Characterization of the single Ca^{2+} -binding site on the Ca^{2+} -ATPase reconstituted with short- or long-chain phosphatidylcholines

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On reconstitution of the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum into bilayers of dimyristoleoylphosphatidylcholine [$\text{di}(\text{C}_{14:1})\text{PC}$] or dinervonylphosphatidylcholine [$\text{di}(\text{C}_{24:1})\text{PC}$] the stoichiometry of Ca^{2+} binding changes from the usual two Ca^{2+} ions bound per ATPase molecule to one Ca^{2+} ion bound per ATPase molecule. For the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$, removal of Ca^{2+} from the Ca^{2+} -bound ATPase results in a decrease in tryptophan fluorescence intensity, as observed for the ATPase in dioleoylphosphatidylcholine [$\text{di}(\text{C}_{18:1})\text{PC}$]. For the ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ removal of Ca^{2+} results in no change in tryptophan fluorescence intensity. In the presence of Mg^{2+} , removal of Ca^{2+} from the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ results in a decrease in tryptophan fluorescence intensity, but for the ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ this results in an increase in intensity. Fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) is the same for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$, but is markedly greater in $\text{di}(\text{C}_{14:1})\text{PC}$, consistent

with a 4-fold increase in the E_1/E_2 equilibrium constant. Addition of Mg^{2+} to NBD-labelled ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ results in an increase in NBD fluorescence, attributed to stronger binding of Mg^{2+} to the E_1 than to the E_2 conformation; addition of Mg^{2+} had no effect on the fluorescence of the NBD-labelled ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$. In the absence of Ca^{2+} , Mg^{2+} increased the tryptophan fluorescence of the ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$, $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$, with the same binding-constant for Mg^{2+} in all three lipids. Addition of Mg^{2+} to the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin resulted in a decrease in fluorescence in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ but had no effect in $\text{di}(\text{C}_{14:1})\text{PC}$. These effects are interpreted in terms of binding of Ca^{2+} at a single outer Ca^{2+} binding-site on the ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$, in a conformation in which the inner site is occluded [in $\text{di}(\text{C}_{14:1})\text{PC}$] or modified in its affinity for Ca^{2+} [in $\text{di}(\text{C}_{24:1})\text{PC}$]. Thapsigargin binds to the ATPase, reducing its affinity for Ca^{2+} both in $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$.

INTRODUCTION

The Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) normally binds two Ca^{2+} ions, and only with two bound Ca^{2+} ions can it be phosphorylated by ATP, leading to transport of Ca^{2+} (de Meis, 1981). However, if the Ca^{2+} -ATPase is reconstituted into bilayers of a short- or long-chain phospholipid such as, respectively, dimyristoleoylphosphatidylcholine [$\text{di}(\text{C}_{14:1})\text{PC}$] or dinervonylphosphatidylcholine [$\text{di}(\text{C}_{24:1})\text{PC}$], then the ATPase binds a single Ca^{2+} ion; the ATPase with a single bound Ca^{2+} can now be phosphorylated by ATP (Michelangelo et al., 1990, 1991; Starling et al., 1993). The question then arises as to the relationship between the two Ca^{2+} binding sites normally found on the ATPase and the single site found on the ATPase after reconstitution with $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$.

The overall process of Ca^{2+} binding fits Scheme 1:

Dissociation of $^{45}\text{Ca}^{2+}$ from the Ca^{2+} -bound ATPase follows a sequential mechanism (Ikemoto et al., 1981; Dupont, 1982; Orlowski and Champeil, 1991; Michelangelo et al., 1990). Studies of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) or fluorescein isothiocyanate (FITC) have detected a

