Sequence Analysis of Phospholamban

IDENTIFICATION OF PHOSPHORYLATION SITES AND TWO MAJOR STRUCTURAL DOMAINS*

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Phospholamban is a regulatory protein in cardiac sarcoplasmic reticulum that is phosphorylated by cAMP- and Ca2+/calmodulin-dependent protein kinase activities. In this report, we present the partial amino acid sequence of canine cardiac phospholamban and the identification of the sites phosphorylated by these two protein kinases. Gas-phase protein sequencing was used to identify 20 NH2-terminal residues. Overlap peptides produced by trypsin or papain digestion extended the sequence 16 residues to give the following primary structure: Ser-Ala-Ile-Arg-Arg-Ala-Thr-Ile-Glu-Met-Pro-Gln-Gln-Ala-Arg-Gln-Asn-Leu-Gln-Asn-Leu-Phe-Ile-Asn-Phe-(Cys)-Leu-Ile-Leu-(Cys)-Leu-Leu-Leu-Ile-.

Phospholamban phosphorylated by either cAMP-dependent or Ca2+-calmodulin-dependent protein kinase was cleaved with trypsin, and the major phosphorylated peptide (comprising >70% of the incorporated 32P label) was purified by reverse-phase high performance liquid chromatography. The identical sequence was revealed for the radioactive peptide obtained from phospholamban phosphorylated by either kinase: Arg-Ala-Ser-Thr-Ile-Glu-Met-Pro-Gln-Gln-. The adjacent residues Ser7 and Thr8 of phospholamban were identified as the unique sites phosphorylated by cAMP- and Ca2+-calmodulin-dependent protein kinases, respectively.

These results establish that phospholamban is an oligomer of small, identical polypeptide chains. A hydrophilic, cytoplasmically oriented NH2-terminal domain on each monomer contains the unique, adjacent residues phosphorylated by cAMP- and Ca2+-calmodulin-dependent protein kinase activities. Analysis by hydrophatic profiling and secondary structure prediction suggests that phospholamban monomers also contain a hydrophobic domain, which could form amphipathic helices sufficiently long to traverse the sarcoplasmic reticulum membrane. A model of phospholamban as a pentamer is presented in which the amphipathic α-helix of each monomer is a subunit of the pentameric membrane-anchored domain, which is comprised of an exterior hydrophobic surface and an interior hydrophilic region containing polar side chains.

Phospholamban is an integral membrane protein of cardiac sarcoplasmic reticulum which is phosphorylated in actively contracting myocardium in response to β-adrenergic stimulation (1). Phospholamban in isolated sarcoplasmic reticulum vesicles is a substrate for soluble cAMP-dependent protein kinase (2, 3), as well as for an endogenous Ca2+/calmodulin-dependent protein kinase (4, 5). Recently, Ca2+-activated, phospholipid-dependent protein kinase (protein kinase C) isolated from rat brain has also been shown to phosphorylate phospholamban (6–8). In vitro studies have correlated the stimulation of Ca2+-dependent ATPase activity and Ca2+ transport by the Ca2+ pump of cardiac sarcoplasmic reticulum vesicles with phosphorylation of phospholamban (1–4, 7, 9–12). These studies have suggested a role for phospholamban in the regulation of the cardiac Ca2+ pump protein, possibly mediated by more than one protein kinase activity, although a physical interaction between phospholamban and the Ca2+ pump has not yet been directly demonstrated. The relative roles of cAMP- and Ca2+-calmodulin-dependent mechanisms of phospholamban phosphorylation in intact myocardium are presently under investigation (1, 13, 14), but understanding of the functional significance of either process will first require detailed knowledge of the structural changes in phospholamban which are induced by phosphorylation.

Evidence has accumulated to indicate that different sites on phospholamban are phosphorylated by cAMP- and Ca2+-calmodulin-dependent protein kinases (4, 7, 8, 15–19), but the exact nature and location of these sites remain controversial. One laboratory has reported that phospholamban is phosphorylated exclusively at serine residues (4, 15) and another that there may actually be two distinct phospholamban proteins of slightly different molecular weights, each bearing a single site phosphorylated by one or the other protein kinase (17). More recent results from our laboratory have supported a model of phospholamban as a 25-kDa oligomer of five identical low molecular weight subunits, each containing one site of phosphorylation for cAMP-dependent protein kinase (at a serine residue) and one site of phosphorylation for Ca2+-calmodulin-dependent protein kinase (at a threonine residue) (8, 18). Recently, Imagawa et al. (70) have also suggested that phospholamban is a pentamer of identical low molecular weight subunits. However, these investigators reported that phospholamban is phosphorylated at two distinct sites by Ca2+-calmodulin-dependent protein kinase. In this latter study, however, phospholamban was detected by phosphorylating it in intact sarcoplasmic reticulum vesicles, and the purified protein was not analyzed (70). To resolve these discrepancies in phospholamban structure reported by different laboratories, it seems essential to purify the protein and determine its sequence in order to identify unambiguously the

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Amino Acid Sequence of Phospholamban

amino acids phosphorylated at various sites. This was one of the goals of the present study.

