Cytoplasmic interactions between phospholamban residues 1–20 and the calcium-activated ATPase of the sarcoplasmic reticulum

Parveen SHARMA*, Valerie B. PATCHELL†, Yuan GAO*, James S. EVANS*¹ and Barry A. LEVINE*^{†2}

*School of Biosciences, University of Birmingham, Birmingham B15 2TT, U.K., and †Department of Physiology, Division of Medical Sciences, School of Medicine, University of Birmingham, Birmingham B15 2TT, U.K.

Phospholamban regulates the activity of the calcium-activated ATPase (CaATPase) of cardiac sarcoplasmic reticulum. Equilibrium fluorescence studies have shown that the N-terminal cytoplasmic region of phospholamban (residues 1–20, domain 1) causes a decrease in the intrinsic tryptophan fluorescence of the CaATPase. The interaction of phospholamban residues 1–20 with the CaATPase also results in spectral changes for the extrinsic chromophore FITC covalently attached to the cytoplasmic region of the calcium pump. The fluorescence changes for both reporter groups correlate with a dissociation constant of $\approx 40 \mu\text{M}$ for the complex between phospholamban residues 1–20 and the CaATPase. Complex formation is notably weaker when phospholamban 1–20 is titrated into the CaATPase in the presence of calcium, with altered conformational effects resulting

from binding. The interaction of domain 1 of phospholamban with the CaATPase is also reduced upon phosphorylation of phospholamban 1–20 at Ser-16. This region of phospholamban 1–20 is shown by isotope-edited NMR study to be involved in interaction with the CaATPase. Binding of the phosphorylated peptide is not abolished, however, indicating that phospholamban 1–20 remains associated with the CaATPase even after phosphorylation. The data provide direct evidence for the interaction between the cytoplasmic regions of phospholamban and the pump, and are discussed in the context of the mechanism for inhibition of cardiac CaATPase activity by phospholamban.

Key words: Ca^{2+} -ATPase, calcium ATPase, fluorescence, NMR, phosphorylation.

INTRODUCTION

Control over intracellular free calcium concentration is crucial to contractile function, with calcium ions being actively pumped away from the contractile protein assembly by a Ca^{2+} -ATPase (CaATPase) located in the sarcoplasmic reticulum (SR). The CaATPase of the SR in both skeletal and cardiac muscle transports 2 mol of calcium into the SR lumen by utilizing 1 mol of ATP as a source of energy during a transport cycle that involves a phosphorylated intermediate of the enzyme [1–3]. The enzyme is a member of the sarcoplasmic/endoplasmic reticulum CaATPase (SERCA) family that comprises a number of isoforms encoded by three separate genes, which are expressed in a tissue-dependent manner [2,3]. The activity of the SR CaATPase of cardiac muscle, isoform 2a, is regulated by phospholamban (PLB), a 52-residue cardiac SR-associated protein. The inhibition of pump activity by PLB is the crucial regulatory mechanism for cardiac contraction [4]. Phosphorylation of PLB at either Ser-16 or Thr-17 modulates this inhibitory ability and results in an increased rate of pumping by the CaATPase.

The mechanism for the regulatory process is unclear. Current consensus stems from a variety of observations by MacLennan et al. [5,6]. The derived model associates the transmembrane region of PLB (residues 31–52) with the ability to lower the Ca^{2+} affinity of the CaATPase through interaction with those transmembrane spans that contribute the cation-coordinating ligands at the two Ca^{2+} -binding sites of the enzyme [2]. The molecular basis by which phosphorylation of the cytoplasmic domain of PLB modulates the functional association between PLB and the CaATPase is not resolved, however. Phosphorylation of residues 16 or 17 of PLB is believed to alter the inhibitory properties of

its transmembrane region through long-range interactions [5]. To elucidate the nature of the conformational relay that must result from phosphorylation of PLB we here focus on the cytoplasmic interactions between PLB and the CaATPase.

Cross-linking studies [7] have indicated the proximity of the N-terminal domain of PLB to a cytoplasmic segment of the CaATPase. Mutations introduced into this sequence of SERCA2a (residues 397–405) compromise the inhibitory ability of PLB [8], whereas mutation of certain residues in the cytoplasmic region of PLB reduce PLB–SERCA2a inhibitory interactions [9]. Cytoplasmic domain fragments of PLB have been found to induce changes in pump activity [10–12] but were not reported to affect the affinity of SERCA2a for Ca^{2+} . To define the molecular mechanism for PLB regulation it is necessary to identify the protein domains involved in the regulatory interactions. Since protein fluorophores are sensitive to conformational change, reporter groups corresponding to different regions of the structure of the CaATPase provide an effective means by which to characterize the interaction of SERCA with the hydrophilic N-terminal region of PLB. We have therefore monitored the response of the intrinsic tryptophan fluorescence of the CaATPase and have also made use of an extrinsic chromophore, FITC, that labels the cytoplasmic region of the enzyme [13]. SERCA isolated from fast skeletal muscle was used in these experiments since it has been shown to be regulated by PLB *in vitro* [14–16] and can function as a substitute for SERCA2a in the heart [17]. We observe that both intrinsic and extrinsic fluorescence properties are altered by interaction with residues 1–20 of PLB (PLB 1–20) shown by NMR study to interact with the CaATPase. The fluorescence data also provide evidence for the transmission of structural change between the

Abbreviations used: CaATPase, Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic reticulum CaATPase; PLB, phospholamban; PLB 1–20, peptide corresponding to residues 1–20 of PLB; PLB 1–20P, PLB 1–20 phosphorylated at Ser-16; F , fluorescence emission intensity; F_0 , initial F ; F_{min} , limiting value of F ; HSQC, heteronuclear single-quantum correlation.