conformation E_2 of the ATPase unable to bind Ca^{2+} at cytoplasmic sites, in equilibrium with the form E_1 with two Ca^{2+} binding sites exposed to the cytoplasm (reviewed in Henderson et al., 1994a,b). It has been suggested that the fluorescence of the NBD-labelled ATPase is sensitive to the E_2 – E_1 conformational change, with high fluorescence in the E_1 state and low fluorescence in the E_2 state (Wakabayashi et al., 1990; Henderson et al., 1994a). Changes in tryptophan fluorescence intensity follow closely the level of Ca^{2+} binding to the ATPase under a variety of conditions and it has been shown that this is consistent with tryptophan fluorescence being sensitive to the $E_1\text{Ca} \rightleftharpoons E_1'\text{Ca}$ conformational change, with the equilibrium constant for this change being close to 1 (Henderson et al., 1994a). The pH and Mg^{2+} -dependence of Ca^{2+} -binding define H^+ and Mg^{2+} binding-constants at the two Ca^{2+} binding sites (Henderson et al., 1994a). Effects of Mg^{2+} on the tryptophan fluorescence-intensity of the ATPase in the absence of Ca^{2+} have been suggested to follow from binding of Mg^{2+} to the second of the two Ca^{2+} binding-sites (Henderson et al., 1994a). pH and Mg^{2+} have also been shown to affect the rate of dissociation of Ca^{2+} from the Ca^{2+} -bound ATPase (Moutin and Dupont, 1991; Henderson et al., 1994b). These effects have been interpreted in terms of binding of H^+ and Mg^{2+} at a 'gating site' on the ATPase. The fluorescence intensity of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (DMC) has been shown to change on binding Mg^{2+} , in a pH-dependent fashion, both in the absence and in the presence

of Ca^{2+} (Stefanova et al., 1992; Henderson et al., 1994b), and it has been suggested that these effects could follow from binding at the gating-site (Henderson et al., 1994b). This model is also consistent with the effects of K^+ on Ca^{2+} binding to the ATPase (Lee et al., 1995).

Here we use these spectroscopic approaches to characterize the Ca^{2+} binding site on the reconstituted ATPase and show that for the Ca^{2+} -ATPase reconstituted with both $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$ the data is consistent with binding of Ca^{2+} to the second Ca^{2+} binding-site only.

MATERIALS AND METHODS

Phospholipids were obtained from Avanti Polar Lipids. The Ca^{2+} -ATPase was purified from skeletal muscle SR as described in East and Lee (1982) and reconstitutions were performed as described in Starling et al. (1993). Phospholipid (10 μmol) was mixed with buffer (400 μl ; 10 mM Hepes/Tris and 15% sucrose, pH 8.0) containing MgSO_4 (5 mM) and potassium cholate (12 mg/ml) and sonicated to clarity in a bath sonicator. ATPase (1.25 mg) in a volume of 20–30 μl was then added and, for $\text{di}(\text{C}_{14:1})\text{PC}$ and dioleoylphosphatidylcholine [$\text{di}(\text{C}_{18:1})\text{PC}$], left for 15 min at room temperature and 45 min at 5 °C to equilibrate before being diluted with buffer (2 ml) and stored on ice until use; for $\text{di}(\text{C}_{24:1})\text{PC}$, samples were equilibrated for 1 h at room temperature.

Binding of $^{45}\text{Ca}^{2+}$ to the ATPase was measured using the double labelling method described in Starling et al. (1993). Maximum observable levels of phosphorylation of the ATPase were determined by incubating the ATPase with 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP and 1 mM CaCl_2 , as described (Starling et al., 1993). Concentrations of protein were estimated by using the extinction coefficient ($1.21 \text{ g}^{-1} \cdot \text{cm}^{-1}$ for a solution in 1% SDS) given by Hardwicke and Green (1974).

The ATPase was labelled with NBD or DMC following the protocols described in Henderson et al. (1994a). Fluorescence measurements were performed at 25 °C using an SLM Aminco 8000C fluorimeter. Measurements of NBD fluorescence were made with excitation and emission wavelengths of 430 nm and 520 nm respectively, with a 450 nm long wavelength cut-off filter (450FLO T-50, Andover Corporation) on the excitation side and a Hoya Y50 500 nm short wavelength cut-off filter on the emission side. Measurements of DMC fluorescence were made with excitation and emission wavelengths of 350 and 425 nm respectively. Tryptophan fluorescence was recorded with an excitation wavelength of 290 nm and emission wavelengths of 315 nm and 340 nm for measurements of the response to Mg^{2+} and Ca^{2+} respectively. All fluorescence measurements were corrected for dilution.

Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+} , Mg^{2+} and H^+ to EGTA given by Godt (1974).