The development of a reproducible method to prepare milligram quantities of highly purified phospholamban (18) has enabled us to determine several physical characteristics of the protein in recent studies (8, 18, 19). These include measurement of phospholamban's susceptibility to phosphorylation (8, 18), its amino acid composition, isoelectric point, and solvent extractability (19), and its sensitivity to proteolytic digestion (8). These characteristics were integrated to form a low resolution model of phospholamban structure in which the protein was described as a pentamer of several small identical subunits. A largely hydrophobic, protease-resistant domain on each subunit appeared to be responsible for holding the multiple subunits together, and a smaller protease-sensitive domain on each subunit appeared to contain all of the relevant sites of phosphorylation (8). We now expand this model based on sequence data obtained from intact purified phospholamban and proteolytic fragments derived from the phosphorylated protein. Our results establish the following points regarding phospholamban structure: 1) Phospholamban monomers contain an identical sequence identical subunits in the phosphorylation sites. 2) The NH2-terminal region of phospholamban is the protease-sensitive domain previously shown to contain the phosphorylation sites; 3) Ser is the amino acid residue phosphorylated by cAMP-dependent protein kinase, and Thr is the amino acid residue phosphorylated by Ca2+/calmodulin-dependent protein kinase. 4) Phospholamban contains a span of at least 20 uncharged residues which forms the previously identified protease-resistant domain involved in stabilization of the protein's oligomeric structure.

EXPERIMENTAL PROCEDURES

Isolation of Dephosphorylated and Phosphorylated Phospholamban from Canine Cardiac Sarcoplasmic Reticulum Vesicles—Phospholamban was purified from canine cardiac sarcoplasmic reticulum vesicles as described previously (19). Selective extraction of the membranes with sodium cholate and adsorption of the solubilized protein to Ca2+ oxalate followed by dialysis at 4 °C yielded a reconstituted particulate fraction enriched 5-fold in phospholamban (19). The protein was solubilized from this reconstituted particulate fraction with use of the detergent Zwittergent 3-14, applied to a column of p-hydroxymercaptoethanesulfonic acid; and adsorption of the solubilized protein to Ca2+ ban was purified from canine cardiac sarcoplasmic reticulum vesicles using 40 mM Pipes/Tris (pH 6.8), 10 mM MgCl2, 8 mM Pipes/Tris (pH 6.8), 10 mM 20 mM EGTA, 20 mM CaCl2, and 104 μg of calmodulin. Phosphorylation was started by adding 2.5 ml of 4 mM Na2ATP containing a trace amount of [γ-32P]ATP. The incubation was conducted for 5 min at 30 °C, and the reaction was then terminated by centrifugation of the suspension in a Ti-70 rotor at 40,000 rpm for 20 min at 4 °C. The reconstituted particulate fraction pellet was then resuspended in 5 ml of 0.25 m sucrose, 30 mM histidine. Phosphorylated phospholamban was solubilized from the reconstituted particulate fraction by addition of 1.4 ml of 10% Zwittergent 3-14 and purified by sulphydryl group affinity chromatography as described previously, using 20 mM dithiothreitol, 0.2% Zwittergent 3-14, 50 mM NaCl, and 10 mM MOPS (pH 7.0) to elute the phosphorylated form of the protein from the mercury column (8). Control experiments verified that phosphorylation of phospholamban in the reconstituted particulate fraction was stimulated greater than 40-fold by addition of calmodulin. All calmodulin-dependent phosphorylation was completely Ca2+-dependent.

Proteolytic Digestion of Phospholamban—Purified preparations of dephosphorylated or phosphorylated phospholamban were cleaved using a 1.5 weight ratio of trypsin or papain to phospholamban. Proteolysis with trypsin was conducted for 18 h at 25 °C in a buffer containing 20 mM TES pH 7.0, 20 mM EGTA, 0.5 mM dithiothreitol, and 0.3% Triton X-100. Phosphorylated phospholamban was solubilized from the mercury column (8). papain was conducted for 18 h at 25 °C in a buffer containing 180 mM Tris/HCl (pH 8.0), 2 mM EDTA, and 1 mM dithiothreitol. Reactions were terminated by centrifugal ultrafiltration of peptides through a YM-10 membrane with use of an Amicon Centrifree. Small peptides were released by proteolytic digestion with trypsin and papain were isolated by application of the Centricon filtrate fraction to a column of DEAE-Sepharose equilibrated with 20 mM MOPS (pH 7.0), 50 mM NaCl, and 0.1% SDS. 30 μl samples of retentate fraction containing 30 μg of proteolyzed phospholamban were injected, and the column was developed isocratically at a flow rate of 0.5 ml/min. Inorganic phosphate (final concentration 1% SDS (final concentration 1% SDS) was added to samples prior to injection. Absorbance of the effluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Initially, fractions containing major, nonradioactive phospholamban fragments were identified by subjecting aliquots of each fraction to SDS-polyacrylamide gel electrophoresis and silver staining, but after correlating the gel results with the 214-nm absorption profile, fractions were pooled on the basis of the spectroscopic profile alone.

