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Binding of Ca^{2+} to the (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum: equilibrium studies

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Equilibrium fluorescence methods have been used to establish a model for Ca^{2+} binding to the (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum and to define the effects of H^+ and Mg^{2+} on Ca^{2+} binding. The basic scheme proposed is: E2 $\rightleftharpoons$ E1 $\rightleftharpoons$ E1Ca $\rightleftharpoons$ E1′Ca $\rightleftharpoons$ E1′Ca_2. The E1 conformation of the ATPase initially has one high-affinity binding site for Ca^{2+} exposed to the cytoplasmic side of the sarcoplasmic reticulum, but in the E2 conformation this site is unable to bind Ca^{2+}; Ca^{2+} does not bind to luminal sites on E2. The second, outer, Ca^{2+}-binding site on the ATPase is formed after binding of Ca^{2+} to the first, inner, site on E1 and the E1Ca $\rightleftharpoons$ E1′Ca conformation change. The pH- and Mg^{2+}-dependence of the E2 $\rightleftharpoons$ E1 equilibrium has been established after changes in the fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole. It is proposed that Mg^{2+} from the cytoplasmic side of the sarcoplasmic reticulum can bind to the first Ca^{2+}-binding site on both E1 and E2. It is proposed that the change in tryptophan fluorescence intensity after binding of Ca^{2+} follows from the E1Ca $\rightleftharpoons$ E1′Ca change. The pH- and Mg^{2+}-dependence of this change defines H^+- and Mg^{2+}-binding constants at the two Ca^{2+}-binding sites. It is proposed that the change in tryptophan fluorescence observed on binding Mg^{2+} follows from binding at the second Ca^{2+}-binding site. Effects of pH and Mg^{2+} on the fluorescence of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin are proposed to follow from binding to a site on the ATPase, the 'gating' site, which affects the affinity of the first Ca^{2+}-binding site for Ca^{2+} and affects the rate of dissociation of Ca^{2+} from the ATPase.

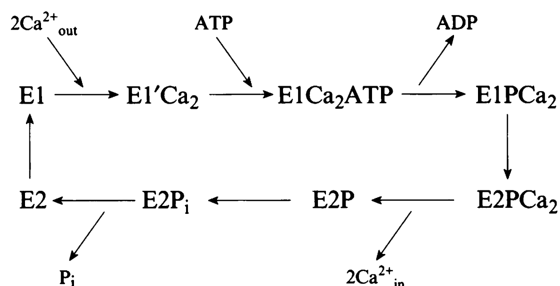
INTRODUCTION

The (Ca^{2+}–Mg^{2+})-ATPase is responsible for the accumulation of Ca^{2+} by the sarcoplasmic reticulum (SR) of skeletal muscle. Binding of Ca^{2+} to the ATPase initiates a change in the catalytic specificity from a conformation in the absence of Ca^{2+} in which the ATPase can be phosphorylated by P_i to one in the presence of Ca^{2+} in which it can be phosphorylated by ATP. Two high-affinity binding sites for Ca^{2+} exist on the ATPase, accessible from the cytoplasmic side of the SR. Site-directed mutagenesis (Clarke et al., 1990) has located the Ca^{2+}-binding sites within transmembrane α-helices of the ATPase, in agreement with fluorescence labelling experiments (Mata et al., 1993). The relationship between the two Ca^{2+}-binding sites on the ATPase is still unclear.

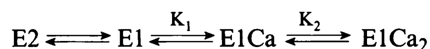
The mechanism of the ATPase is usually discussed in terms of Scheme 1 (de Meis, 1981). In the absence of any ligands, the ATPase exists in one of two conformations, E1 or E2. In the E1 conformation, the ATPase can bind two Ca^{2+} ions on the cytoplasmic side of the SR. After binding of MgATP, the ATPase is phosphorylated to give E1PCa_2. After a conformation change to E2PCa_2, Ca^{2+} dissociates into the lumen of the SR, the ATPase dephosphorylates and recycles to E1. In the original formulation of the model, all E2 states of the ATPase (phosphorylated and non-phosphorylated) were postulated to have two low-affinity binding sites for Ca^{2+}, facing the lumen of the SR (de Meis, 1981). A variety of kinetic experiments have, however, suggested that the unphosphorylated E2 state cannot bind Ca^{2+} (Petithory and Jencks, 1988).