¹ Permanent address: Lawrence University, Appleton, WI 54912-0599, U.S.A.

² To whom correspondence should be addressed, at the School of Biosciences (e-mail b.a.levine@bham.ac.uk).

cytoplasmic and membrane-associated regions of the pump since the interaction of PLB 1-20 with the CaATPase is sensitive to calcium co-ordination by the enzyme.

MATERIALS AND METHODS

Calcium-pump preparations

SERCA 1a from rabbit fast skeletal muscle was purified following the method of Michelangeli and Munkonge [18] with the following modifications. (i) The initial extract was centrifuged for 10 min at 5000 *g* rather than for 30 min at 5500 *g*. (ii) The second centrifugation was carried out for 90 min at 40 000 *g* rather than 105 min at 30 000 *g*. (iii) The centrifugation step claimed to eliminate actin and myosin was replaced by filtration through tightly packed glass wool. (iv) Final centrifugation was for 90 min at 40 000 *g* instead of 120 min at 30 000 *g*. The final product migrated on SDS/PAGE as a single band with an apparent molecular mass of 100 kDa. The concentration of the protein preparation was estimated using the method described by Hardwicke and Green [19]. The CaATPase activity of the preparation was assayed by measurement of the initial rate of release of inorganic phosphate. SERCA preparations were stored at -70°C as a suspension in 1 M KCl/0.25 M sucrose/50 mM phosphate buffer, pH 8.0, prior to the fluorescence experiments that were carried out within 2 weeks of the final purification step. Frozen SR preparations were found to retain ATPase activity for at least 3 months.

FITC labelling of the CaATPase

A sample of the CaATPase at a concentration of 2 mg/ml was incubated with 20 μM FITC in the presence of 0.2 M sucrose, 80 mM KCl, 25 mM Tris/HCl, pH 7.6, 25 mM glycine/NaOH, pH 9.2, 5 mM MgCl_2 and 0.1 mM EGTA. The reaction was allowed to proceed for 10 min at room temperature [20]. To stop the labelling reaction and to remove any free FITC, the sample was transferred to a Sephadex G-25 column pre-incubated with ice-cold stopping solution containing 80 mM KCl, 20 mM Mops, pH 6.8, and 5 mM MgCl_2 . Fractions of 2 ml were collected every 4 min. Final protein concentrations were estimated using the procedure described by Hardwicke and Green [19] monitoring the absorbance at a wavelength of 280 nm. The labelled product was visualized by its migration on SDS/PAGE as a band with an apparent molecular mass of 100 kDa.

Fluorescent labelling of the CaATPase by FITC was virtually abolished in the presence of saturating concentrations of ATP. The site of labelling was checked by digestion of the CaATPase using trypsin, which specifically cleaves the enzyme at two sites; first at Arg-505, producing two fragments, with subsequent cleavage of the N-terminal 55 kDa product at Arg-198 to yield 22 kDa and 33 kDa subfragments [21]. The FITC fluorescent label was not detectable in these subfragments of the initial 55 kDa cleavage product but was seen in the residual protein band corresponding to the initial C-terminal fragment consistent with the reported site of primary labelling by FITC at Lys-515.

The cytoplasmic location of the FITC label was probed by quenching using acrylamide. As judged by the respective Stern-Volmer [22] quenching coefficients, the observed reduction in FITC fluorescence was comparable with that found for the emission intensity due to tryptophans of the CaATPase. The tryptophan residues of the CaATPase do not constitute a homogeneous population [23,24], but inspection of the crystal structure [25] indicates that the sole cytoplasmic tryptophan, Trp-552, displays a surface exposure similar to that of Lys-515.

Purification and phosphorylation of PLB 1-20

The peptide comprising cardiac PLB 1-20 (MDKVYQLTRSAIRRASTIEM) was synthesized using Fmoc (fluorenylmethoxycarbonyl) polyamide chemistry by Alta Bioscience (University of Birmingham, Birmingham, UK) and the same peptide incorporating ^{15}N -labelling of Val-4, Ala-11, Ala-15 and Met-20 was obtained from Syntem (Montpellier, France). The purity and identity of both peptide samples was checked by HPLC on a Vydac C_{18} column using a water/acetonitrile gradient followed by MS and spectral characterization by proton NMR spectroscopy. The peptide was freeze-dried and stored at -20°C prior to use. To achieve phosphorylation of Ser-16, the PLB peptide, PLB 1-20 (5 mg in 1 ml of 50 mM Tris/HCl, pH 7.7, 1 mM cAMP, 10 mM MgCl_2 and 5 mM ATP) was incubated with 200 μg of bovine cAMP-dependent protein kinase (Sigma; 1–2 units of activity/ μg) at 30°C for 60 min, in which time phosphorylation was complete as judged by electrophoresis on acid urea gels. The phosphorylated peptide was purified by HPLC as described previously [26] and the identity of Ser-16 as the phosphorylated residue was confirmed by proton NMR spectroscopy. The peptide was then freeze-dried prior to use.