Small phosphorylated peptides released from phospholamban by tryptic digestion were isolated by high performance liquid chromatography (Beckman) using an Altech TSK-3000 size exclusion column equilibrated with 20 mM MOPS (pH 7.0), 50 mM NaCl, and 0.1% SDS. 50 μl samples of retentate fraction containing 30 μg of purified phospholamban were injected, and the column was developed isocratically at a flow rate of 0.5 ml/min. Inorganic phosphate (final concentration 1% SDS (final concentration 1% SDS) was added to samples prior to injection. Absorbance of the effluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Initially, fractions containing major, nonradioactive phospholamban fragments were identified by subjecting aliquots of each fraction to SDS-polyacrylamide gel electrophoresis and silver staining, but after correlating the gel results with the 214-nm absorption profile, fractions were pooled on the basis of the spectroscopic profile alone.

Small phosphorylated peptides released from phospholamban by tryptic digestion were isolated by high performance liquid chromatography using an Altech reverse-phase C18 column equilibrated with 0.05% trifluoroacetic acid in water (solvent A) and developed with a gradient of 0.05% trifluoroacetic acid in methanol (solvent B) according to the following program: 0–10 min, 0% B; 10–20 min, 0–35% B; 20–30 min, 35–65% B; 30–110 min, 65% B. Typically, 50 μl of puritified phospholamban filtrate fraction was detected and eluted at a flow rate of 0.5 ml/min. Fractions from the reverse-phase column were collected at 1-min intervals and analyzed for 32P by Cerenkov counting. Radioactive fractions were further examined by thin layer electrophoresis and autoradiography (8).

Small phosphorylated peptides released from phospholamban by papain were isolated by application of the Centricon filtrate fraction to a column (1 × 18 cm) of DEAE-Sepharose equilibrated with 10 mM MOPS (pH 7.0) and 0.1% Zwittergent 3-14. The NH2-terminal peptide released by papain was eluted from the column in the same buffer containing 50 mM MOPS (pH 7.1).

Automated Sequence Analysis—Amino acid sequences were determined using an Applied Biosystems Model 470A gas-phase protein sequenator, essentially as described by Hewick et al. (21). Phenylthiohydantoin were analyzed by reverse-phase high performance liquid chromatography using a Waters Nova-Pak column and the gradient elution system described in Waters Applications Brief M5300. A Waters high performance liquid chromatography system including two M510 pumps, a WISP 710B autoinjector, and an M440 dual channel absorbance detector was used. The detector was set to monitor the sum of the absorbances at 254 and 280 nm (for peak detection of phenylthiohydantoins) and 313 nm (for qualitative detection of breakdown products of serine-phenylthiohydantoin and threonine-phenylthiohydantoin). The recovery of phenylthiohydantoins at each cycle was measured with an integrative recorder (Waters...
Phenylthiohydantoin were usually obtained in low yields, but they were often recovered in low yields.

Phosphoamino Acid Analysis—$^{32}$P-labeled phospholamban or isolated phosphorylated peptides were hydrolyzed for 4 or 7 h in 6 N HCl at 110 °C in evacuated tubes. The samples were lyophilized and examined by thin layer electrophoresis as described below. Phosphoserine and phosphothreonine standards were visualized with ninhydrin. Amino acid analyzers by the method of Spackman et al. (25).

Curbozymethylation—3–4 μg of extensively dialyzed phospholamban or ribonuclease in 10 mM MOPS (pH 7.5) were carboxymethylated with iod0$^{2-}$H]acetic acid (135 mCi/mmol) in the presence of cysteic acid (135 mCi/mmol) in the presence of the cationic detergent hexadecylpyridinium chloride at a 2% concentration. Although improved reduction and carboxymethylation of membrane proteins have been reported to occur in cationic detergent solutions (22), we obtained similar results when reactions occurred upon boiling in SDS. Samples were alkylated directly without prior incubation at 37 °C for 1 h in the dark. Reaction products were subjected to high performance liquid chromatography were oxidized with performic acid (24), dried, and hydrolyzed with 6 N HCl in evacuated tubes for 22 h at 110 °C. The resulting amino acids were analyzed on a Beckman Model 119CL amine acid analyzer by the method of Spackman et al. (25).