The existence of two unliganded conformations (E1 and E2) for the ATPase is controversial. It has been shown that a wide range of experiments on the rate of phosphorylation of the ATPase by ATP can be interpreted without the need for a second conformation (Stahl and Jencks, 1987; Petithory and Jencks, 1988). However, a variety of spectroscopic experiments suggest that the ATPase does not exist in a single conformational state in the absence of Ca^{2+}. Thus it has been shown that the fluorescence of the ATPase labelled with fluorescein isothiocyanate (FITC) decreases on addition of Ca^{2+} and increases on addition of vanadate (an analogue of phosphate). These changes could follow directly from the binding of Ca^{2+} or vanadate, or, in terms of Scheme 1, could reflect a transition between the E2 and E1 states of the ATPase, with the ATPase being in the E1 state in the presence of Ca^{2+} and in the E2 state in the presence of vanadate. The observation that the relative magnitudes of the fluorescence responses to Ca^{2+} and vanadate were pH-sensitive was taken as evidence in favour of the latter model, with the E2–E1 equilibrium being pH-sensitive, low pH favouring the E2 conformation (Pick, 1982; Pick and Karlish, 1982). The value of the equilibrium constant E1/E2 was estimated to be 0.5 at pH 7.2 (Froud and Lee, 1986a). Wakabayashi et al. (1990a,b) have suggested that the fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) is also sensitive to the E2–E1 change and can be used to study the effect of pH on this transition, giving values for the E1/E2 equilibrium constant comparable with those estimated from the fluorescence of FITC-labelled ATPase (see below). As the fluorescence of the NBD probe is itself independent of pH, the pH-dependence of the fluorescence of NBD-labelled ATPase must either reflect the effect of an ionization in a region close to the probe on the ATPase (a localized change) or a more global change in conformation as in the E2–E1 model. The observation that inhibitors of the ATPase, such as thapsigargin or *t*-butyl hydroquinone (the effects of which on the kinetics of the ATPase are consistent with binding to the E2 conformation), produce the same effect on the fluorescence of NBD-labelled ATPase as vanadate or low pH is consistent with a global change on the ATPase (Wictome et al., 1992a,b). This is also consistent with the observed changes in

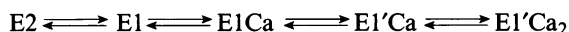
Abbreviations used: Ado*PP*[NH]*P*, adenosine 5′-[$\beta\gamma$-imido]triphosphate; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; di($C_{14:1}$)PC, dimyristoleoylphosphatidylcholine; FITC, fluorescein isothiocyanate; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; SR, scaroplasmic reticulum.



Scheme 1



Scheme 2



Scheme 3

fluorescence of FITC-labelled ATPase on addition of thapsigargin (Sagara et al., 1992).

A pH-dependent conformational change between two conformations E2 and E1, with only the latter being able to bind Ca^{2+} at high-affinity sites, will have important consequences for Ca^{2+} binding to the ATPase. The binding of Ca^{2+} to the ATPase has been shown to be co-operative (Dupont, 1976; Ikemoto et al., 1978; Dupont and Leigh, 1978; Guillain et al., 1980; Inesi et al., 1980; Champeil et al., 1983; Gould et al., 1986; Froud and Lee, 1986a; Inesi, 1987) consistent with Scheme 2.

To explain the observed co-operativity in Ca^{2+} binding (Hill coefficient close to 2) the equilibrium constant E1/E2 would have to be less than about 0.01 if the two Ca^{2+}-binding sites were of equal intrinsic affinity (binding constants $K_1 = K_2$). However, such a value for the ratio E1/E2 is inconsistent with the values obtained from studies of FITC-labelled and NBD-labelled ATPase (Pick, 1982; Froud and Lee, 1986a). The observed co-operativity of Ca^{2+} binding could, however, be explained if the intrinsic affinity of the second site for Ca^{2+} was greater than that for the first site ($K_2 > K_1$). This, combined with the observation that the dissociation of Ca^{2+} from the ATPase is sequential (Ikemoto et al., 1981; Dupont, 1982; Inesi, 1987; Moutin and Dupont, 1991; Orlowski and Champeil, 1991), suggests Scheme 3 for Ca^{2+} binding, with a single binding site for Ca^{2+} exposed to the cytoplasm on E1, the second higher-affinity site forming on transition to E1′Ca after binding of the first Ca^{2+}.