Fluorescence measurements

All fluorescence emission spectra were obtained using a Perkin-Elmer LS50B fluorescence spectrometer interfaced to a computer and equipped with a magnetic stirrer and a thermostatically controlled cell holder (21°C). The excitation wavelengths for tryptophan and FITC were 280 nm and 496 nm respectively. Fluorescence emission intensity (F) values were corrected for the corresponding solvent emission fluorescence values and the dilution effects ($< 5\%$) resulting from the titration carried out. The fluorescence intensity changes shown are values corresponding to 340 nm and 520 nm for tryptophan and FITC respectively. Acrylamide quenching of the emission fluorescence of FITC-labelled SERCA was monitored upon addition of small aliquots of a stock solution (dilution effects $< 5\%$) over an acrylamide concentration range up to 50 mM. This range was selected following a control study of the minimal concentration required to achieve a 75% reduction in the fluorescence emission of free tryptophan studied at the same concentration as the CaATPase used for the quenching experiments. This control study was carried out since the collisional quenching of the chromophores on the enzyme was expected to be lower than that of free tryptophan given the smaller diffusion coefficient of the CaATPase and would therefore result in a decreased quenching constant. The observed quenching of the FITC and tryptophan emission of the CaATPase was analysed using a Stern-Volmer plot [22] since the extent of quenching showed a linear dependence on the acrylamide concentration in the range used.

For the titrations with the PLB peptides the sample volume of the CaATPase (2 mg of enzyme/ml) was 1.8 ml of a solution containing 80 mM KCl and 5 mM MgCl_2 buffered with 20 mM Mops, pH 6.8. Titration with PLB 1-20 was carried out by addition of small aliquots of a stock solution of each peptide (8 mg/ml in the same buffer) up to a total concentration of 250 μM peptide to ensure saturation of the fluorescence changes observed. The concentration of PLB peptide added could also be calibrated from the appearance of fluorescence emission associated with Tyr-6 of the N-terminal, cytoplasmic sequence of PLB. This emission, with a maximum at 308 nm, overlapped that observed for tryptophan and this precluded the reliable quantification of changes in CaATPase tryptophan emission in the presence of concentrations of PLB peptide greater than 100 μM . For this reason, the binding affinity values derived from the

measurement of changes in tryptophan emission were obtained from at least two separate experiments in each case and were generated by the use of a linear plot, $1/(F_0 - F)$ versus (concentration of added peptide)⁻¹ (where F_0 is the initial F value). The accuracy of the derived K_d values was gauged from the R^2 value (> 0.95) obtained for the linear fit and the requirement that a derivative linear representation of the experimental data, $(F_0 - F_{\min})/(F_0 - F)$ versus (concentration of added peptide)⁻¹ (where $F_{\min}$ is the limiting value of F), obtained by iterative fit extrapolate to an intercept value of 1, as expected at very high concentrations of the peptide. No interference from PLB tyrosine emission occurred when monitoring changes in FITC emission. The FITC emission intensity was found to plateau at high concentrations of PLB 1-20 and the binding-affinity values derived were obtained by curve fitting of the fluorescence data to a 1:1 binding curve in combination with the use of a reciprocal linear plot, $(F_0 - F_{\min})/(F_0 - F)$ versus (concentration of added peptide)⁻¹. The accuracy of the K_d values was again gauged from the R^2 value (> 0.95) obtained for the linear fit and the requirement that iterative fit of the linear representation of the experimental data extrapolate to an intercept value of 1.

NMR spectroscopy experiments

¹H-NMR spectra were obtained using a spectrometer operating at 600 MHz (Varian Unityplus). The freeze-dried ¹⁵N-labelled peptide was dissolved in 90% water/10% ²H₂O buffered with 20 mM deuterated Tris/HCl, pH 7.5, for titration with the SERCA1a preparation. The absence of non-specific association has been demonstrated in earlier studies using a randomized peptide sequence showing also that the NMR spectrum of PLB 1-20 was unaffected by the presence of phospholipids alone [26]. Two-dimensional ¹H-¹⁵N heteronuclear single-quantum correlation (HSQC) spectra of the ¹⁵N-labelled peptide were acquired at a sample temperature of 12 °C using enhanced-sensitivity pulsed-field gradient methods [27]. ¹⁵N-decoupling was carried out using the GARP (globally optimized alternating-phase rectangular pulses [28]) sequence. Solvent suppression for the ¹H-¹⁵N spectra was achieved by the use of gradients. Data-set sizes were 512 increments of 2000 complex points with spectral widths of 12 p.p.m. in the ¹H dimension and 40 p.p.m. in the ¹⁵N dimension. The ¹H carrier was placed on the water signal and the ¹⁵N carrier was placed on 98 p.p.m.

RESULTS

PLB 1-20 interacts with the cytoplasmic domain of the CaATPase

In order to utilize spectral monitors at different locations of the CaATPase when investigating the interaction with the cytoplasmic region of PLB, parallel measurements of the intrinsic tryptophan and the extrinsic FITC fluorescence emission properties of the FITC-labelled CaATPase were made as a function of added PLB 1-20. FITC was selected as the extrinsic chromophore since it has been shown to label Lys-515, which lies in the nucleotide-binding pocket of the CaATPase [13,25]. Labelling by FITC was virtually abolished by saturating concentrations of ATP and, consistent with its cytoplasmic location, FITC emission was observed to be sensitive to collisional quenching by acrylamide.

Initial experiments were carried out with the FITC-labelled CaATPase. Titration with PLB 1-20 resulted in a monotonic decrease of the fluorescence intensity of FITC monitored at 520 nm (Figure 1). This fluorescence change plateaued at an overall emission-intensity reduction of 73%, with no apparent change in the spectral location of the fluorescence maximum.