Thin Layer Electrophoresis—$^{32}$P-labeled peptides and phosphorylated amino acids were resolved by this thin layer electrophoresis on Kodak cellulose chromatogram sheets. Electrophoresis was conducted in a Savant chamber for 1 h at 500 V in a solution of 88% formic acid/glacial acetic acid/water (v/v, 2.5.7.59) (pH 1.9). After electrophoresis, the gels were covered in a plastic wrap, and autoradiograms were developed. For quantitation, radioactive spots were scraped from the sheets and counted in a liquid scintillation counter.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the slightly modified buffer system of Porzio and Carson (26). Gels were 1.5 mm thick 0.75-mm thick slab gels were used for silver staining (28). SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the slightly modified buffer system of Porzio and Carson (26) described previously (27). 8% slab gels were used to identify intact phospholamban during purification, whereas 10% slab gels were used to separate nonradioactive fragments of phospholamban following phosphorylation. Slab gels to be stained with Coomassie Blue, and, after extensive washing to remove nonspecific $^{32}$H label from the gel, the protein bands were cut out and solubilized by incubation in 90% hydrogen peroxide at 50 °C overnight. Radioactivity was quantitated by liquid scintillation counting, and quenching was corrected for by use of the external standard method.

Amino Acid Analysis—Lyophilized samples of phospholamban or the large tryptic fragment of phospholamban purified by size exclusion chromatography were oxidized with performic acid (24), dried, and hydrolyzed with 6 N HCl in evacuated tubes for 22 h at 110 °C. The resulting amino acids were analyzed on a Beckman Model 119CL amine acid analyzer by the method of Spackman et al. (25).

Materials—Cholic acid, p-hydroxymercuribenzoate-agarose, hexacycliryzin chloride, the catalytic subunit of cAMP-dependent protein kinase (isolated from beef heart), calmodulin (isolated from bovine brain), phosphoserine, phosphothreonine, trypsin, and papain were purchased from Sigma. Trifluoroacetic acid was obtained from Pierce, and [γ-$^{32}$P]ATP and iod0$^{2-}$H]acetic acid were purchased from Amersham Corp. Twitriterg 2-14 (N-tetradecyl-N,N,N,N-dimethyl-3-ammonio-1-propanesulfonate) was purchased from Behring Diagnostics.

RESULTS

In previous studies we have presented data suggesting that phospholamban is a pentameric protein of $M_\text{r} \approx 25,000$, which, upon boiling in SDS, is dissociated into identical subunits of $M_\text{r} \approx 5,000$ (8, 18, 19). Each subunit is phosphorylated at different sites by cAMP- and Ca$^{2+}$/calmodulin-dependent protein kinases (9, 18). As a result of phosphorylation, there is a large change in phospholamban's apparent isoelectric point, from pl = 10 (dephosphorylated) to pl = 6.7 (phosphorylated) (19). To identify the sites of phosphorylation, phospholamban and to learn more about the protein's structure, we subjected phospholamban to automated Edman degradation. The results from gas-phase protein sequencing are shown in Fig. 1. Direct sequencing of phospholamban allowed identification of 20 residues (Fig. 1, Intact phospholamban, residues 1–20) before low yields prevented conclusive recognition of subsequently released residues. It should be noted that the identification of residues 1 and 2 using intact phospholamban was somewhat tentative due to the presence of high background contamination in these cycles. However, a peptide produced by papain digestion of phospholamban was later isolated by anion-exchange chromatography. Sequence analysis of this peptide yielded Ser-Ala-Ile-Arg-Ang-Ala-Ser-Thr-Ile-Glu-Met-Pro-Pro, confirming the sequence identified using intact phospholamban. It is apparent that residues 4–8 define a potential site of phosphorylation on phospholamban, in that a pair of serines is followed after 1 residue by a serine and threonine (see "Discussion"). Proteolytic fragments of $^{32}$P-labeled phospholamban were made so that phosphorylated peptides could be sequenced. Cleavage of phosphorylated phospholamban with chymotrypsin produced a major 21-kDa nonradioactive fragment (Fig. 2, lane 3, PLB2) and several smaller phosphopeptides (8). As shown previously (8), the major nonradioactive 21-kDa fragment (Fig. 2, lane 3, PLB2) exhibited the characteristic dissociation into subunits (Fig. 2, lane 4, PLB4) induced by boiling in SDS. Rapid separation of >90% of the small radioactive peptides from this large fragment was effected by filtration of the reaction products through a YM-10 membrane with use of an Amicon Centricon. The filtrate, containing the $^{32}$P-labeled peptides, was then fractionated by reverse-phase high performance liquid chromatography.

