The hydrophilic domain of phospholamban inhibits the Ca^{2+} transport step of the Ca^{2+} -ATPase

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The peptide MEKVQYLTRSAIRRASTIEMPQQAR-Cys corresponding to residues 1–25 of phospholamban was found to inhibit the ATPase activity of skeletal muscle Ca^{2+} -ATPase, but to have no effect on the Ca^{2+} -dependence of its activity. The peptide was found to decrease the rate of the Ca^{2+} transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$) by a factor of 2.4. The rate of this same step was decreased by poly(L-Arg) by a factor of 2.2. The peptide shifted

the E2–E1 equilibrium of the ATPase towards E1 by a factor of 4 due to stronger binding to the E1 than to the E2 conformation of the ATPase; dissociation constants for binding to E1 and E2 were estimated as 3 and 10 μM respectively. The peptide had no effect on the level of phosphorylation by P_i in the absence of Ca^{2+} or on the rate of phosphorylation by ATP in the presence of Ca^{2+} .

INTRODUCTION

Phospholamban is a small protein of 52 amino acids which inhibits the Ca^{2+} -ATPase of cardiac sarcoplasmic reticulum (Tada, 1992). Although skeletal muscle sarcoplasmic reticulum contains no phospholamban, the skeletal muscle Ca^{2+} -ATPase is inhibited by phospholamban when they are co-reconstituted (Szymanska et al., 1990; Tada, 1992; Vorherr et al., 1992). Phospholamban contains a hydrophilic N-terminal domain (Met-1 to Asn-30) and a hydrophobic C-terminal domain (Leu-31 to Leu-52) which is likely to be a transmembrane α -helix. The hydrophilic domain contains two regions, an N-terminal region from Met-1 to Pro-21 predicted to be α -helical and a less structured region (Gln-22 to Asn-30) linking the N-terminal region to the transmembrane α -helix (Tada, 1992). Binding of the hydrophilic domain of phospholamban to cardiac Ca^{2+} -ATPase has been shown to reduce the maximum rate of hydrolysis of ATP with no effect on the affinity of the ATPase for Ca^{2+} , whereas binding of the hydrophobic domain decreases the affinity of the ATPase for Ca^{2+} with no effect on the maximum rate of hydrolysis (Sasaki et al., 1992).

The kinetics of the ATPase are usually discussed in terms of the E1–E2 model shown in Scheme 1. It is proposed that in the

Scheme 1

E1 conformation the ATPase possesses two outward-facing Ca^{2+} -binding sites of high affinity. After binding of Ca^{2+} and ATP the enzyme is phosphorylated to give E1PCa_2 which can undergo a conformation change to E2PCa_2 from which Ca^{2+} is lost to the lumen of the sarcoplasmic reticulum. Dephosphoryl-

ation of E2P then allows recycling to E1 (de Meis, 1981). We have shown that a variety of polycationic compounds, including spermine, inhibit the skeletal muscle Ca^{2+} -ATPase by reducing the rate of the $\text{E1PCa}_2 \rightarrow \text{E2P}$ step (Hughes et al., 1994). Spermine also shifts the E2–E1 equilibrium towards E1 and inhibits phosphorylation of the ATPase by P_i by competition with the Mg^{2+} which is essential for the reaction. Here we show that binding of the hydrophilic domain of phospholamban to the ATPase also decreases the rate of the $\text{E1PCa}_2 \rightarrow \text{E2P}$ step and shifts the E2–E1 equilibrium towards E1 but has no effect on phosphorylation of the ATPase by P_i .

MATERIALS AND METHODS

Sarcoplasmic reticulum from rabbit skeletal muscle and purified Ca^{2+} -ATPase were prepared as described in East and Lee (1982). ATPase activities were measured using a coupled enzyme assay as described (East and Lee, 1982). Ca^{2+} concentrations were calculated using the binding constants for EGTA, Ca^{2+} , Mg^{2+} and H^+ given by Godt (1974). Poly(L-Arg) (molecular-mass range 5000–15000 Da) was obtained from Sigma.

The peptides MEKVQYLTRSAIRRASTIEMPQQAR-Cys [PLN(1–25)] and RRASTIEMPQQAR-Cys [PLN(13–25)] corresponding to residues 1 to 25 and 13 to 25 respectively of rabbit heart phospholamban were synthesized with a C-terminal cysteine by the method of Merrifield (1986) and checked for purity by h.p.l.c. on a reversed-phase C8 column.