It is likely that Mg^{2+} will be able to bind to the two high-affinity binding sites for Ca^{2+} on the ATPase in competition with Ca^{2+} (Gould et al., 1986). However, Moutin and Dupont (1991) have reported that binding of Mg^{2+} to the ATPase increases the rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase, implying the Mg^{2+} can also bind to site(s) on the ATPase other than at the Ca^{2+}-binding sites. Binding of Mg^{2+} to the Ca^{2+}-bound ATPase has also been detected fluorimetrically, from changes in the fluorescence of the ATPase labelled at Cys-344 with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) (Stefanova et al., 1992). Here we study this effect of Mg^{2+} in some detail. Ca^{2+} binding to the ATPase can be studied directly using $^{45}Ca^{2+}$ or indirectly by following changes in tryptophan fluorescence of the ATPase (Dupont and Leigh, 1978; Guillain et al., 1980, 1981; Verjovski-Almeida and Silva, 1981; Champeil et al., 1983; Fernandez-Belda et al., 1984; Scofano et al., 1985; Moutin and Dupont, 1991). We show that studies of the effects of pH and Mg^{2+} on equilibrium binding of Ca^{2+} to the ATPase can be combined with studies of the E2–E1 equilibrium using NBD-labelled ATPase to define binding constants for Scheme 3. In the following paper (Henderson et al., 1994), we show that this same model is consistent with studies of the rates of Ca^{2+} binding and dissociation.

MATERIALS AND METHODS

Br-DMC was obtained from Molecular Probes. SR from rabbit skeletal muscle and the purified (Ca^{2+}–Mg^{2+})-ATPase were prepared as described in Stefanova et al. (1992). For some experiments, SR was washed with deoxycholate to remove luminal proteins such as calsequestrin by the method of Meissner et al. (1973). SR was diluted to 2.5 mg/ml in buffer (10 mM Tris/HCl, pH 7.4, 0.3 M sucrose, 0.5 M KCl, 1 mM EDTA, 1.25 mM $MgCl_2$, 10 μM $CaCl_2$, 0.5 mg/ml deoxycholate) and incubated on ice for 10 min. The sample was then centrifuged at 110000 ***g*** at 4 °C for 75 min and the pellet resuspended in a small volume of wash buffer (5 mM Hepes/KOH, pH 7.4, 0.3 M sucrose, 0.5 M KCl, 5 mM $MgCl_2$, 10 μM $CaCl_2$) and the sample frozen until use.

The ATPase was labelled with NBD by the protocol of Wakabayashi et al. (1990a) with some modifications. ATPase or SR (20 mg of protein) was suspended in buffer (10 ml; 13 mM MOPS/NaOH, pH 7.0, 0.2 M KCl, 1 mM $CaCl_2$) containing 0.6 mg/ml adenosine 5′-[$\beta\gamma$-imido]triphosphate (Ado*PP*[NH]*P*). Then 120 μl of a stock solution of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in ethanol (5 mg/ml) was added with mixing and the mixture incubated in the dark for 1 h at 25 °C. The reaction was then stopped by diluting into 4 vol. of ice-cold buffer (10 mM histidine/HCl, pH 6.8, 0.1 M KCl, 0.3 M sucrose) and centrifuged at 37000 ***g*** for 30 min at 4 °C. The pellet was resuspended in buffer (4.5 ml; 20 mM Mops/NaOH, pH 7.0, 0.1 M NaCl, 0.3 M sucrose) containing 0.3 mg/ml Ado*PP*[NH]*P*. Dithiothreitol (0.5 ml; 100 mM) was then added followed by 10 min incubation in the dark at 25 °C. The reaction was stopped by dilution into 8 vol. of ice-cold buffer (20 mM Mops/NaOH, pH 7.0, 0.1 M NaCl, 0.3 M sucrose) and centrifuged for 30 min at 37000 ***g*** at 4 °C. The pellet was resuspended in buffer and frozen until used. Sealed SR vesicles were loaded with 20 mM Ca^{2+} by incubation on ice for 2 h.

The ATPase was labelled with Br-DMC as described in Stefanova et al. (1992).

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 and 520 nm respectively. To reduce the signal due to scattered light, the excitation beam was passed through a 450 nm long-wavelength cut-off filter (450FLO T-50, Andover Corporation) and the emission was passed through a Hoya Y50 500 nm short-wavelength cut-off filter. 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 and emission wavelengths of 315 and 340 nm for measurements of the response to Mg^{2+} and Ca^{2+} respectively.