To determine the sequence surrounding the site of phospholamban phosphorylated by cAMP-dependent protein kinase, the limit tryptic phosphopeptide obtained from 250 μg of phosphorylated phospholamban was isolated by high performance liquid chromatography. The small phosphopeptides in the filtrate were injected on an Alttech reverse-phase C18 column and eluted with a gradient (0.5%/min) of methanol (Fig. 3). One major phosphopeptide constituted 75% of released phosphopeptide and was trypsin released at 53% methanol (peak 3); three smaller phosphopeptides eluted at 47, 49, and 55% methanol and constituted 5, 4, and 16% of released $^{32}$P label, respectively (Fig. 3). Thin layer electrophoresis of the radioactive fractions demonstrated two mobility forms (Fig. 3, inset); the high mobility peptides could be converted to the lower mobility forms with a higher concentration of trypsin (8), indicating that the lower mobility form was the limit tryptic peptide.

The sequence for the major limit tryptic phosphopeptide (Fig. 3, peak 3) phosphorylated by cAMP-dependent protein kinase was determined to be Arg-Ala-Ser-Thr-Ile-Glu-Met-Pro-Gln-Gln (Fig. 1, residues 5–14). The finding of arginine residue 5) and not alanine (residue 6) as the NH2-terminal residue of this limit tryptic peptide could be explained by the inhibition of tryptic hydrolysis at sites near phosphorylated residues, reported by others (29–31). Phosphoamino acid analysis of this radioactive peptide indicated only phosphoserine (Fig. 4), and so it was concluded that serine in the third position of this peptide (Fig. 1, residue 7) was the residue phosphorylated by cAMP-dependent protein kinase.

To localize the site in phospholamban phosphorylated by the endogenous Ca$^{2+}$/calmodulin-dependent protein kinase, phospholamban was first phosphorylated in the reconstituted particulate fraction in the presence of Ca$^{2+}$ and calmodulin.
**Amino Acid Sequence of Phospholamban**

**FIG. 1. Partial amino acid sequence of canine cardiac phospholamban.** The sequence of 36 residues of phospholamban was obtained by gas-phase protein sequencing of intact phospholamban and overlapping, enzymatically derived peptides (bars denote sequence obtained from each peptide). The serine phosphorylated by cAMP-dependent protein kinase (cAMP PK) and the threonine phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinase (Ca/CaM PK) are noted.

**FIG. 2. Cleavage of phosphorylated phospholamban with trypsin.** 250 \(\mu\)g of purified phospholamban phosphorylated by the catalytic subunit of cAMP-dependent protein kinase was incubated for 16 h with 50 \(\mu\)g of trypsin. The resulting large, nonradioactive fragment of phospholamban was purified by high performance liquid chromatography using an Alttech TSK-3000 size exclusion column, and SDS-polyacrylamide gel electrophoresis and silver staining was then performed on an aliquot of the pooled, purified sample. Lane 1 shows the 25-kDa, high molecular mass form of intact phospholamban (PLB\(_h\)), and lane 3 shows the purified 21-kDa tryptic fragment that was sequenced. Lane 4 shows that this tryptic fragment exhibits the characteristic dissociation into subunits induced by boiling in SDS, similar to control phospholamban (lane 2). PLB\(_l\) designates the dissociated, low molecular weight form of phospholamban. The panel on the right shows an autoradiogram of the same gel, illustrating removal of the \(^{32}\)P label by trypsin.

Fig. 5 shows that calmodulin markedly stimulated phosphorylation of phospholamban in this fraction and that the labeled phosphoprotein was completely dissociated by boiling in SDS prior to electrophoresis. A phosphoprotein of \(M_r = 55,000\), which may represent the autophosphorylated type II calmodulin kinase (see “Discussion”), is also evident on the autoradiogram. Phosphorylated phospholamban was solubilized from the reconstituted particulate fraction in Zwittergent 3-14 and purified by sulfhydryl group affinity chromatography (8, 19). The phosphorylated protein was then digested with trypsin, the products were filtered, and the small phosphopeptides fractionated by reverse-phase high performance liquid chromatography as described above for the peptides phosphorylated by cAMP-dependent protein kinase. In this case, the major phosphopeptide released by trypsin from phospholamban phosphorylated by the Ca\(^{2+}\)/calmodulin-de-
Phosphoserine (Fig. 4) peptide compared with the more rapid, complete release of proteolyzed 32P-labeled phospholamban peptides through the retentate fraction after filtration of the small peptides released by proteolysis were obtained by filtration through a YM-10 membrane as described in the text. Samples (~2000 Cerenkov cpn) of the unfractonated peptides were hydrolyzed in 6 N HCl for 4 or 7 h, and the products were resolved by thin layer electrophoresis (pH 1.9) and visualized by autoradiography. Nonradioactive phosphoamino acid standards were visualized with ninhydrin. Ser-P, phosphoserine; Thr-P, phosphothreonine; Pi, inorganic [32P]phosphate.