RESULTS

Fluorescence of the NBD-labelled ATPase

The fluorescence intensity of NBD-labelled ATPase increases with increasing pH (Figure 1). Fluorescence intensities were, within experimental error, the same for the ATPase reconstituted with $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ or with a mixture of $\text{di}(\text{C}_{14:1})\text{PC}$ and cholesterol at a molar ratio of 1:1. Higher fluorescence intensities were, however, observed at all pH values for the ATPase reconstituted with $\text{di}(\text{C}_{14:1})\text{PC}$, the effect being most

Figure 1 Effect of phospholipid structure on the fluorescence intensity of NBD-labelled ATPase

Shown are the fluorescence intensities in buffer containing 0.3 mM EGTA in the absence of Mg^{2+} for NBD-labelled ATPase reconstituted in: (○), $\text{di}(\text{C}_{18:1})\text{PC}$; (△), $\text{di}(\text{C}_{24:1})\text{PC}$; (▽) a 1:1 molar ratio of $\text{di}(\text{C}_{14:1})\text{PC}$ and cholesterol; (□), $\text{di}(\text{C}_{14:1})\text{PC}$. Fluorescence intensities for the ATPase reconstituted in $\text{di}(\text{C}_{14:1})\text{PC}$ were identical in the presence or absence of 20 mM Mg^{2+} . 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. In the presence of Ca^{2+} , the fluorescence intensity for all systems was 1.54, independent of pH. The solid lines are simulations calculated as described in the text, assuming fluorescence intensities of 1.1 and 2.0 for E_2 and E_1 states respectively.

marked at intermediate pH values (Figure 1). Addition of Mg^{2+} to NBD-labelled ATPase reconstituted with either $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ results in an increased fluorescence intensity, the data fitting to a single binding site for Mg^{2+} with K_d values of 9.2 ± 1.5 and 8.9 ± 1.9 mM in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$ respectively, at pH 7.0 (Figure 2). A similar result was reported previously for the ATPase in SR vesicles (Henderson et al., 1994a) but addition of 20 mM Mg^{2+} had no effect on the

Figure 2 Effect of Mg^{2+} on the fluorescence intensity of reconstituted NBD-labelled ATPase

Shown are the percent fluorescence changes observed on addition of Mg^{2+} in 150 mM Mops/80 mM Tris at pH 7.0 to the ATPase reconstituted with: (○), $\text{di}(\text{C}_{18:1})\text{PC}$; (□), $\text{di}(\text{C}_{24:1})\text{PC}$. The solid lines are best fits to the data for a single Mg^{2+} binding-site giving K_d values of 9.2 ± 1.4 and 8.9 ± 1.9 mM for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$, respectively. The broken line is a simulation calculated using the binding constants given in Henderson et al. (1994a), assuming a maximum fluorescence change of 11%.

fluorescence intensity for NBD-labelled ATPase in di(C_{14:1})PC at any pH value (see legend to Figure 1).

Scheme 2

As described in Henderson et al. (1994a) the effect of pH on the fluorescence intensity of NBD-labelled ATPase in SR vesicles is consistent with Scheme 2 with values for K_1 , K_{H6} and K_{H7} of 4.0 M^{-1} , $5.0 \times 10^5 \text{ M}^{-1}$ and $3.0 \times 10^8 \text{ M}^{-1}$ respectively, assuming that E_1 and E_2 are states of high and low fluorescence, respectively. These same constants fit the data for the ATPase reconstituted with di(C_{18:1})PC or di(C_{24:1})PC, and the data for the ATPase reconstituted with di(C_{14:1})PC fit the same scheme but with the value of K_1 increased to 16.0, with the fluorescences for the E_1 and E_2 states being unaffected by changes in the lipid (Figure 1). The effect of Mg²⁺ on the fluorescence of NBD-labelled ATPase in SR vesicles was interpreted in terms of Mg²⁺ binding to the first, inner Ca²⁺ binding-site on the ATPase, with stronger binding to E_1 than E_2 resulting in a shift towards E_1 and the observed increase in fluorescence intensity (Henderson et al., 1994a). As shown in Figure 2, the observed effect of Mg²⁺ on the ATPase in di(C_{18:1})PC or di(C_{24:1})PC can be fitted using the binding constant for Mg²⁺ given in Henderson et al. (1994a).

Binding of ⁴⁵Ca²⁺ to the ATPase

The level of Ca²⁺ binding we observe to the ATPase in SR vesicles is typically $\approx 7 \text{ nmol/mg}$ of protein which, combined with a maximum level of phosphoenzyme formed from ATP of 3.5 nmol/mg of protein, corresponds to two Ca²⁺ ions bound per ATPase molecule (Starling et al., 1993). The maximal level of Ca²⁺ binding to the ATPase reconstituted with di(C_{14:1})PC or

Figure 3 Effect of phospholipid structure on the binding of ⁴⁵Ca²⁺ to the reconstituted ATPase

