Volume 309, number 3, 413-416 FEBS 11554 September 1992

Evidence for a phosphorylation-induced conformational change in phospholamban cytoplasmic domain by CD analysis

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Received 20 July 1992

Phospholamban (PLB), an integral membrane protein of cardiac sarcoplasmic reticulum (SR), is described as the regulator of the Ca^{2+}-ATPase pump, via its phosphorylation–dephosphorylation of Ser-16. Recently it has been shown that a direct interaction between the N-terminal hydrophilic domain of PLB and Ca^{2+}-ATPase may be one of the mechanisms of regulation. In order to show that this interaction could be modulated by a phosphorylation-induced conformational change in PLB, we ran CD studies on the synthetic peptide PLB(2-33) in its phosphorylated and non-phosphorylated forms, at various pHs, concentrations and in the absence or presence of trifluoroethanol. The results show a clear difference in structure of the phosphorylated and non-phosphorylated peptide.

Phospholamban; Synthetic peptide; Phosphorylation; Circular dichroism

1. INTRODUCTION

Phospholamban (PLB) is an integral membrane protein of cardiac sacroplasmic reticulum (SR) and is known to modulate the activity of the Ca^{2+}-ATPase via its phosphorylation by cAMP-dependent protein kinase [1,2].

It was recently shown, by reconstitution studies, that PLB in its dephosphorylated form is an inhibitor of the Ca^{2+}-ATPase and that its phosphorylation by cAMP-PK reverses the inhibition [3–5]. Furthermore, studies in the intact heart have shown that β-adrenergic stimulation is associated with PLB phosphorylation [6].

PlB is an amphiphilic polypeptide of 52 amino acids and can be divided into two domains: (i) the cytoplasmic hydrophilic N-terminal domain which contains Ser-16, the phosphorylation site for cAMP-PK, and (ii) the hydrophobic C-terminal region that forms an α-helical transmembrane region.

Although biochemically well characterized, the molecular mechanism by which PLB regulates the Ca^{2+}-ATPase activity has not yet been elucidated. Different studies have led to the hypothesis that Ca^{2+} uptake in the SR is modulated by electrostatic interactions between PLB and Ca^{2+}-ATPase [7,8]. More recently it has been shown that a direct interaction between the hydrophilic domain of PLB and Ca^{2+}-ATPase may be one of the mechanisms of regulation [5,9,10,11]. This interaction could be modulated by a conformational change in the hydrophilic domain of PLB via its phosphorylation, as suggested by Huggins et al. [12] when they showed that the sensitivity of PLB to digestion by proteases was greatly reduced by phosphorylation.

In order to obtain direct evidence that phosphorylation of Ser-16 of PLB could induce a conformational change in its hydrophilic domain, we ran circular dichroism (CD) studies on the synthetic peptide PLB(2-33) in its phosphorylated and non-phosphorylated forms. Our results clearly show that a conformational change can be observed upon phosphorylation of the peptide.

2. MATERIALS AND METHODS

2.1. *Synthesis of PLB(2-33)*

Peptide PLB(2-33) was synthesized manually using solid-phase peptide synthesis. Amino acids were coupled as Boc derivatives (Neosystem Laboratories). BOP was used as the coupling reagent. Synthesis was carried out on 4-methyl benzhydrylamine resin (Applied Biosystems) to produce the C-terminal amidated peptide. Coupling reactions were performed in 25% DMF/DCM (v/v) with a three-fold excess of protected Boc-amino acids and BOP, in the presence of DIEA (8 eq.). Acetylation of the N-terminal residue was performed using a mixture of acetic anhydride and DIEA. Cleavage of the peptide was performed using the 'low-high' HF procedure, i.e. anhydrous HF in the presence of dimethyl sulphur, *p*-cresol and thiocresol.

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Abbreviations: PLB, phospholamban; SR, sarcoplasmic reticulum; CD, circular dichroism; Boc, tert-butyloxycarbonyl; BOP, benzotriazoyl-*N*-oxy-tris (dimethylamino) phosphonium hexafluorophosphate; DIEA, *N*-ethyl-diisopropylamine; DCM, dichloromethane; DMF, dimethylformamide; HF, hydrogen fluoride; TFE, trifluoroethanol; FAB-MS, fast atom bombardment mass spectrometry.