The titration data (Figure 2) enable the derivation of a dissociation constant (K_d) of 45 μ M for the interaction observed between PLB 1-20 and the CaATPase as monitored by FITC fluorescence (Table 1). Complementary data were obtained by monitoring the CaATPase tryptophan emission at 340 nm during the titration with PLB 1-20. The fluorescence decrease observed also followed a hyperbolic saturation curve that was characterized by a derived K_d of 47 μ M obtained by linear transformation of the binding data (Table 1).

Confirmation that the interaction detected was not compromised by FITC labelling was obtained from titrations carried out using unmodified SERCA preparations. The spectral response of the tryptophan fluorescence closely matched that observed using FITC-labelled CaATPase (Figure 2) with a derived K_d of 40 μ M (Figure 3 and Table 1). Since a peptide corresponding to a randomized sequence of PLB 1-20 did not interact with the CaATPase preparation [26], the overall correspondence of the data provides direct evidence for complex formation between PLB 1-20 and SERCA. The fluorescence changes observed indicate further that the binding of the N-terminal region of PLB induces environmental and conformational changes in different regions of the large cytoplasmic domain of the CaATPase.

Ca²⁺-modulated response of the nucleotide-binding pocket of the CaATPase upon interaction with PLB 1-20

The nucleotide-binding site of the CaATPase appears relatively insensitive to the binding of calcium since, as observed previously [29,30], the addition of calcium to FITC-labelled CaATPase led to only a small decrease ($\approx 5\%$) in FITC fluorescence. To investigate whether calcium binding influences the cytoplasmic interaction with PLB 1-20 detected by the chromophore attached to Lys-515, the FITC-labelled SERCA preparation preincubated with 2 μ M Ca²⁺ was titrated with PLB 1-20. FITC fluorescence decreased monotonically with added PLB 1-20, corresponding to a K_d value of 80 μ M (Figure 2), and plateaued at a larger overall reduction in emission intensity ($\Delta F_{\max}/F_0$ of some 90%) than was observed upon binding of PLB 1-20 in the absence of calcium (Table 1). These data indicate that the binding of PLB 1-20 still occurs in the presence of calcium, but suggest that the interaction causes a different conformational response in the nucleotide-binding pocket when the CaATPase is in the calcium-bound state. The concurrent observation that calcium binding led to a reduction in the affinity of PLB 1-20 for the CaATPase was reflected in the PLB peptide-dependent decrease of both FITC and tryptophan fluorescence emission observed for the calcium-bound state of the CaATPase (Figure 2 and Table 1). This observation suggests that the determinants and/or kinetics of the interaction of PLB 1-20 with the cytoplasmic domain of SERCA have been altered by the transition to the calcium-bound state of the enzyme.

These observations provide direct evidence for the calcium dependence of the interaction between the cytoplasmic regions of PLB and the CaATPase and, further, indicate that calcium binding modulates but does not abolish the physical association between SERCA and PLB 1-20. Since phosphorylation at Ser-16 abolishes the ability of PLB to inhibit CaATPase activity, it was of interest to determine the impact of phosphorylation on the association between PLB 1-20 and the cytoplasmic region of SERCA.

Cytoplasmic interaction of PLB 1-20 with the CaATPase is retained upon PLB phosphorylation

Titration with PLB 1-20 phosphorylated at Ser-16 (PLB 1-20P) was carried out by monitoring the intrinsic tryptophan

Figure 1 FITC fluorescence changes upon addition of PLB 1-20 into FITC-labelled CaATPase

The fluorescence spectrum, with excitation at 496 nm, was recorded at 21 °C for a total sample volume of 1.8 ml. The emission spectra obtained at different concentrations of the peptide, PLB 1-20, are shown and labelled 1–5 corresponding to: (1) SERCA in absence of peptide and (2–5) SERCA in the presence of (2) 37 μM PLB 1-20, (3) 112.8 μM PLB 1-20, (4) 150.4 μM PLB 1-20 and (5) 233.12 μM PLB 1-20.

Figure 2 Plot of the intrinsic tryptophan and extrinsic FITC fluorescence changes observed upon titration of PLB 1-20 into the different SERCA preparations

The normalized fluorescence changes for the two fluorophores are expressed as $(F_0 - F)/F_0$. Δ , Tryptophan fluorescence (338 nm) of unmodified SERCA in the absence of calcium; $\blacklozenge$, FITC fluorescence of SERCA (518 nm) in the absence of calcium; $\blacksquare$, FITC fluorescence of SERCA in the presence of calcium; $\square$, tryptophan fluorescence of unmodified SERCA in the presence of calcium. The solid curves drawn through the experimental points are the fits obtained to a 1:1 binding equation (in the absence of calcium, $K_d = 45 \mu\text{M}$, Δ and $\blacklozenge$; in the presence of calcium, $K_d = 85 \mu\text{M}$, $\square$ and $\blacksquare$). Shown as dotted lines are the predicted binding curves at two different K_d values that illustrate the extremities of the range of values compatible with the experimental titration curves obtained in the absence and presence of calcium. These predicted curves indicate that the experimental datasets obtained in the absence and presence of calcium are characteristic of distinguishably different K_d values, as reported with both fluorophores.