Shown is the binding of ⁴⁵Ca (nmol/mg protein) to the ATPase reconstituted with: (○), di(C_{18:1})PC; (△), di(C_{14:1})PC; (□), di(C_{24:1})PC. Ca²⁺ binding was measured at pH 7.2 in 20 mM Hepes/Tris, 20 mM Mg²⁺, 25 °C, at $1.8 \mu\text{M}$ ATPase. Ca²⁺ binding measured for the ATPase reconstituted with di(C_{14:1})PC (▲), or di(C_{24:1})PC (■), in the presence of $1.8 \mu\text{M}$ thapsigargin. The continuous lines represent simulations calculated as described in the text assuming maximum Ca²⁺ binding-levels of 7.5 nmol/mg protein for the ATPase in di(C_{18:1})PC and 3.75 nmol/mg protein in di(C_{14:1})PC and di(C_{24:1})PC.

di(C_{24:1})PC is half that observed for the ATPase reconstituted with di(C_{18:1})PC (Figure 3). The Ca²⁺ affinity of the ATPase reconstituted with di(C_{14:1})PC is slightly higher than that for the ATPase reconstituted with di(C_{18:1})PC, whereas that in di(C_{24:1})PC is slightly less (Figure 3).

The sesquiterpene lactone thapsigargin has been shown to reduce the affinity of the Ca²⁺-ATPase for Ca²⁺ (Wictome et al., 1992, 1994). As shown in Figure 3, this is also observed for the ATPase reconstituted with di(C_{14:1})PC or di(C_{24:1})PC where at a 1:1 molar ratio of thapsigargin to ATPase, the K_d for Ca²⁺ decreases to $\approx 0.4 \text{ mM}$.

Tryptophan fluorescence changes

Addition of EGTA to the Ca²⁺-bound ATPase in SR vesicles in the absence of Mg²⁺ results in a decrease in tryptophan fluorescence intensity (Dupont et al., 1988). The decrease in fluorescence intensity is shown in Figure 4 as a function of pCa at pH 6.0, 7.2 and 8.0 for the ATPase reconstituted with di(C_{18:1})PC.

Figure 4 Ca²⁺-dependence of tryptophan fluorescence intensity for the reconstituted ATPase

The figure shows the decrease in tryptophan fluorescence intensity of the reconstituted ATPase on addition of EGTA to the given pCa value: (a) pH 6.0 (in 130 mM Mes/50 mM Tris); (b) pH 7.2 (in 150 mM Mops/80 mM Tris); and (c) pH 8.0 (in 100 mM Tris/27 mM Mes). Open symbols (○, □, △) in the absence of Mg²⁺, filled symbols (●, ■, ▲) in the presence of 20 mM Mg²⁺. ATPase reconstituted with: (○, ●), di(C_{18:1})PC; (□, ■), di(C_{24:1})PC; (△, ▲), di(C_{14:1})PC. The lines are simulations calculated as described in the text.

Figure 5 Effect of Mg^{2+} on the tryptophan fluorescence intensity of the Ca^{2+} -ATPase

Shown is the increase in tryptophan fluorescence intensity on addition of Mg^{2+} to the ATPase reconstituted in: ($\circ$), $\text{di}(\text{C}_{18:1})\text{PC}$; ($\square$), $\text{di}(\text{C}_{14:1})\text{PC}$; ($\triangle$), $\text{di}(\text{C}_{24:1})\text{PC}$. The buffer was 150 mM Mops/80 mM Tris, pH 7.2, containing 0.3 mM EGTA. The continuous lines are best fits to a simple binding equation with K_d values of 2.6 ± 0.6 , 2.7 ± 0.8 and 3.1 ± 1.5 mM for $\text{di}(\text{C}_{18:1})\text{PC}$, $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$ respectively.

Changes of comparable magnitude are observed for the ATPase reconstituted in $\text{di}(\text{C}_{24:1})\text{PC}$ although concentrations of Ca^{2+} resulting in half maximal fluorescence changes are higher for the ATPase reconstituted with $\text{di}(\text{C}_{24:1})\text{PC}$ at all pH values. Addition of EGTA to the Ca^{2+} -bound ATPase reconstituted with $\text{di}(\text{C}_{14:1})\text{PC}$ results in no change in tryptophan fluorescence intensity in the absence of Mg^{2+} (Figure 4).

In the presence of Mg^{2+} , higher concentrations of Ca^{2+} are required for half maximal changes in fluorescence intensity for the ATPase reconstituted with $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ and the magnitude of the change is reduced (Figure 4). As reported previously (Michelangelo et al., 1990), in the presence of Mg^{2+} , addition of EGTA to the Ca^{2+} -bound ATPase reconstituted with $\text{di}(\text{C}_{14:1})\text{PC}$ results in an increase in fluorescence intensity. As shown in Figure 4 the magnitudes of the observed changes are comparable at pH 8.0 and 7.2, but no significant change in tryptophan fluorescence intensity is observed at pH 6.0.