2.2. *Purification of the synthetic peptide*

The crude synthetic peptide was fractionated on a Fractogel TSK HW-40 (F) (Merck) column using 10% acetic acid as eluent. Fractions containing the peptide were pooled, lyophilized and purified by HPLC on a Waters Associates System. Absorbance was monitored at 206 nm. A Waters PrepPak C8 Delat Pak column (300 Å, 25 × 100 mm) was used. Elution was performed at a flow rate of 20 ml/min, using a linear gradient over 20 min from 20% CH_3CN/0.1% TFA to 40% CH_3CN/0.1% TFA.

2.3. *Phosphorylation of PLB(2-33)*

Peptide PLB(2-33) (1.5 mg) was phosphorylated using the catalytic subunit of cAMP-protein kinase (Sigma) (500 U) in a medium containing KCl (0.1 M), $MgCl_2$ (1 mM), EDTA (0.1 mM), Tris (20 mM), ATP (10^{-2} M) in a total volume of 1 ml. After incubation at 30°C for 1 h, the reaction was stopped by boiling. Peptide P-PLB(2-33) was purified by HPLC as described for peptide PLB(2-33).

2.4. *Characterization of PLB(2-33) and P-PLB(2-33)*

Amino acid analysis was performed on both peptides. After hydrolysis with 6 N HCl (110°C, 24 h) amino acids were derivatized with phenylisothiocyanate and analyzed as described by the Waters Picotag manual.

PLB(2-33) and P-PLB(2-33) were characterized by measurement of their molecular mass by fast atom bombardment mass spectrometry (FAB-MS) performed on a VG Analytical ZAB HF double focusing mass spectrometer. (i) PLB (2-33), m/z = 3,831.13 (calc. 3,831.45); (ii) P-PLB(2-33), m/z = 3,911.53 (calc. 3,911.42).

2.5. *Circular dichroism experiments*

The CD spectra of both peptides were obtained using a Cary 60 instrument, calibrated with d(+)10-camphor sulphonic acid.

Peptides were solubilized in phosphate buffer (5 mM), at different pHs (4.0, 7.4, 9.0) and in the absence or in the presence of TFE (10, 15, 20 and 40%). Peptide concentrations were determined by amino acid analysis.

The CD data were recorded in a 1 mm quartz cell at 20°C and were expressed in terms of ellipticity units per mole of peptide residues (Θ in deg·cm^2·dmol^{-1}).

3. RESULTS AND DISCUSSION

For our study we synthesized peptide PLB(2-33) (Fig. 1) acetylated as its N-terminus as native PLB, and amidated at its C-terminus to avoid artefactual electrostatic interactions and also to mimic the hydrophobic character of the C-terminal domain of PLB. The phosphorylated peptide was obtained by enzymatic phosphorylation with cAMP-PK.

We then examined the CD spectra of the phosphorylated peptide (P-PLB(2-33)) and non-phosphorylated peptide (PLB(2-33)) under various conditions of pH, concentration and in the presence and absence of TFE. Peptide concentrations were determined by amino acid analysis.

The CD spectra of the non-phosphorylated peptide (Fig. 2) at a concentration of 5×10^{-5} M in phosphate

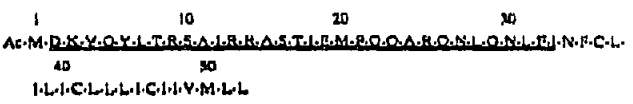

Fig. 1. PLB sequence. The sequence of the synthetic peptide, PLB(2-33), is underlined.

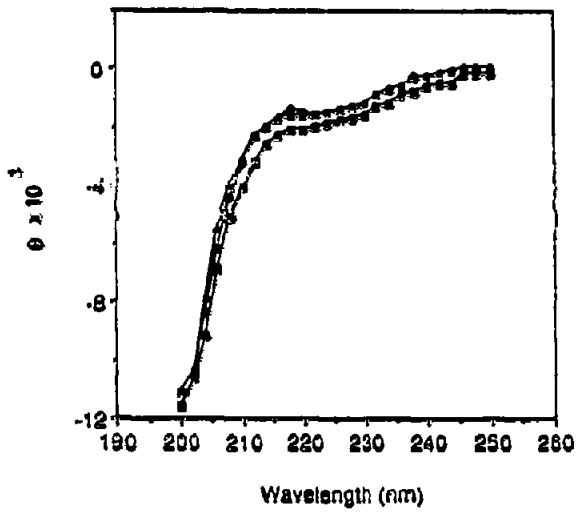

Fig. 2. CD spectra of PLB(2-33) at a concentration of 5×10^{-5} M, in phosphate buffer (5 mM) at pH 4 (▲), pH 7.4 (●) and pH 9.0 (■). The data are expressed in terms of mean residue ellipticity, Θ, in deg·cm^2·dmol^{-1}.

buffer (5 mM) at different pHs (4.0, 7.4 and 9.0) indicated the absence of a well-defined ordered structure. No pH-induced change was observed. The broad negative shoulder at about 220 nm and the low intensity of the negative band at 200 nm suggest that the peptide is not completely random. The same results were observed for the phosphorylated peptide (data not shown).