The sequence determined for this peptide was identical to the one obtained from phospholamban phosphorylated by cAMP-dependent protein kinase: Arg-Ala-Ser-Thr-Ile-Glu-Met-Pro-Gln-Gln—(Fig. 1, residues 5–14). Phosphoamino acid analysis of the peptide phosphorylated by Ca2+/calmodulin-dependent protein kinase, however, indicated only phosphothreonine (Fig. 4), consistent with previous observations using intact sarcoplasmic reticulum vesicles (8). (The slower release of phosphothreonine during partial acid hydrolysis of this peptide compared with the more rapid, complete release of phosphoserine (Fig. 4) probably reflects the more interior location of threonine within the sequence and the more slowly hydrolyzed peptide bond between threonine and isoleucine.)

Thus Thr(1) (Fig. 1) is the amino acid residue phosphorylated by the Ca2+/calmodulin-dependent protein kinase intrinsic to cardiac membranes.

To extend the known sequence of phospholamban, the large nonradioactive fragment produced by tryptic cleavage (Fig. 2, lanes 3 and 4, silver stain) was sequenced. This large fragment was obtained from the retentate fraction after filtration of tryptolyzed 32P-labeled phospholamban peptides through the Amicon Centricon. Prior to sequencing the nonradioactive, major tryptic fragment, it was further purified by high performance liquid chromatography using an Alttech TSK-3000 size exclusion column. Control experiments revealed that, with this type of chromatography, noncleaved phosphorylated phospholamban (Mr = 27,000) eluted at 16 min, the large nonradioactive tryptic fragment (Mr = 21,000) eluted at 18 min, and small phosphorylated peptides eluted at 25 min (data not shown).

The sequence of the major, nonradioactive tryptic fragment of phospholamban (Fig. 2, lanes 3 and 4, silver stain) was determined to be Gln-Asn-Leu-Gln-Asn-Leu-Phe-Ile-Asn-Leu-(Cys)-Leu-Ile-Leu-Ile-(Cys)-Leu-Leu-Leu-Ile-Val-Gln-Asn-Leu-Gln-Asn-Leu-Phe-Ile-Asn-Leu-(Cys)-Leu-Ile-Leu-Ile-Asn-Leu-Phe-Ile-Asn-Leu-Gln-Asn-Leu-Phe-Ile-Asn-Leu-(Cys)-Leu-Ile-Leu-Ile-(Cys)-Leu-Leu-Leu-Ile-(Fig. 1, residues 17–36). At cycles 11 and 16 of the major tryptic fragment no sign of any phenylthiohydantoin was found (Fig. 1, residues 27 and 32). The most plausible explanation for this observation is that residues 27 and 32 were cysteine residues, since cysteine is known to have an unstable phenylthiohydantoin, and amino acid analysis of intact phospholamban indicated 3 cysteine residues per 50 amino acid residues (19). Consistent with this, the amino acid composition of the purified, large tryptic fragment of phospholamban revealed a high molar percentage of cysteine residues (Table I). The sequence of phospholamban recently deduced from a cDNA clone by Tada and co-workers2 confirmed the assignment of Cys-SH at positions 11 and 16 in this peptide. The value in the peptide (Table I) not contained in the observed sequence (Fig. 1) suggested that the sequence continued beyond the 20 residues identified. Comparison of the observed sequence of the large tryptic fragment of phospholamban with the sequence obtained from the intact molecule places this fragment at residues 17–36 (Fig. 1). The hydrophobic nature of this fragment probably explains its resistance to several proteases (8).

The possibility of disulfide bonds in phospholamban was examined in two ways. To determine whether the phospholamban monomers were linked by intersubunit disulfide bonds, extensively dialyzed phospholamban was heated at 50°C for 1 h in 2% SDS in the presence or absence of 20 mM CaM

polyacrylamide gels and subjecting them to liquid scintillation counting. The results (Table 11) show that labeling of ribonuclease, included as a control,

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**Table I**

**Amino Acid Sequence of Phospholamban**

The 21-kDa tryptic peptide of phospholamban was purified by high performance liquid chromatography as described under “Experimental Procedures.” Incorporated radioactivity was quantitated by cutting labeled protein bands from iod0[2-~H]acetic acid in the presence of 2% hexadecylpyridinium chloride as described under “Experimental Procedures.” Quench correction was by the external standard method.

**Table II**

**Carboxymethylation of intact phospholamban and ribonuclease**

Phospholamban and ribonuclease were carboxymethylated with iodo[2-3H]acetic acid in the presence of 2% hexadecylpyridinium chloride as described under “Experimental Procedures.” Incorporated radioactivity was quantitated by cutting labeled protein bands from polyacrylamide gels and subjecting them to liquid scintillation counting. Quench correction was by the external standard method.