Addition of Mg^{2+} to the Ca^{2+} -ATPase in SR vesicles in the absence of Ca^{2+} results in an increase in tryptophan fluorescence intensity, when fluorescence is excited at 290 nm and observed at 315 nm (Guillan et al., 1982; Henderson et al., 1994a). As shown in Figure 5, addition of Mg^{2+} to the ATPase reconstituted with $\text{di}(\text{C}_{18:1})\text{PC}$, $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ all result in increases in fluorescence intensities, with K_d values of 2.6 ± 0.6 , 2.7 ± 0.8 and 3.1 ± 1.5 mM respectively. As shown, the magnitude of the fluorescence change is less for the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$ or $\text{di}(\text{C}_{14:1})\text{PC}$ than in $\text{di}(\text{C}_{18:1})\text{PC}$ (Figure 5).

Effect of Mg^{2+} on DMC-labelled ATPase

Addition of Mg^{2+} to DMC-labelled ATPase in SR vesicles in the absence of Ca^{2+} results in a decrease in DMC fluorescence intensity, with the magnitude of the decrease increasing with decreasing pH; in the presence of Ca^{2+} , the pH dependence of the fluorescence change is much less marked (Stefanova et al., 1992; Henderson et al., 1994a). These effects have been attributed to binding to a 'gating' site according to Scheme 3 with the binding constant for H^+ at the gating site being different for the Ca^{2+} -free forms E_2 and E_1 and for the Ca^{2+} -bound forms E_1Ca , $\text{E}_1'\text{Ca}$ and $\text{E}_1'\text{Ca}_2$ ($K_{\text{H}3} = 1.25 \times 10^7 \text{ M}^{-1}$ and $K_{\text{H}2} = 5.0 \times 10^8 \text{ M}^{-1}$), and the

binding constants K_6 , K_7 , K_8 and K_9 for Mg^{2+} all being equal (500 M^{-1}) (Henderson et al., 1994a).

Scheme 3

Figure 6 Effect of Mg^{2+} on the fluorescence intensity of DMC-labelled ATPase

DMC-labelled ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PC}$ ($\circ$, $\square$, $\triangle$) or $\text{di}(\text{C}_{24:1})\text{PC}$ ($\bullet$, $\blacksquare$, $\blacktriangle$) was incubated in 40 mM Tris/maleate at pH 6.0 ($\circ$, $\bullet$), 7.0 ($\square$, $\blacksquare$) or 8.0 ($\triangle$, $\blacktriangle$) in 1 mM EGTA ($\circ$, $\bullet$) or 1 mM Ca^{2+} ($\square$, $\blacksquare$) and the effect of Mg^{2+} on coumarin fluorescence intensity determined. (c) Effect of the addition of 2.0 mM Mg^{2+} in 40 mM Tris/maleate, pH 6.0, containing 1 mM EGTA on the fluorescence of DMC-labelled ATPase reconstituted in: A, $\text{di}(\text{C}_{18:1})\text{PC}$; B, $\text{di}(\text{C}_{24:1})\text{PC}$; C, $\text{di}(\text{C}_{14:1})\text{PC}$.

Effects of Mg²⁺ on the fluorescence of DMC-labelled ATPase reconstituted with either di(C_{18:1})PC or di(C_{24:1})PC are identical with those observed for the ATPase in the native SR membrane, both in the presence and in the absence of Ca²⁺ (Figure 6). By contrast, addition of Mg²⁺ to the DMC-labelled ATPase reconstituted with di(C_{14:1})PC resulted in no observable change in fluorescence (Figure 6).

DISCUSSION

The phospholipid fatty acyl chain length that produces maximum ATPase activity for the Ca²⁺-ATPase of skeletal muscle SR is 18 carbons (Lee and East, 1993). On reconstitution with a phospholipid containing short or long fatty acyl chains, as in di(C_{14:1})PC and di(C_{24:1})PC respectively, ATPase activities are low and the stoichiometry of Ca²⁺ binding changes from the usual two Ca²⁺ ions bound per ATPase molecule to one Ca²⁺ ion bound per ATPase molecule. The effects of di(C_{14:1})PC and di(C_{24:1})PC on the ATPase are, however, different, since phosphorylation of the ATPase by ATP is considerably slower in di(C_{14:1})PC than in di(C_{24:1})PC (Michelangeli et al., 1991). Further, the effects of di(C_{14:1})PC on activity and Ca²⁺-binding can be reversed by the addition of a variety of hydrophobic molecules, including cholesterol, whereas such molecules have no effect on the ATPase reconstituted with di(C_{24:1})PC (Michelangeli et al., 1990, 1991; Lee et al., 1991; Starling et al., 1993).