According to secondary structure prediction, several groups have suggested a mainly helical structure for the hydrophilic cytosolic domain of PLB. The first twenty residues were predicted to form an α-helical amphipathic structure, while the following ten-residue segment seemed to be less structured [13,14].

To confirm the existence of the helical structure and to compare the conformational behaviour of peptides PLB(2-33) and P-PLB(2-33), we have examined their CD spectra in phosphate buffer at pH 7.4 in the presence of TFE, as TFE is known to stabilize pre-existing structures [15,16]. The results are shown in Fig. 3A and B.

The CD spectra analysis of both peptides showed that increasing amounts of TFE induced a higher α-helical content, characterized by the two negative bands at about 208 and 220 nm. In 40% TFE, the spectra became clearly helical and the negative band at 220 nm reached -13.1×10^3 deg·cm^2·dmol^{-1} and -8.9×10^3 deg·cm^2·dmol^{-1} for the non-phosphorylated and the phosphorylated peptide, respectively. Using at least-squares curve fitting procedure for the quantitative analysis of the CD spectra [17] it was estimated that the α-helix content at 40% of TFE was 33% for PLB(2-33) and 25% for P-PLB(2-33). These results are in agreement with the predicted helical structure for the N-terminal domain of PLB and also show that the non-phosphorylated pep-

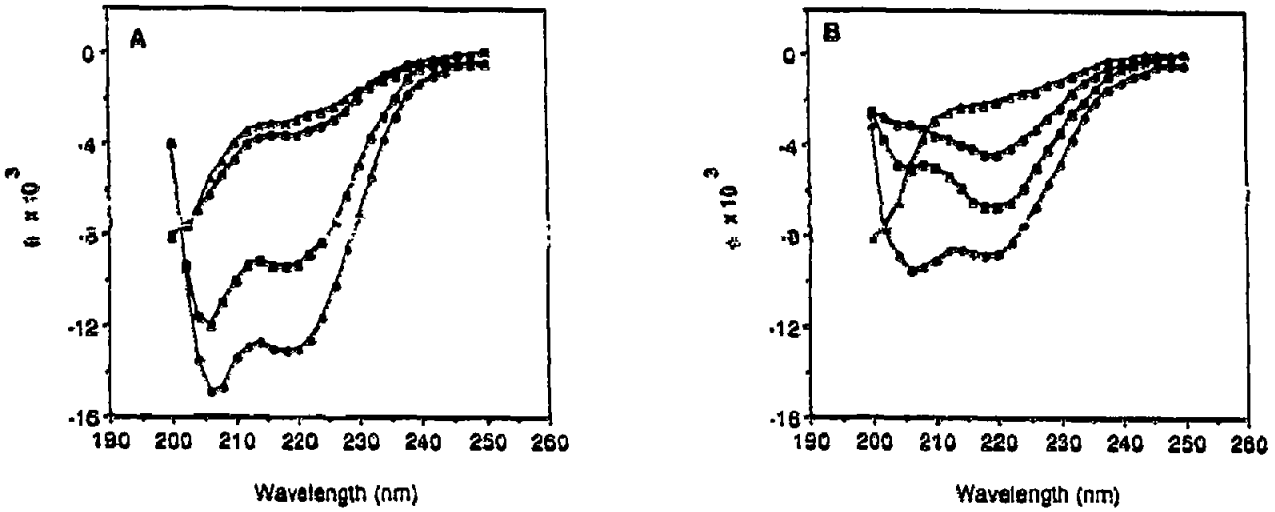

Fig. 3. CD spectra of PLC(2-33) (A) and P-PLB(2-33) (B) at a concentration of 5×10^{-5} M in phosphate buffer, pH 7.4, containing 10% TFE (▲), 15% TFE (●), 20% TFE (■) and 40% TFE (◆).

tide has a more pronounced α-helical character than the phosphorylated one.

Comparing the behaviour of both peptides in 15% TFE, monitored by the CD curve around 200 nm, it was found that PLB(2-33) was still in a random coil conformation, while P-PLB(2-33) had more structure (Fig. 4). The same results were found with both peptides, at a five-fold higher concentration, in phosphate buffer alone (Fig. 5).