The time dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined using a Biologic rapid filtration device at room temperature (typically 20 °C). Sarcoplasmic reticulum vesicles in buffer (0.2 mg of protein/ml; 150 mM Mes/Tris, pH 6.0, 20 mM MgSO_4 , 100 μM $^{45}\text{CaCl}_2$, 500 μM [^3H]sucrose) containing 4% (w/w protein) A23187, were loaded on a Millipore HAWP (0.45 μm pore size) filter and then perfused for the chosen times with the same buffer but containing 2 mM ATP and 100 μM $^{40}\text{CaCl}_2$. The filter was dried overnight in air and then counted in Optiphase HiSafe 3. The amount of [^3H]sucrose trapped on the filter was used to calculate the wetting volume of the filter, and the amount of $^{45}\text{Ca}^{2+}$ calculated to be in

Figure 1 Effect of peptide PLN(1-25) on the ATPase activity of uncoupled sarcoplasmic reticulum vesicles

The figure shows ATPase activity (i.u./mg of protein) as a function of peptide concentration at 10 μM Ca^{2+} . The insert shows activity as a function of free Ca^{2+} concentration at 25 μM peptide. Activities were measured at pH 7.2 and 25 $^{\circ}\text{C}$ in the presence of 2.1 mM ATP, 100 mM KCl, 0.036 μM ATPase and 4.0 $\mu\text{g}/\text{ml}$ A23187: ATPase alone ($\bullet$) and in the presence of peptide ($\circ$).

this volume was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound to the ATPase. A correction was also applied for $^{45}\text{Ca}^{2+}$ non-specifically bound to the filter, typically amounting to 1 nmol of Ca^{2+} bound/mg of protein.

Steady-state measurements of phosphorylation by [^{32}P]P_i were carried out in 150 mM Mes/Tris, pH 6.3, containing 5 mM EGTA, 1 mM P_i and 5 mM Mg^{2+} , at 25 $^{\circ}\text{C}$ and the required concentration of peptide, at a protein concentration of 0.1 mg/ml. Samples were incubated for 10 s and then quenched with 12% (v/v) trichloroacetic acid/0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed three times with quenching solution and then counted. The time dependence of phosphorylation of the ATPase by [γ - ^{32}P]ATP at 25 $^{\circ}\text{C}$ was determined using a Biologic QFM-5 system as described in Starling et al. (1994). The ATPase (0.2 mg/ml) incubated in buffer (pH 7.2, 40 mM Hepes/Tris, 5 mM Mg^{2+} , 100 mM KCl, 100 μM Ca^{2+}) was mixed in a 1:1 ratio with the same buffer containing 50 μM [γ - ^{32}P]ATP to give a final concentration of ATP of 25 μM , followed by quenching with 12% (v/v) trichloroacetic acid/0.2 M phosphoric acid.

The ATPase was labelled with nitrobenzo-2,1,3-oxadiazole (NBD) as described previously (Wictome et al., 1992). Measurements of NBD fluorescence were performed at 25 $^{\circ}\text{C}$ using an SLM Amino 8000C fluorimeter with excitation and emission wavelengths of 430 and 520 nm respectively.

Structures were modelled using QUANTA.

RESULTS

The peptide PLN(1-25) which corresponds to the first 25 residues of phospholamban, when added to uncoupled sarcoplasmic reticulum vesicles prepared from skeletal muscle, inhibits ATPase activity (Figure 1). As shown in Figure 1, the peptide had no significant effect on the Ca^{2+} -dependence of activity, either at the sites of high affinity resulting in stimulation of activity or at the sites of low affinity resulting in inhibition of activity. Poly(L-Arg)

Figure 2 Effect of peptide on the ATP-induced rate of release of $^{45}\text{Ca}^{2+}$ from the ATPase

