membrane protein of *Escherichia coli* K-12, stable in dodecyl sulfate solution containing 8 M urea, have already been reported (Yu et al., 1979).

Only upon combination of ionic and nonionic detergents is phospholamban finally dissociated into monomers. The purified material easily aggregates into high molecular weight forms, and there is no evidence that the dimeric structure does indeed correspond to the native state of the activator within the membrane plane.

The distribution of phospholamban between the pump-bound, the dimeric, and the monomeric forms is one of the explanations available for the low yield of our diagonal purification procedure. This procedure is extremely simple and operationally easy, and it provides in a matter of days enough material for the preliminary characterization of the protein as reported herein.

Phospholamban is only phosphorylated on serine residues and contains the arginyl residues that were expected from its ability to serve as substrate of cAMP-dependent protein kinase (see Cohen, 1978, for a review). The portion of the polypeptide chain that is available at the cytosolic surface of the SR membrane is accessible not only to the different kinases involved in the activation of the pump (Le Peuch et al., 1979) but also to proteases that remove the phosphopeptides, at variance with the reported inability of trypsin to remove the protein-bound ^{32}P (Tada et al., 1975). This observation paves the way to the isolation of phosphopeptides from phosphorylated SR vesicles. Even though dodecyl sulfate gel electrophoresis points to a monomer molecular weight of 11 000, the amino acid analysis would also be compatible with one residue each of tyrosine and histidine, i.e., with a minimal molecular weight of 5500. Only a more thorough chemical characterization (e.g., cyanogen bromide cleavage) will permit a clear-cut definition of the chain length.

The molecule must exhibit a highly hydrophobic domain which allows reinsertion into the lipid bilayer of liposomes without sonication. Both dephosphorylation and rephosphorylation of liposome-bound phospholamban proceed more slowly than for the "native" protein in cardiac SR vesicles (Le Peuch et al., 1979). Even though these experiments provide evidence for the presence of the phosphorylated sites at the outer surface of the liposomes, they suggest that the phospholamban conformation is altered either as a consequence of the purification procedure or, more likely, by absence of interacting Ca^{2+},Mg^{2+}-ATPase.

This and several other observations reported herein point to the crucial importance of the interaction of the pump and its activator, in terms of both tertiary structure and biological activity.

Availability of phospholamban in a pure form will hopefully permit reconstitution experiments designed to test the hypothesis according to which dephosphophospholamban modifies the kinetic parameters of the cardiac pump otherwise similar to those of the skeletal muscle pump (Hicks et al., 1979).

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