From these data, it appears that, in the presence of TFE, peptides PLB(2-33) and P-PLB(2-33) have different conformational behaviours and that the phosphorylated peptide adopts an ordered secondary structure at a lower concentration of TFE than the non-phosphorylated form, but that the latter is able to assume a more α-helical secondary structure.

The negatively charged phosphate group (pK_2 = 5.9) introduced on Ser-16 of PLB(2-33) probably induces a conformational change by electrostatic interactions, modifying the intrapeptide hydrogen bonds. As TFE is supposed to lead to an increase of electrostatic interactions due to the lowering of the dielectric constant [15,16], it is possible that it contributes partly to the observed difference in ellipticity between PLB(2-33) and P-PLB(2-33). Nevertheless, the CD data, obtained at high peptide concentration in the absence of TFE, confirm that phosphorylation of peptide PLB(2-33) does induce a conformational change.

Simmerman et al. [18], in a study of detergent-solubilized PLB, reported that phosphorylation does not significantly alter PLB secondary structure. However, our results show a clear difference in structure of the

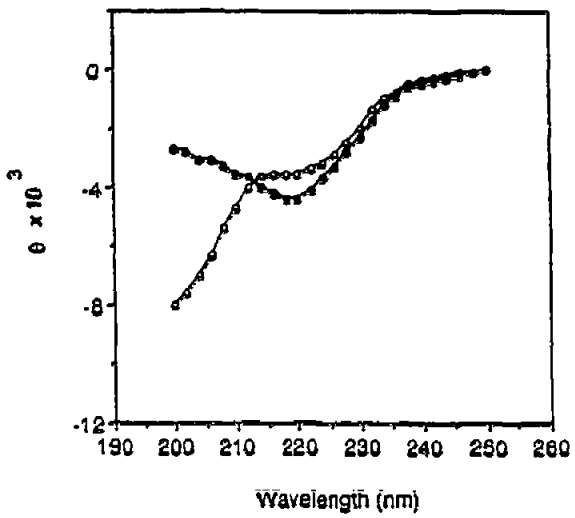

Fig. 4. CD spectra of PLB(2-33) (○) and P-PLB(2-33) (●) at a concentration of 5×10^{-5} M in 5 mM phosphate buffer, pH 7.4, containing 15% TFE.

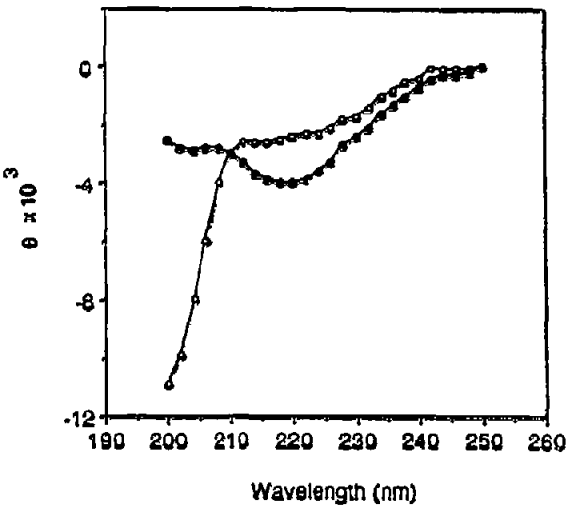

Fig. 5. CD spectra of PLB(2-33) (○) and P-PLB(2-33) (●) at a concentration of 25×10^{-5} M in 5 mM phosphate buffer, pH 7.5.

phosphorylated and non-phosphorylated peptide PLB(2-33). We suggest, therefore, that such a localized conformational change in the cytoplasmic N-terminal domain, not detected in secondary structure studies of the whole protein, may indeed occur. These results would support the hypothesis that PLB regulates, at least partly, the Ca^{2+}-ATPase via its phosphorylation by direct interaction between the cytosolic part of both proteins. Recently, however, Sasaki et al. [10] have shown that PLB suppresses the Ca^{2+}-ATPase at two different sites, the cytoplasmic domain and the transmembrane domain. They suggested that a conformational change of the PLB transmembrane domain was induced by phosphorylation of Ser-16 via a conformational change of the cytosolic domain. ^2H NMR experiments are now under way to compare the effects of PLB and phosphorylated PLB on the lipid bilayer.

Acknowledgements: We are indebted to Dr. B. Luu for scientific support and to Dr. T. Barkas for carefully reading the manuscript. We gratefully acknowledge Prof. Seelig (Biozentrum-Basel) for CD spectropolarimeter facilities. A part of this research was supported by a FEBS post-doctoral fellowship to E. Terzi.

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