Sarcoplasmic reticulum vesicles (0.2 mg/ml; 1.2 μM ATPase) were first equilibrated in pH 6.0 buffer (150 mM Mes/Tris, 20 mM Mg^{2+}) containing 100 μM $^{45}\text{Ca}^{2+}$ and 0.5 mM [^3H]sucrose in the presence of 4% (w/w protein) A23187 either in the absence ($\circ$) of peptide or in the presence ($\square$) of (a) 25 μM PLN(1-25), or (b) 10 μM poly(L-Arg). Vesicles (0.1 mg of protein) were then absorbed on to Millipore filters. The loaded filter was perfused for the given times with the same buffer containing 100 μM unlabelled Ca^{2+} , 0.5 mM [^3H]sucrose and 2 mM ATP in the absence ($\circ$) or presence ($\square$) of (a), PLN(1-25) and (b) poly(L-Arg). The solid lines represent fits to single-exponential decays with the parameters given in the text.

also inhibited ATPase activity, with a maximal inhibition of 41% and an IC_{50} of 0.2 μM , based on an average molecular mass of 10000 (results not shown). The peptide PLN(13-25) had no detectable effect on ATPase activity (results not shown).

Orlowski and Champeil (1991) have shown that the rate of Ca^{2+} dissociation from the phosphorylated ATPase can be measured by pre-equilibrating the ATPase in leaky vesicles with $^{45}\text{Ca}^{2+}$ and then perfusing them on Millipore filters with $^{40}\text{Ca}^{2+}$ and ATP. The experiment relies on the rate of phosphorylation of the ATPase being much faster than the rate of dissociation of $^{45}\text{Ca}^{2+}$ from the unphosphorylated ATPase so that the observed rate of loss of $^{45}\text{Ca}^{2+}$ gives the rate of the $\text{E1PCa}_2 \rightarrow \text{E2P}$ step in Scheme 1 (Orlowski and Champeil, 1991). In the absence of peptide, the dissociation of Ca^{2+} fits to a single-exponential process with a rate constant of $29.8 \pm 4.0 \text{ s}^{-1}$. In the presence of 25 μM PLN(1-25) the rate of dissociation of Ca^{2+} is reduced and fits to a rate constant of $12.1 \pm 3.0 \text{ s}^{-1}$ (Figure 2a). In the presence

Figure 3 Effect of pH and PLN(1–25) on the fluorescence intensity of NBD-labelled ATPase

Shown are the fluorescence intensities of NBD-labelled ATPase in the absence (○) or presence (□) of 35 μM PLN(1–25). The lines are simulations, calculated using the parameters in Henderson et al. (1994) with values for the equilibrium constant E1/E2 of 4.0 and 16.0 in the absence and presence of PLN(1–25) respectively. Buffers were as follows: pH 6.0, 130 mM Mes/50 mM Tris; pH 6.5, 164 mM Mes/82 mM Tris; pH 7.0, 150 mM Mops/80 mM Tris; pH 7.5, 140 mM Mops/82 mM Tris; pH 8.0, 100 mM Tris/27 mM Mes; pH 8.5, 100 mM Tris/27 mM Mes. All contained 0.3 mM EGTA.

Scheme 2

Table 1 Phosphorylation of the Ca^{2+} -ATPase by P_i in the presence of PLN(1–25)

Sarcoplasmic reticulum vesicles were incubated in 150 mM Mes/Tris, pH 6.3, at 25 °C in the presence of 5.0 mM EGTA, 5 mM Mg^{2+} , 1 mM P_i and the given concentration of PLN(1–25) and the level of phosphorylation (nmol of [EP]/mg of protein) determined.

Concentration of PLN(1–25) (μM)	[EP] (nmol/mg of protein)
0	1.58
5	1.53
15	1.64
20	1.65
25	1.71

of 10 μM poly(L-Arg) the rate of dissociation of Ca^{2+} fits to a rate constant of $13.3 \pm 2.0 \text{ s}^{-1}$ (Figure 2b).

Effects of PLN(1–25) on the E2–E1 equilibrium of the ATPase were studied by making use of the conformational sensitivity of

Figure 4 Effect of PLN(1–25) on the fluorescence intensity of NBD-labelled ATPase at pH 7.0

NBD-labelled ATPase was incubated in 50 mM Hepes/Tris, pH 7.0, containing 0.3 mM EGTA and the change in fluorescence intensity recorded on addition of PLN(1–25). The solid line shows a fit to a single binding site with model $K_d = 7.4 \mu\text{M}$.