**DISCUSSION**

This is the first report of the amino acid sequence of the sites phosphorylated by cAMP-dependent protein kinase and Ca2+/calmodulin-dependent protein kinase in a well-characterized, intrinsic membrane protein. The Ca2+/calmodulin-dependent protein kinase which phosphorylates phospholamban in cardiac sarcoplasmic reticulum membranes probably belongs to the type II family of Ca2+/calmodulin-dependent kinases, which have now been identified in several different tissues. These kinases have broad substrate specificities, undergo autoprophosphorylation at 50-60-kDa subunits, and exist in both soluble and particulate forms (32-35). Soluble activities, recently purified from heart (36-37), phosphorylate phospholamban and show similarities to the particulate activity phosphorylating phospholamban in both free and junctional cardiac sarcoplasmic reticulum vesicles (5, 38, 39). The soluble and particulate activities characterized from canine heart both appear to undergo autophosphorylation at 55-kDa subunits (5, 37, 38).

Comparison of the phospholamban sequence surrounding the phosphorylation sites with sequences from other, nonmembrane proteins phosphorylated by cAMP- and Ca2+/calmodulin-dependent protein kinases reveals similarities (Table III). The site phosphorylated in phospholamban by cAMP-dependent protein kinase contains the most common pattern of residues recognized by this kinase (Table III, sequences 2-7), in which the phosphorylated residue is separated by 1 amino acid from a pair of basic residues, most frequently 2 arginines (51, 52). The basic residues are located NH2-terminal to the phosphorylated site. The site phosphorylated in phospholamban by the Ca2+/calmodulin-dependent protein kinase is similar to sites in proteins phosphorylated by other Ca2+/calmodulin-dependent protein kinases (Table III, sequences 10-14) in that the phosphorylated residue is two positions removed from the COOH terminus of 1 or 2 basic residues (53, 54). Although the phospholamban sequence is unusual in that a threonine residue, rather than a serine, is phosphorylated by the Ca2+/calmodulin-dependent protein kinase, this observation is consistent with the specificity of the Ca2+/calmodulin-dependent protein kinase as determined with the use of synthetic peptide substrates (55). A phosphorylated residue two positions removed from the nearest basic residue can also be recognized secondarily by cAMP-dependent protein kinase (Table III, sequences 6, 8, 9, 12, 13). However, we obtained no evidence in any of our experiments for phosphorylation of Thr10 of phospholamban by cAMP-dependent protein kinase. A reason for this may be that, in this specific configuration, threonine is inferior to serine as a substrate for cAMP-dependent protein kinase. Alternatively, higher order structure has been demonstrated by others to play a role in the expression of potential phosphorylation sites within a sequence (44, 56). The NH2-terminal region of phospholamban may be stabilized in a way to present favorably only Ser for phosphorylation by cAMP-dependent protein kinase, while nevertheless maintaining sufficient flexibility for recognition by the kinase (57).

dithiothreitol, or heated at 100 °C for 5 min in 2% SDS in the presence or absence of 20 mM dithiothreitol. SDS-polyacrylamide gel electrophoresis revealed that phospholamban was only dissociated into subunits by heating at 100 °C, and that dithiothreitol was not required for dissociation to occur. No dissociation was observed at 50 °C, whether or not dithiothreitol was present (data not shown). These results strongly suggest that the 25-kDa form of phospholamban is not stabilized by intersubunit disulfide bonds (19).

To determine whether the phospholamban monomers contained an internal disulfide bond, the protein was derivatized with iodo[2-3H]acetic acid, and the reaction products were subjected to polyacrylamide gel electrophoresis; protein bands were identified by staining, and the bands were cut out and solubilized for liquid scintillation counting. The results (Table II) show that labeling of ribonuclease, included as a control, was greatly increased by prior treatment of the protein with dithiothreitol, as expected from the known high content of disulfide bonds in this protein. In contrast, labeling of phospholamban was not enhanced by prior treatment with dithiothreitol. When expressed per milligram of total protein reacted, the cysteine content of phospholamban was found to be comparable to that of reduced ribonuclease, as predicted from the known amino acid compositions of the two proteins. These results confirm that phospholamban is a cysteine-rich protein and demonstrate that the cysteine residues in each phospholamban monomer are probably present in the reduced, sulfhydryl form.
The significance of adjacent amino acid residues on phospholamban which are phosphorylated uniquely and exclusively by two different kinases is presently unknown. Although adjacent residues on other proteins which are phosphorylated by cAMP-dependent protein kinase have been catalogued (Table III, substrates 6 and 7), and phosphorylation of the same residue on a protein by different kinases is also well known (Table III, substrates 12-14), this is the first report of a membrane protein bearing adjacent residues phosphorylated by two different kinases. It is known that occupancy at one phosphorylation site on phospholamban does not prevent phosphorylation at the other site (4, 8, 18), although the kinetics of this multiple phosphorylation have not been rigorously examined, nor has the susceptibility of the two sites to different phosphatase activities been studied in any detail. Determination of phosphorylation effects on the secondary and tertiary structure of phospholamban may be helpful in elucidating the functional significance of these two adjacent phosphorylation sites.