fluorescence of unmodified SERCA. The addition of PLB 1-20P led to a decrease of tryptophan emission, indicating that the cytoplasmic interaction with the CaATPase had not been

abolished by phosphorylation of Ser-16. The fluorescence change observed was greater than that found upon binding of PLB 1-20 and was characterized by a larger K_d (60 μM) than was observed

Table 1 Fluorescence emission changes upon interaction of PLB 1-20 with the CaATPase

$\Delta F_{\max}/F_0 = (F_0 - F_{\min})/F_0$, which is the magnitude of the change in fluorescence intensity at saturating concentrations of PLB 1-20, expressed as a fraction of F_0 , derived by linear fitting of the binding data (Figure 3). The K_d values quoted were obtained from at least two separate experiments in each case and were derived by curve fitting of the fluorescence data to a 1:1 binding curve in combination with the use of a reciprocal linear plot, $\Delta F_{\max}/(F_0 - F)$ versus (concentration of added peptide)⁻¹, as shown in Figures 2 and 3.

Fluorescence emission	Sample	Ca ²⁺	$\Delta F_{\max}/F_0$	K_d (μ M)
FITC	FITC-labelled CaATPase in the presence of PLB 1-20	—	73 ± 5%	44 ± 10
Tryptophan	FITC-labelled CaATPase in the presence of PLB 1-20	—	37 ± 7%	47 ± 10
Tryptophan	CaATPase in the presence of PLB 1-20	—	32 ± 8%	40 ± 10
FITC	FITC-labelled CaATPase in the presence of PLB 1-20	2 μ M	90 ± 5%	83 ± 10
Tryptophan	CaATPase in the presence of PLB 1-20	2 μ M	34 ± 9%	80 ± 10
Tryptophan	CaATPase in the presence of phosphorylated PLB 1-20	—	76 ± 10%	60 ± 10

Figure 3 Linear representation of the binding data obtained upon titration of SERCA with PLB 1-20

The slope of this reciprocal plot yields the K_d value for each experimental condition and was obtained by iterative fit of the data ($R^2 > 0.95$), shown as solid lines that are labelled with the corresponding K_d value derived. Extrapolation of the linear plots of the experimental data shows an intercept value of 1, as would be expected at very high concentrations of the peptide. The dissociation constants derived in each case are given in Table 1. Δ , Tryptophan fluorescence of unmodified SERCA in the absence of calcium; $\blacklozenge$, FITC fluorescence of SERCA in the absence of calcium; $\blacksquare$, FITC fluorescence of SERCA in the presence of calcium; $\square$, tryptophan fluorescence of unmodified SERCA in the presence of calcium; $\bullet$, tryptophan fluorescence of unmodified SERCA titrated with PLB 1-20P.

for the binding of unphosphorylated PLB 1-20 (Figure 3 and Table 1). These data indicate that phosphorylation of Ser-16 results in reduced interaction affinity and hence altered kinetics of complex formation with the CaATPase.

The observations above suggested that the mechanism for the reversible regulatory influence of PLB phosphorylation may primarily involve an altered interaction of its cytoplasmic N-terminal region with a contact site on SERCA crucial to the formation of the phosphoenzyme, which follows calcium activation of the CaATPase. We therefore studied by NMR chemical-shift mapping whether the C-terminal segment of PLB 1-20 encompassing the PLB phosphorylation-site sequence participated in the contacts made with the calcium pump.

The phosphorylation site on the cytoplasmic domain of PLB is involved directly in interaction with the CaATPase

The introduction of a restricted number of ¹⁵N-labelled residues in the sequence of PLB 1-20 during synthesis provided sequence-specific reporter groups that would not alter the binding properties of the peptide. Use of this peptide allows resolution of the signals of the labelled residues by isotope-edited experiments and the consequent application of the chemical-shift mapping approach, where changes in backbone NH chemical shifts in PLB 1-20 are monitored via two-dimensional ¹H-¹⁵N HSQC spectra during titration with the CaATPase. The approach is particularly useful for direct observation of resonances that undergo binding-dependent chemical-shift perturbations. This strategy has been

Figure 4 Sections of the two-dimensional ¹H-¹⁵N HSQC spectra of the ¹⁵N-labelled (Ala 11, Ala 15) PLB 1-20 in the absence (A) and presence (B) of the CaATPase

Spectrum (B) was acquired at a molar ratio 5:1 peptide/CaATPase. Under these conditions some 20% of the PLB 1-20 is bound (see Table 1).

used previously in studies of protein–protein contacts (e.g. [31]) and in the study of the interaction between peptides based on the sequence of PLB and SERCA [26]. By the use of isotope-edited ^1H - ^{15}N HSQC NMR it proved possible to monitor any spectral changes for the backbone amide protons of Ala-11 and Ala-15 individually upon complex formation with the intact CaATPase. The spectra (Figure 4) show the chemical-shift change experienced by Ala-15 while the amide resonance of Ala-11 is found to show a smaller shift perturbation upon interaction with SERCA at the relative molar ratios used. The shift alterations in both the ^1H and ^{15}N frequencies indicate that Ala-15 must be involved in contacts with the surface of the CaATPase. Thus phosphorylation of Ser-16 of PLB is likely to hinder directly the association with SERCA of the C-terminal region of PLB 1-20.