Figure 5 Effect of PLN(1–25) on the rate of phosphorylation of the Ca^{2+} -ATPase with ATP

Shown is the rate of phosphorylation of the ATPase by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence (○) or presence (□) of 25 μM PLN(1–25). The ATPase was incubated in buffer (pH 7.2, 40 mM Hepes/Tris, 5 mM Mg^{2+} , 100 mM KCl, 100 μM Ca^{2+}) in the absence or presence of 25 μM PLN(1–25) and then mixed in a 1:1 ratio with the same buffer containing 50 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and either no (○) or 25 μM PLN(1–25) (□), to give a final ATP concentration of 25 μM . The lines represent fits to single exponentials with the parameters given in the text.

the ATPase labelled with NBD (Wakabayashi et al., 1990; Wictome et al., 1992). As shown in Figure 3, the fluorescence emission intensity of NBD-labelled ATPase increases with increasing pH. It has been shown that this effect of pH is consistent with Scheme 2 for the E2–E1 equilibrium, with values for K_{H6} , K_{H7} and K_1 of $5 \times 10^5 \text{ M}^{-1}$, $3.0 \times 10^8 \text{ M}^{-1}$, and 4.0 respectively (Henderson et al., 1994), and for the sample used to obtain the data shown in Figure 3, fluorescence intensities of 2.80 and 2.22 for the E1 and E2 states respectively. Fluorescence intensities observed in the presence of 35 μM PLN(1–25) are higher than in the absence of PLN(1–25) at all pH values and, as shown in

Figure 3, the data fit to the same model but with a value for the equilibrium constant E1/E2 of 16.0. The concentration-dependence of the effect of PLN(1–25) at pH 7.0 fits to a K_d value of $7.4 \mu\text{M}$ (Figure 4); the data show that the effect of PLN(1–25) is almost saturated at $35 \mu\text{M}$, which is the concentration used to obtain the data in Figure 3.

PLN(1–25) had no significant effect on the rate of phosphorylation of the ATPase when the ATPase incubated in the presence of Ca^{2+} was mixed with ATP; rates of phosphorylation were 87 ± 21 and $71 \pm 18 \text{ s}^{-1}$ respectively in the absence and presence of $25 \mu\text{M}$ PLN(1–25) (Figure 5). PLN(1–25) also had no significant effect on the level of phosphorylation of the ATPase by P_i observed at pH 6.3 in the absence of Ca^{2+} , at a Mg^{2+} concentration of 5 mM (Table 1).

DISCUSSION

The activity of the SERCA2 isoform of the Ca^{2+} -ATPase expressed in smooth muscle, slow skeletal muscle and cardiac muscle is modulated by interaction with phospholamban (Eggermont et al., 1990). Phospholamban is a homopentameric protein which in the unphosphorylated form interacts with the Ca^{2+} -ATPase, reducing the maximal rate of the ATPase and reducing the affinity of the ATPase for Ca^{2+} (Morris et al., 1991; Sasaki et al., 1992). Phosphorylation of phospholamban by cyclic AMP-, cyclic-GMP-, or Ca^{2+} /calmodulin-dependent protein kinases prevents interaction with the Ca^{2+} -ATPase (Eggermont et al., 1990). The activity of the SERCA1 isoform of the Ca^{2+} -ATPase found in the sarcoplasmic reticulum of fast skeletal muscle is modulated by interaction with phospholamban if both the ATPase and phospholamban are co-expressed in COS cells (Toyofuku et al., 1993), even though phospholamban is not found in the sarcoplasmic reticulum of fast skeletal muscle. The activity of the SERCA3 isoform of the Ca^{2+} -ATPase is, however, not modulated by interaction with phospholamban when co-expressed in COS cells with phospholamban (Toyofuku et al., 1993).

As shown in Figure 1, the peptide PLN(1–25), corresponding to the hydrophilic domain of phospholamban, inhibits ATP hydrolysis by the Ca^{2+} -ATPase of skeletal muscle; a maximum inhibition of 44 % was observed with half maximal inhibition at $5 \mu\text{M}$ PLN(1–25). Sasaki et al. (1992) obtained up to 37 % inhibition of the ATPase activity of cardiac Ca^{2+} -ATPase with a peptide corresponding to residues 1–32 of phospholamban, maximal inhibition being observed with $110 \mu\text{M}$ peptide. Kim et al. (1990) and Vorherr et al. (1992) observed much smaller effects of similar peptides on Ca^{2+} uptake into sealed sarcoplasmic reticulum vesicles measured in the presence of oxalate, but Ca^{2+} uptake in the presence of oxalate is known to be at least partly dependent on the rate of oxalate transport across the membrane

(Stefanova et al., 1991) and the oxalate transporter is unlikely to be affected by phospholamban. Although the molar ratio of peptide:ATPase required for inhibition is relatively high (half maximal inhibition at a molar ratio of 136:1 in Figure 1) this presumably reflects the relatively high water solubility of the polar peptide.