For the purpose of designing incisive experiments to probe phospholamban secondary and tertiary structure, a model is instructive. The current results from sequence analysis are consistent with the findings of Wegener et al. (8) that cAMP-dependent protein kinase phosphorylates 1 serine (identified now as Ser) and that Ca\(^2\+)/calmodulin-dependent protein kinase phosphorylates an adjacent threonine residue (Thr). The NH\(_2\)-terminal location of the phosphorylated region and the hydrophobicity of the protease-resistant fragment explain the cleavage patterns observed by Wegener et al. (8).

Hydropathic profiling (58) of phospholamban (Fig. 6) supports a membrane-embedded location for the protease-resistant domain, whereas the NH\(_2\)-terminal domain is hydrophilic, free from the membrane, and available for phosphorylation. The average hydropathy of residues 17-36 of phospholamban is calculated to be +1.9, which, according to Kyte and Doolittle (58), gives a high probability that this 20-amino acid stretch will be embedded in the sarcoplasmic reticulum membrane.

The model that emerges from these studies is of a molecule which spans the sarcoplasmic reticulum once, with a polar, phosphorylatable head on the cytoplasmic side of the membrane. The embedded domain is most probably formed by the protease-resistant fragment containing residues 17-36, which may assume a helical configuration sufficiently long to traverse the membrane. Although the potential for \(\beta\)-sheet in this region is predicted to be greater than the \(\alpha\)-helix potential (\((\mathcal{P})_\alpha = 1.03, (\mathcal{P})_\beta = 1.25\) (59), the helix is the predominant form observed in lipid solutions, despite the considerable potential for \(\beta\)-sheet occasionally predicted for membrane-bound sequences by empirical methods (60, 61). A helical wheel diagram (62) indicates that an \(\alpha\)-helix of phospholamban residues 17-36 would be amphipathic (63) (Fig. 7). Five such helices oriented with the polar faces inward could form a pentameric transmembrane domain with a hydrophobic exterior presenting Leu, Ile, and Phe residues to the lipid bilayer, and a largely hydrophilic interior, containing several Gin and Asn residues (Figs. 7 and 8).

An attractive feature of such a model is the assemblage in the interior of the transmembrane complex of polar groups contributed by the side chains of Gin\(^1\), Asn\(^18\), Gin\(^20\), and Asn\(^23\), which could form a network of hydrogen bonds responsible for the strong stabilization of the phospholamban pentamer (8). Hydrogen bonding between transmembrane helices...
FIG. 7. Helical wheel diagram of phospholamban residues 17-36. The protease-resistant domain of phospholamban (residues 17-36) is represented as an amphipathic α-helix viewed end-on from the COOH-terminal end (62). The vertical line roughly separates the polar (Interior) and nonpolar (Exterior) faces of the helix. Five such helices together could form the pentameric transmembrane domain of phospholamban, in which the nonpolar face of each helix is oriented to the exterior (in contact with phospholipid hydrocarbon tails) and the polar face is oriented to the interior (sequestering polar side chains from lipid contact).

of glycoporphin A (64), bacteriorhodopsin (65, 66), and the alamethicin channel (67) have been similarly proposed to stabilize those structures. In this model of phospholamban, the side chains of Asn18, Cys27, and Cys32 would form bands of polar groups within the interior of the structure which, along with the side chains of Gln17, Asn18, Gln20, and Asn21, could provide a means to solvate the interior by hydrogen bonding to water molecules. Planes of polar groups lining the interior of transmembrane helical domains have been proposed to preserve the aqueous channels of alamethicin (67) and the acetylcholine receptor (68). The exterior surface of the phospholamban transmembrane domain would be entirely hydrophobic, displaying only side chains of leucine, isoleucine, and phenylalanine (Figs. 7 and 8). Whether or not phospholamban exhibits any kind of channel activity is currently unknown, but is presently under investigation in our laboratory.

While this manuscript was under review, we learned that Tada and co-workers had determined the complete amino acid sequence of phospholamban by recombinant DNA and gas-phase sequencing methods (69). The results from both laboratories are in complete agreement for the sequence of 36 residues that we have presented here. In addition, Tada et al. (69) identified 9 more residues at the NH2 terminus of phospholamban and 7 other residues at the COOH terminus. Therefore, the segment of 36 residues that we have presented here corresponds to positions 10-45 of the Tada sequence (69), and the sites phosphorylated by the cAMP- and the Ca2+/calmodulin-dependent protein kinase correspond, respectively, to the serine at position 16 and the threonine at position 17 with respect to the entire molecule (69). The amino acid compositions determined for intact phospholamban (19) and the major tryptic peptide (Table I) are also in good agreement with the amino acid compositions predicted from the complete sequence of the protein (69). Development of more detailed models of phospholamban structure and function should be facilitated by knowledge of the protein’s sequence and sites of phosphorylation.

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