DISCUSSION

The inhibition of SERCA2a activity by PLB is indicated by a variety of mutagenic and biochemical studies to be effected through both cytoplasmic and transmembrane regions of the CaATPase [6,32]. While the transmembrane region of PLB has been shown to reduce the apparent Ca^{2+} affinity of the CaATPase and is hence an essential component in the mechanism of inhibition of Ca^{2+} transport [32,33], the inferred involvement of the cytoplasmic region of PLB in its regulatory activity has not been clearly defined. Mutation of hydrophobic and charged residues in the region between residues 2 and 18 of PLB has been found to prevent inhibition of the CaATPase by PLB when co-expressed in COS cells [9]. These observations emphasise the part played by cytoplasmic contacts in the regulation of pump activity by PLB. The spectroscopic investigation here into the mechanistic

contribution of the N-terminal region of PLB demonstrates the interaction of PLB 1-20 with SERCA and identifies the hydrophilic region close to the phosphorylation site as involved in complex formation between PLB 1-20 and the CaATPase.

The sensitivity of both FITC and intrinsic tryptophan fluorescence to interaction with PLB 1-20 indicates that binding elicits conformational effects in different regions of the cytoplasmic domain of the CaATPase. Interestingly, the comparable affinity of interaction obtained by titration of SERCA and FITC-labelled SERCA indicates that modification of Lys-515 does not detectably alter the ability of PLB 1-20 to bind to the CaATPase. The fluorescence data demonstrate further directly that the association of SERCA with PLB 1-20 is modified, but not disrupted, by phosphorylation of Ser-16 of PLB. The possibility that PLB 1-20P maintains contact with the CaATPase has been inferred previously from studies of the intact proteins [34] and protein fragments [26]. These observations together suggest that it is the cytoplasmic residues of PLB proximal to its phosphorylation site that provide the key phosphorylation-sensitive contacts with the CaATPase.

A possible phosphorylation-sensitive cytoplasmic PLB contact region on the CaATPase is believed to encompass residues 397–405 [7,26]. This segment of the enzyme, located on a solvent-exposed loop on the nucleotide-binding domain that also contains Lys-515 and Trp-552 (Figure 5), appears to be crucial for enabling inhibitory control by PLB [8]. The binding of PLB 1-20 is seen from the FITC fluorescence data to result in a readily detectable change in the environment of Lys-515 in the nucleotide-binding pocket, suggesting relay through the molecular framework of the cytoplasmic region of the CaATPase linking the nucleotide-binding site to the interaction site(s) on the enzyme involved in the binding of PLB 1-20. This linkage is

Figure 5 Schematic representation of the cytoplasmic region of SERCA based on the crystal structure of the calcium-bound form of the enzyme ([25], PDB identifier 1eul)

(A) Residues 330–737 showing the relative locations of Lys-400 and Lys-515, the site of FITC labelling. Lys-515 lies ≈ 0.4 nm from residue 421 at the end of the helical region, which links the nucleotide-binding pocket to the loop residues 397–406 that are recognized to be influential in mediating the interaction between the CaATPase and PLB [8]. (B) The nucleotide-binding domain, residues 360–604, is shown with residues 394–422 depicted as solid ribbons. The side chains of Lys-397, Ile-402, Lys-515 and Trp-552 are highlighted to indicate their relative dispositions.

consistent with the suggested functional relationship between PLB binding and the nucleotide site of the CaATPase [35]. A structural basis for this relay can be rationalized from the observed topology of the nucleotide-binding domain of SERCA comprised of residues 360–600 [25]. The PLB contact residues 397–405 are located immediately preceding a helical turn-loop-helix motif. The residues that participate in the helix (SERCA residues 407–421) act as a cylindrical plug that is sandwiched in the middle of the domain with one end of the helix adjacent to Lys-515 (Figure 5). In the calcium-bound conformation of the CaATPase the side chain of Lys-515 is ≈ 0.4 nm from Asn-421. The binding of PLB 1-20 at the exposed loop could thereby be relayed directly to the surroundings of the FITC label.

Further, within ≈ 1.5 nm from the 397–405 loop lies Trp-552. Inspection of the crystal structure [25] indicates that the sole cytoplasmic tryptophan, Trp-552, displays a surface exposure similar to that of the FITC-labelled residue, Lys-515. The tryptophan fluorescence change upon interaction of SERCA with both PLB 1-20 and PLB 1-20P is found to be of the same magnitude. A direct conformational linkage between residues 397–405 and Trp-552 that could affect this fluorescence response is not readily apparent from the structure of the calcium-bound CaATPase, however (Figure 5B). This raises the interesting possibility that the N- and C-terminal regions of PLB 1-20 span distinct binding sites on the cytoplasmic region of the CaATPase and that the alteration in intrinsic fluorescence originates from an environmental/conformational transition induced by the contact with SERCA of the N-terminal, largely hydrophobic, region of PLB. This possibility, in keeping with the impact of mutations in the N-terminal of PLB [9], is also consistent with the suggestion that PLB 1-20 may straddle separate segments of the cytoplasmic domain of SERCA [26] and the observation that deacetylation of the N-terminal residue affects the ability of PLB residues 1-25 to inhibit the maximal activity of SERCA1 [12]. The fluorescence changes here observed upon interaction of PLB 1-20 and PLB 1-20P with the CaATPase therefore imply that phosphorylation of PLB relieves dual-sited cytoplasmic contacts between PLB and the calcium pump.

On the basis of the fluorescence data it is not feasible to exclude the possibility that the extreme N-terminal region of PLB binds to a different location on the CaATPase upon phosphorylation of Ser-16. The maintained sensitivity of tryptophan fluorescence to binding of phosphorylated PLB 1-20 and the probable primary contribution to this fluorescence change from Trp-552 suggest, however, that the N-terminal region of PLB retains attachment either in the vicinity of Trp-552 on the nucleotide-binding domain of SERCA or at a site that is structurally linked to the environment of Trp-552.