Ca²⁺-binding to the ATPase is sequential, as described in Scheme 1, with Ca²⁺ first binding to an 'inner' Ca²⁺ binding-site of low affinity, this leading to a conformation change on the ATPase and the appearance of a second, 'outer', Ca²⁺ binding-site of higher affinity. Rapid kinetic studies suggest binding of Ca²⁺ in a narrow, channel-like structure (Ikemoto et al., 1981; Dupont, 1982; Michelangeli et al., 1990; Orlowski and Champeil, 1991), consistent with modelling studies (Lee et al., 1994). It is necessary to postulate a lower affinity for the inner than for the outer Ca²⁺ site, both to account for the observed co-operativity of Ca²⁺ binding and to account for the effect of Ca²⁺ concentration on the rate of dissociation of Ca²⁺ from the Ca²⁺-bound ATPase (Henderson et al., 1994b). Binding of H⁺, Mg²⁺ and K⁺ at a 'gating' site has been proposed to explain the effects of these ions on the rate of dissociation of Ca²⁺ from the Ca²⁺-bound ATPase (Henderson et al., 1994a,b; Lee et al., 1995). These sites are illustrated in Figure 7.

A number of schemes could be proposed that would result in binding of a single Ca²⁺ ion to the ATPase:

Schemes 4 and 5 postulate Ca²⁺ binding to the first, inner Ca²⁺ binding-site. In Scheme 4 this is not followed by the conformation change ($E_{1in}Ca \rightleftharpoons E_{1in}'Ca$) that would create the second, outer, Ca²⁺ binding-site. In Scheme 5 binding to the inner site is followed by the normal conformation change, but to produce an outer Ca²⁺ binding-site that is unable to bind Ca²⁺. The other two Schemes (6 and 7) propose Ca²⁺ binding to the outer Ca²⁺

Figure 7 Schematic diagram of the Ca²⁺, Mg²⁺ and K⁺ binding sites on the Ca²⁺-ATPase

Shown are the inner and outer Ca²⁺ binding-sites on the Ca²⁺-ATPase. Binding of Ca²⁺ is in competition with H⁺ and Mg²⁺ and, at the inner site, with K⁺. Mg²⁺, H⁺ and K⁺ binding at a 'gating' site affects the affinity of the ATPase for Ca²⁺ and the rate of binding and dissociation of Ca²⁺ (Henderson et al., 1994a; Lee et al., 1984b). Protonation of a further site on the ATPase affects the E₂-E₁ equilibrium (Henderson et al., 1994a).

binding-site and not to the inner site. In Scheme 6 it is proposed that the first, inner, binding-site exists in E₁ and that E₁ is in equilibrium with a form E₁' in which the outer Ca²⁺ binding-site is formed normally. Alternatively, in Scheme 7 it is proposed that E₂ is in equilibrium not with E₁ but with E₁', the form with a blocked inner Ca²⁺ binding-site but a normal outer Ca²⁺ binding-site.

The changes in tryptophan fluorescence intensity seen on binding Ca²⁺ to the native ATPase follow from the E₁Ca $\rightleftharpoons$ E₁'Ca transition (Henderson et al., 1994a). Schemes 4 and 7 would then not be expected to show a change in Trp fluorescence on binding Ca²⁺. Scheme 4 would give low-affinity Ca²⁺ binding since the inner Ca²⁺ binding site is postulated to be of lower affinity than the outer site (Henderson et al., 1994a). Scheme 5, although still postulating binding of Ca²⁺ to the inner binding site, could result in higher affinity for Ca²⁺ than Scheme 4 if the equilibrium constant $E_{1in}'Ca/E_{1in}Ca$ were greatly in favour of E_{1in}'Ca. Measurement of the pH-dependence of Ca²⁺-binding to the ATPase in di(C_{14:1})PC and di(C_{24:1})PC allows a distinction to be made between Scheme 5 and Schemes 6 and 7, since the pH-dependencies of Ca²⁺-binding at the inner and outer binding-sites have been shown to be different (Henderson et al., 1994a).