As reported by Sasaki et al. (1992) for the cardiac Ca^{2+} -ATPase, PLN(1–25) had no significant effect on the Ca^{2+} -dependence of ATPase activity (Figure 1), only on maximal rate. We made a similar observation for spermine and other polycationic compounds (Hughes et al., 1994). Inhibition by spermine was found to follow from a decrease in the rate of the $\text{E1PCa}_2 \rightarrow \text{E2P}$ transition, with no effect on the rate of phosphorylation or dephosphorylation (Hughes et al., 1994). As shown in Figure 2(a), $25 \mu\text{M}$ PLN(1–25) reduces the rate of the $\text{E1PCa}_2 \rightarrow \text{E2P}$ transition by a factor of 2.4. Poly(L-Arg), which decreases ATPase activity by up to 41 %, causes a decrease in the rate of this transition by a factor of 2.2 at a concentration of $10 \mu\text{M}$ (Figure 2b).

PLN(1–25) also shifts the E2–E1 equilibrium for the ATPase towards E1, the data being consistent with a 4-fold increase in the equilibrium constant E1/E2 (Figure 3). In terms of Scheme 3, this implies 4-fold stronger binding of PLN(1–25) to the E1 conformation than to the E2 conformation, with binding to E1 or E2 being independent of pH. From Scheme 3, the effective dissociation constant K^{eff} for PLN(1–25) to the ATPase is given by:

$$(1/K^{\text{eff}}) = [(1/K^{\text{E2}}) + K_1(1/K^{\text{E1}})]/(1 + K_1)$$

where K^{E2} and K^{E1} are dissociation constants for PLN(1–25) for E2 and E1 respectively. With $K^{\text{E2}} = 4.0 \times K^{\text{E1}}$ and $K^{\text{eff}} = 7.4 \mu\text{M}$ (Figure 4), K^{E2} and K^{E1} become 10 and $3 \mu\text{M}$ respectively. The value of K^{eff} is comparable with the concentration of PLN(1–25) causing half-maximal inhibition of ATPase activity ($5 \mu\text{M}$ at pH 7.2) making it likely that both effects follow from binding at one site. Spermine was also found to bind more strongly to the E1 than to the E2 conformation of the ATPase (Hughes et al., 1994).

Although PLN(1–25) shifted the E2–E1 equilibrium towards E1 (Figure 3) it had no significant effect on the Ca^{2+} -dependence of ATPase activity (Figure 1). A corresponding observation was made in studies of the effects of nonylphenol on the activity of the ATPase where nonylphenol shifted the E2–E1 equilibrium towards E2, but again with no effect on the Ca^{2+} -dependence of activity (Michelangeli et al., 1990). Simulations show that the dependence of ATPase activity on the concentration of Ca^{2+} is only slightly affected by the value of the equilibrium constant E1/E2 as strong binding of MgATP to E1 strongly favours E1MgATP over E2 (Michelangeli et al., 1990).

Spermine was found to decrease phosphorylation of the ATPase by P_i in the absence of Ca^{2+} , by competition with Mg^{2+} (Hughes et al., 1994). As shown in Table 1, PLN(1–25) had no significant effect on the measured level of phosphorylation by P_i at concentrations inhibiting ATPase activity. This would suggest that the effects of spermine were less specific than those of PLN(1–25), and that the effects of spermine on phosphorylation by P_i followed from binding at a site other than that affecting the E2–E1 and $\text{E1PCa}_2 \rightarrow \text{E2P}$ steps.