As well as being modulated by phosphorylation, the interaction of SERCA with PLB 1-20 is shown here to be sensitive to calcium binding to the CaATPase, as reported by the environmental change experienced by the FITC label. A much greater fluorescence emission decrease of the chromophore occurs when PLB 1-20 interacts with the calcium-bound state of SERCA, indicating that the binding of PLB 1-20 relays a different conformational response to the nucleotide-binding pocket in the presence of calcium. It has been proposed that calcium binding at transmembrane locations of SERCA is relayed to result in weakened interactions between the constituent subdomains of the cytoplasmic region of the CaATPase [25,35,36]. This inter-subdomain rearrangement could account for the diminished SERCA affinity observed for PLB 1-20. Further, the indications that PLB 1-20 forms extended contacts with the CaATPase suggest the possibility that the cytoplasmic region of PLB functions to constrain the relative orientation of the nucleotide-

binding and phosphorylation subdomain elements of SERCA required for phosphoenzyme formation upon nucleotide binding. Phosphorylation of PLB would then act to relieve the constraints and hence facilitate the subdomain organization that is needed for efficient phosphoenzyme formation upon calcium binding by the CaATPase.

This work was supported by the British Heart Foundation and the Wellcome Trust. P.S. is in receipt of a studentship from the Biotechnology and Biological Sciences Research Council and J.S.E. thanks Lawrence University (Appleton, WI, U.S.A.) for granting a sabbatical leave and the School of Biosciences, University of Birmingham, Birmingham, U.K., for enabling participation in this work.

REFERENCES

- Inesi, G. (1985) Mechanism of calcium transport. *Annu. Rev. Physiol.* **47**, 573–601
- MacLennan, D. H., Rice, W. J. and Green, N. M. (1997) The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca^{2+} -ATPases. *J. Biol. Chem.* **272**, 28815–28818
- Møller, J. V., Juul, B. and le Maire, M. (1996) Structural organisation, ion transport, and energy transduction of P-type ATPases. *Biochim. Biophys. Acta* **1286**, 1–51
- Simmerman, H. K. B. and Jones, L. R. (1998) Phospholamban: protein structure, mechanism of action, and role in cardiac function. *Physiol. Rev.* **78**, 921–947
- Kimura, Y., Kurzydowski, K., Tada, M. and MacLennan, D. H. (1996) Phospholamban regulates the Ca^{2+} -ATPase through intramembrane interactions. *J. Biol. Chem.* **271**, 21726–21731
- Kimura, Y., Kurzydowski, K., Tada, M. and MacLennan, D. H. (1997) Phospholamban inhibitory function is activated by depolymerization. *J. Biol. Chem.* **272**, 15061–15064
- James, P., Inui, M., Chiesi, M. and Carafoli, E. (1989) Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. *Nature (London)* **342**, 90–92
- Toyofuku, T., Kurzydowski, K., Tada, M. and MacLennan, D. H. (1994) Amino acids Lys-Asp-Asp-Lys-Pro-Val⁴⁰² in the Ca^{2+} -ATPase of cardiac sarcoplasmic reticulum are critical for functional association with phospholamban. *J. Biol. Chem.* **269**, 22929–22932
- Toyofuku, T., Kurzydowski, K., Tada, M. and MacLennan, D. H. (1994) Amino acids Glu² to Ile¹⁸ in the cytoplasmic domain of phospholamban are essential for functional association with Ca^{2+} -ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **269**, 3088–3094
- Sasaki, T., Inui, M., Kimura, Y., Kuzuya, T. and Tada, M. (1992) Molecular mechanism of regulation of the Ca^{2+} pump ATPase by phospholamban in cardiac sarcoplasmic reticulum. *J. Biol. Chem.* **267**, 1674–1679
- Hughes, G., East, J. M. and Lee, A. G. (1994) The hydrophilic domain of phospholamban inhibits the Ca^{2+} transport step of the Ca^{2+} -ATPase. *Biochem. J.* **303**, 511–516
- Starling, A. P., Sharma, R. P., East, G. M. and Lee, A. G. (1996) The effect of N-terminal acetylation on Ca^{2+} -ATPase inhibition by phospholamban. *Biochem. Biophys. Res. Commun.* **226**, 352–355
- Mitchinson, C., Wilderspin, A. F., Trinnaman, B. J. and Green, N. M. (1982) Identification of a labelled peptide after stoichiometric reaction of fluorescein isothiocyanate with the Ca^{2+} -dependent adenosine triphosphate of sarcoplasmic reticulum. *FEBS Lett.* **146**, 87–92
- Szymanski, G., Kim, H. W., Cuppoletti, J. and Kranias, E. G. (1990) Regulation of the skeletal sarcoplasmic-reticulum Ca^{2+} pump by phospholamban in reconstituted phospholipid-vesicles. *Membr. Biochem.* **9**, 191–202
- Voherr, T., Chiesi, M., Schwaller, R. and Carafoli, E. (1992) Regulation of the calcium ion pump of sarcoplasmic reticulum: reversible inhibition by phospholamban and by the calmodulin binding domain of the plasma membrane calcium ion pump. *Biochemistry* **31**, 371–376
- Hughes, G., Starling, A. P., Sharma, R. P., East, G. M. and Lee, A. G. (1996) An investigation of the mechanism of inhibition of the Ca^{2+} -ATPase by phospholamban. *Biochem. J.* **318**, 973–979
- Ji, Y., Loukianov, E., Loukianova, T., Jones, L. R. and Periasamy, M. (1999) SERCA1a can functionally substitute for SERCA2a in the heart. *Am. J. Physiol.* **276**, H89–H97
- Michelangelo, F. and Munkonge, F. M. (1991) Methods of reconstitution of the purified sarcoplasmic reticulum (Ca^{2+} - Mg^{2+})-ATPase using bile salt detergents to form membranes of defined lipid to protein ratios or sealed vesicles. *Anal. Biochem.* **194**, 231–236
- Hardwicke, P. M. D. and Green, N. M. (1974) The effect of delipidation on the adenosine triphosphate of sarcoplasmic reticulum. *Eur. J. Biochem.* **42**, 183–193