The data obtained for the ATPase reconstituted with di(C_{14:1})PC are consistent with Scheme 7. In the absence of Mg²⁺, addition of EGTA to the Ca²⁺-bound ATPase in di(C_{14:1})PC has no effect on tryptophan fluorescence intensity (Figure 4), as predicted for Scheme 7. In the absence of Ca²⁺, addition of Mg²⁺ to the ATPase results in an increase in tryptophan fluorescence intensity, attributed to binding of Mg²⁺ to the outer Ca²⁺ binding-site (Henderson et al., 1994a). Although the magnitude of the change observed on addition of Mg²⁺ to the ATPase in di(C_{14:1})PC is smaller than for the ATPase in di(C_{18:1})PC, the measured affinity for Mg²⁺ is the same (Figure 5). The effective K_d for the native ATPase for Mg²⁺ is 2.4 mM at pH 7.2 (Henderson et al., 1994a), compared with experimental values of 2.6 ± 0.6 and 2.7 ± 0.8 mM for the ATPase in di(C_{18:1})PC and di(C_{14:1})PC, respectively (Figure 5). These results predict that, in the presence of Mg²⁺, addition of EGTA to the Ca²⁺-bound ATPase in di(C_{14:1})PC would result in an increase in tryptophan fluorescence intensity arising from the displacement

of Ca^{2+} from the outer Ca^{2+} binding site by Mg^{2+} , as observed experimentally (Figure 4). The Ca^{2+} -dependence of the change in tryptophan fluorescence intensity (Figure 4) can be simulated using the binding-constants for the outer Ca^{2+} binding-site determined for the native ATPase (Henderson et al., 1994a), combined with the equilibrium constant E_2/E_1' derived from measurements with NBD-labelled ATPase, as described below. Data on $^{45}\text{Ca}^{2+}$ binding to the ATPase reconstituted in $\text{di}(\text{C}_{14:1})\text{PC}$ can also be simulated with these same constants in terms of Scheme 7 (Figure 3).

The effect of $\text{di}(\text{C}_{14:1})\text{PC}$ on the E_2/E_1' equilibrium of the ATPase has been determined using NBD-labelled ATPase. Fluorescence of the NBD-labelled ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ is pH-sensitive (Figure 1), the data fitting to Scheme 2 using the constants established for the native ATPase (Henderson et al., 1994a). In $\text{di}(\text{C}_{14:1})\text{PC}$, higher NBD fluorescence is observed than in $\text{di}(\text{C}_{18:1})\text{PC}$ at all pH values in the absence of Ca^{2+} , although in the presence of Ca^{2+} , fluorescence intensities are the same on reconstitution with either $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{18:1})\text{PC}$. The experimental data for $\text{di}(\text{C}_{14:1})\text{PC}$ can be reproduced in terms of Scheme 2, assuming an increase in the equilibrium constant E_1/E_2 by a factor of 4, with no effect on the H^+ binding constants, and assuming that the fluorescence intensities of the E_1 (or E_1') and E_2 states are the same in $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PC}$ (Figure 1). Measurements using FITC-labelled ATPase have also detected a shift towards E_1 on reconstitution with $\text{di}(\text{C}_{14:1})\text{PC}$; an increase in equilibrium constant by a factor of 10 was estimated (Froud et al., 1986), in reasonable agreement with the value given here, given the greater difficulty of the measurements with FITC-labelled ATPase arising from the intrinsic pH-dependence of the fluorescence of the FITC group (Froud and Lee, 1986).

The presence of cholesterol at a 1:1 molar ratio with $\text{di}(\text{C}_{14:1})\text{PC}$ increases the Ca^{2+} binding stoichiometry from one Ca^{2+} ion bound per ATPase molecule to the normal two (Starling et al., 1993) and also reverses the effect of $\text{di}(\text{C}_{14:1})\text{PC}$ on NBD fluorescence (Figure 1). We have reported previously that addition of cholesterol to the ATPase reconstituted with $\text{di}(\text{C}_{14:1})\text{PC}$ reverses the effect of $\text{di}(\text{C}_{14:1})\text{PC}$ on tryptophan fluorescence (Starling et al., 1993).

Binding of Mg^{2+} to native, NBD-labelled ATPase increases fluorescence, attributed to binding of Mg^{2+} at the inner Ca^{2+} binding-site, with higher affinity in E_1 than E_2 (Henderson et al., 1994a). Addition of Mg^{2+} to NBD-labelled ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ had no effect on fluorescence (Figure 1), suggesting that Mg^{2+} is unable to bind to the inner Ca^{2+} binding-site.