PLN(1–25) had no effect on the rate of phosphorylation of the ATPase by ATP (Figure 5); spermine was also observed to have no effect on the rate of this step (Hughes et al., 1994). We conclude that the effects of PLN(1–25) are likely only to be on the E2–E1 and $\text{E1PCa}_2 \rightarrow \text{E2P}$ steps of the ATPase. An effect of PLN(1–25) on the equilibrium constant for just one step of the reaction sequence is not possible: it is not possible to change the

Scheme 3

Figure 6 The structures of spermine and of the hydrophilic region of phospholamban

Shown is a comparison of the structures of spermine (left) and residues 2–23 of phospholamban (right). Spermine is shown in a fully extended conformation, with distances between nitrogen atoms given in Å ($1 \text{ Å} = 0.1 \text{ nm}$). For phospholamban just the α -carbon backbone and residues Arg-9, Arg-13, and Arg-14 are shown for clarity. Residues 2–23 of phospholamban have been modelled as a slightly distorted α -helix, in an energy-minimized conformation with separations between nitrogens of residues Arg-9 and Arg-13, and Arg-14 (as shown by the broken lines) of 6.8 and 5.2 Å respectively.

equilibrium constant for just one step in the reaction sequence because the product of the equilibrium constants around the ATPase cycle must (for leaky vesicles) be a constant, equal to the equilibrium constant for the hydrolysis of ATP. An increase in the equilibrium constant E1/E2 requires a decrease in the equilibrium constant E2P/E1PCa₂, if these are the only two steps modified by binding of PLN(1–25). A decrease in the equilibrium constant E2P/E1PCa₂ would be consistent with the observed decrease in the rate of the E1PCa₂ → E2P step.

We conclude that the hydrophilic domain of phospholamban can bind to the Ca^{2+} -ATPase of cardiac or skeletal muscle and inhibit ATPase activity by decreasing the rate of the Ca^{2+} -transport step. The inhibition of this same step by spermine (Hughes et al., 1994) or poly(L-Arg) (Figure 2) implies that cationic sites on phospholamban are involved in the interaction. Since the peptide PLN(13–25) had no detectable effect on ATPase activity (results not shown), cationic residues in the region 1–12 are presumably important; this is in agreement with the results of Sasaki et al. (1992) who found no inhibition of ATPase

activity with a peptide corresponding to residues 8–47 of phospholamban and with the results of Toyofuku et al. (1993) who showed that residues between Glu-2 and Ile-18 were important for interaction with the ATPase. Since spermine and spermidine inhibit ATPase activity with similar potencies, but *N*¹-acetylspermidine causes no inhibition (Hughes et al., 1994), it is likely that binding of spermine to the ATPase involves three of the four nitrogen centres (Figure 6). It has been suggested that the hydrophilic domain of phospholamban between residues 2 and 23 can adopt an α -helical conformation (Tada, 1992). Figure 6 shows an energy-minimized structure for this region of phospholamban modelled as a slightly distorted α -helix with a separation between the nitrogens of Arg-9, Arg-13 and Arg-14 equal to that between the terminal and central nitrogens of spermine.

Two regions on the Ca^{2+} -ATPase have been identified as being important for interaction with phospholamban (James et al., 1989; Toyofuku et al., 1993). The first is a region between Asp-370 and Lys-400, close to the residue on the ATPase (Asp-351) phosphorylated by ATPase. This region of the ATPase has been shown, by using anti-peptide antibodies, to be surface exposed (Mata et al., 1992), consistent with its proposed interaction with phospholamban (James et al., 1989; Toyofuku et al., 1993). Toyofuku et al. (1993) have shown that the nucleotide-binding/hinge region between Arg-467 and Arg-743 is also involved in the interaction with phospholamban. Anti-peptide antibody-binding studies have shown considerable surface exposure in this region of the ATPase (Mata et al., 1992). Tada (1992) has suggested that the hydrophilic domain of phospholamban consists of an α -helical structure between Met-1 and Pro-21 and a less ordered structure between Gln-22 and Asn-30. If the region between Gln-22 and Asn-30 is fully extended, it would have an end-to-end length of 30 Å. The separation between Pro-21 and Arg-9 in the structure shown in Figure 6 is 17 Å. Thus the maximum height above the membrane surface of the binding-site region proposed in Figure 6 would be approx. 47 Å. We have estimated heights of Cys-344, Glu-439 and Cys-670/Cys-674 above the membrane surface to be 45, 70, and 54 Å respectively (Mata et al., 1993; Stefanova et al., 1993; Baker et al., 1994). Thus the proposed structure for the binding-site region on phospholamban is compatible with the suggested sites of interaction on the ATPase.

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