- 20 Inesi, G. and Teruel, J. A. (1988) Roles of phosphorylation and nucleotide binding domains in calcium transport by sarcoplasmic reticulum adenosine triphosphate. *Biochemistry* **27**, 5885–5890
- 21 Pick, U. (1981) Interaction of fluorescein isothiocyanate with nucleotide-binding sites of the Ca-ATPase from sarcoplasmic reticulum. *Eur. J. Biochem.* **121**, 187–195
- 22 Lehrer, S. S. (1971) Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **10**, 3254–3263
- 23 Gomezfernandez, J. C., Baena, M. D., Teruel, J. A., Villalain, J. and Vidal, C. J. (1985) A fluorescence quenching study of tryptophan residues of calcium ATPase from sarcoplasmic-reticulum. *Biochem. Soc. Trans.* **13**, 244–245
- 24 DeForesta, B., Champeil, P. and LeMaire, M. (1990) Different classes of tryptophan residues involved in the conformational changes characteristic of the sarcoplasmic reticulum Ca^{2+} -ATPase cycle. *Eur. J. Biochem.* **194**, 383–391
- 25 Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature (London)* **405**, 647–655
- 26 Levine, B. A., Patchell, V. B., Sharma, P., Gao, Y., Bigelow, D. J., Yao, Q., Goh, S., Colyer, J., Drago, G. A. and Perry, S. V. (1999) Sites on the cytoplasmic region of phospholamban involved in interaction with the calcium-activated ATPase of the sarcoplasmic reticulum. *Eur. J. Biochem.* **264**, 905–913
- 27 Bax, A., Ikura, M., Kay, L. E., Torchia, D. A. and Tschudin, R. (1990) Comparison of different modes of two-dimensional reverse-correlation NMR for the study of proteins. *J. Magn. Reson.* **86**, 304–318
- 28 Shaka, A. J., Barker, P. B. and Freeman, R. (1985) Computer-optimized decoupling scheme for wideband applications and low-level operation. *J. Magn. Reson.* **64**, 547–552
- 29 Pick, U. (1981) Dynamic interconversions of phosphorylated and non phosphorylated intermediates of the Ca-ATPase from sarcoplasmic reticulum followed in a fluorescein-labelled enzyme. *FEBS Lett.* **123**, 131–136
- 30 Froud, R. J. and Lee, A. G. (1986) Conformational transitions in the Ca^{2+} - Mg^{2+} -activated ATPase and the binding of Ca^{2+} ions. *Biochem. J.* **237**, 197–206
- 31 Buchko, G. W., Daughdrill, G. W., de Lorimier, R., Rao, S., Isern, N. G., Lingbeck, J. M., Taylor, J. S., Wold, M. S., Gochin, M., Spicer, L. D. et al. (1999) Interactions of human nucleotide excision repair protein XPA with DNA and RPA70 Delta C327: chemical shift mapping and N-15 NMR relaxation studies. *Biochemistry* **38**, 15116–15128
- 32 Michio, A., McKenna, E., Kurzydowski, K., Tada, M. and MacLennan, D. H. (2000) Physical interactions between phospholamban and sarco(endo)plasmic reticulum Ca^{2+} -ATPases are dissociated by elevated Ca^{2+} , but not by phospholamban phosphorylation, vanadate, or thapsigargin, and are enhanced by ATP. *J. Biol. Chem.* **275**, 15034–15038
- 33 Toyofuku, T., Kurzydowski, K., Tada, M. and MacLennan, D. H. (1993) Identification of regions in the Ca^{2+} -ATPase of sarcoplasmic reticulum that affect functional association with phospholamban. *J. Biol. Chem.* **268**, 2809–2815
- 34 Coll, K. E., Johnson, Jr, R. G. and McKenna, E. (1999) Relationship between phospholamban and nucleotide activation of cardiac sarcoplasmic reticulum Ca^{2+} adenosine triphosphate. *Biochemistry* **38**, 2444–2451
- 35 Ogawa, H., Stokes, D. L., Sasabe, H. and Toyoshima, C. (1998) Structure of the Ca^{2+} pump of sarcoplasmic reticulum: a view along the lipid bilayer at 9-Å resolution. *Biophys. J.* **75**, 41–52
- 36 Negash, S., Huang, S. and Squier, T. C. (1999) Rearrangement of domain elements of the Ca-ATPase in cardiac sarcoplasmic reticulum membranes upon phospholamban phosphorylation. *Biochemistry* **38**, 8150–8158

Received 16 October 2000/14 December 2000; accepted 20 February 2001