Measurements with the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$ are consistent with Scheme 6. In the absence of Mg^{2+} , addition of EGTA to the Ca^{2+} -bound ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$ results in a decrease in tryptophan fluorescence intensity, with a magnitude comparable to that seen for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ (Figure 4). Thus in this system, the ATPase is able to undergo a transition comparable to the $E_1\text{Ca} \rightleftharpoons E_1'\text{Ca}$ transition in the native ATPase, consistent with Schemes 5 or 6 above. We were unable to fit the experimental data as a function of pH in terms of Scheme 5 but, as shown in Figure 4, a good fit could be obtained in terms of Scheme 6. In the calculation of Ca^{2+} -binding in terms of Scheme 6, the binding constants for the outer Ca^{2+} binding-site were as given in Henderson et al. (1994a) for the native ATPase. The fluorescence of NBD-labelled ATPase is the same in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ (Figure 2), suggesting that the equilibrium constant E_2/E_1' is the same in the two lipids. It is also necessary to account for the effects of H^+ binding at the gating site (Scheme 3). Scheme 3 with $K_{H2} > K_{H3}$ implies stronger binding of Ca^{2+} to the protonated forms of the ATPase ($E_1\text{H}$ and $E_1\text{HMg}$) than to the non-

protonated forms (E_1 and $E_1\text{Mg}$), that is, $K_{C1c} = K_{C1b} > K_{C1a} = K_{C1d}$. The effects of Mg^{2+} on the fluorescence of DMC-labelled ATPase in the absence or presence of Ca^{2+} are the same for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ (Figure 6), suggesting that an equivalent scheme to Scheme 3 applies to the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$, with protein-binding constants K_{H3} for the E_2 and E_1 states, and K_{H2} for the E_1' and $E_{1out}'\text{Ca}$ states. The effect of Ca^{2+} on the tryptophan fluorescence of the ATPase can be simulated assuming a value for the equilibrium constant E_1'/E_1 of 0.015 (Figure 4).

Although the effect of Mg^{2+} on tryptophan fluorescence intensity is smaller for the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$ than in $\text{di}(\text{C}_{18:1})\text{PC}$, the dependence on Mg^{2+} concentration is the same (Figure 5). Both the Ca^{2+} -dependence of tryptophan fluorescence in the presence of Mg^{2+} (Figure 4) and of $^{45}\text{Ca}^{2+}$ -binding (Figure 3) can be simulated with an equilibrium constant of 0.015 for E_1'/E_1 .

The effect of Mg^{2+} on the fluorescence of NBD-labelled ATPase is the same in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$ (Figure 2). This implies that Mg^{2+} is able to bind to the first Ca^{2+} binding-site on the ATPase in $\text{di}(\text{C}_{24:1})\text{PC}$ even though this site cannot bind Ca^{2+} . A small structural change at the first Ca^{2+} binding site could lead to a marked reduction in affinity for Ca^{2+} with little effect on Mg^{2+} binding. Since the co-ordination number of Ca^{2+} bound to proteins is generally 7 compared with 6 for Mg^{2+} (Strynadka and James, 1989) a possible change giving this result would be rotation of a carboxylate group from bidentate co-ordination to monodentate co-ordination.

As shown in Figure 6, although the effect of Mg^{2+} on the fluorescence of DMC-labelled ATPase is the same in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$, Mg^{2+} has no effect on the fluorescence of the labelled ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$. This suggests a more major change in the conformation of the ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ than in $\text{di}(\text{C}_{24:1})\text{PC}$.

Finally, it has been reported that binding of thapsigargin to the ATPase results in a large reduction in affinity for Ca^{2+} , attributed to stronger binding to the E_2 conformation (Wictome et al., 1992). As shown in Figure 3, a marked reduction in Ca^{2+} affinity is seen on binding thapsigargin to the ATPase reconstituted in both $\text{di}(\text{C}_{14:1})\text{PC}$ and $\text{di}(\text{C}_{24:1})\text{PC}$. Further, addition of thapsigargin at pH 8.0 to NBD-labelled ATPase reconstituted in $\text{di}(\text{C}_{14:1})\text{PC}$ results in a large decrease in fluorescence intensity (data not shown), as observed for the native ATPase and attributed to a shift of the ATPase to the E_2 conformation. (Wictome et al., 1992). Thus the thapsigargin binding-site is maintained despite the change in the Ca^{2+} binding-sites on reconstitution with $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$.

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