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

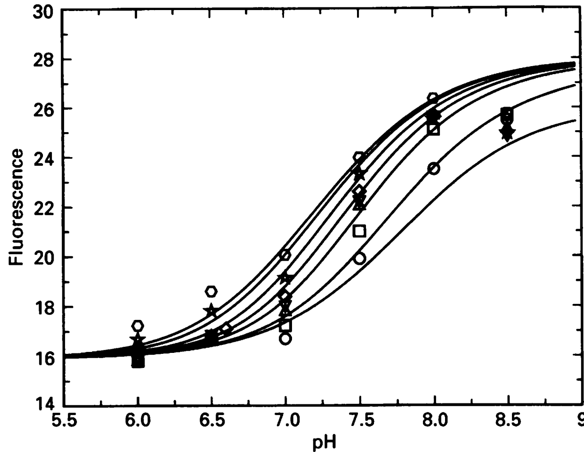
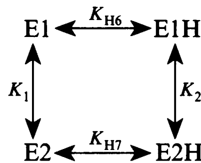


Figure 1 Effects of pH and Mg^{2+} on the fluorescence intensity of NBD-labelled ATPase

NBD-labelled ATPase was incubated in 3 mM Mes/Tris, pH 6.0, buffer containing 1 mM EGTA and no Mg^{2+} (○) or Mg^{2+} at the following concentrations (mM); 1 (□); 5 (△); 10 (▽); 20 (◇); 50 (★); 100 (○). The pH was increased in 0.5 pH unit steps by addition of a concentrated Tris solution. Fluorescence intensities were corrected for dilution. The curves are simulations calculated by using the parameters in Table 1, assuming relative fluorescence intensities of 15.8 and 28.6 for E2 and E1 forms respectively.



Scheme 4

Table 1 Reaction steps and equilibrium constants for binding of Ca^{2+}, Mg^{2+} and H^+ to the (Ca^{2+}–Mg^{2+})-ATPase

The superscript c denotes binding of Mg^{2+} and H^+ to the Ca^{2+}-binding sites and the superscript g denotes binding of Mg^{2+} and H^+ to the 'gating' site. K_{C1} is the effective binding constant for Ca^{2+} at the first site (Scheme 5) calculated from K_{C1a}, K_{C1b}, K_{C1c} and K_{C1d} (Scheme 6).

Reaction step	Equilibrium constant symbol	Value
E2→E1	K_1	4.0
E1→E1H	K_{H6}	5.0×10^5 M^{-1}
E2→E2H	K_{H7}	3.0×10^8 M^{-1}
E1→E1H^C	K_{H4}	5.0×10^9 M^{-1}
E2→E2H^C	K_{H5}	5.0×10^9 M^{-1}
E1→E1Mg^C	K_{Mg1}*	K_{C1}/2000
E2→E2Mg^C	K_{Mg2}	$K_{Mg1}\times0.21$
E1→E1Ca	K_{C1a}	8.8×10^6 M^{-1}
E1H→E1HCa	K_{C1b}	3.5×10^8 M^{-1}
E1HMg→E1HMgCa	K_{C1c}	3.5×10^8 M^{-1}
E1Mg→E1MgCa	K_{C1d}	8.8×10^6 M^{-1}
E1→E1Mg^g	K_6	500 M^{-1}
E1Ca→E1MgCa^g	K_9	500 M^{-1}
E1→E1H^g	K_{H3}	1.25×10^7 M^{-1}
E1Ca→E1HCa^g	K_{H2}	5.0×10^8 M^{-1}
E1H→E1HMg^g	K_7	500.0 M^{-1}
E1HCa→E1HMgCa^g	K_8	500.0 M^{-1}
E1Ca→C1′Ca	K_{C3}	1.0
E1′Ca→E1′Ca_2	K_{C2x}	1.3×10^{10} M^{-1}
E1′CaH^C→E1′Ca_2H^C	K_{C2y}	2.2×10^6 M^{-1}
E1′Ca→E1′CaH^C	K_{H8}	2.0×10^{10} M^{-1}
E1′Ca→E1′CaMg^C	K_{Mg3}	7.8×10^5 M^{-1}

* Value for the NBD-labelled ATPase.

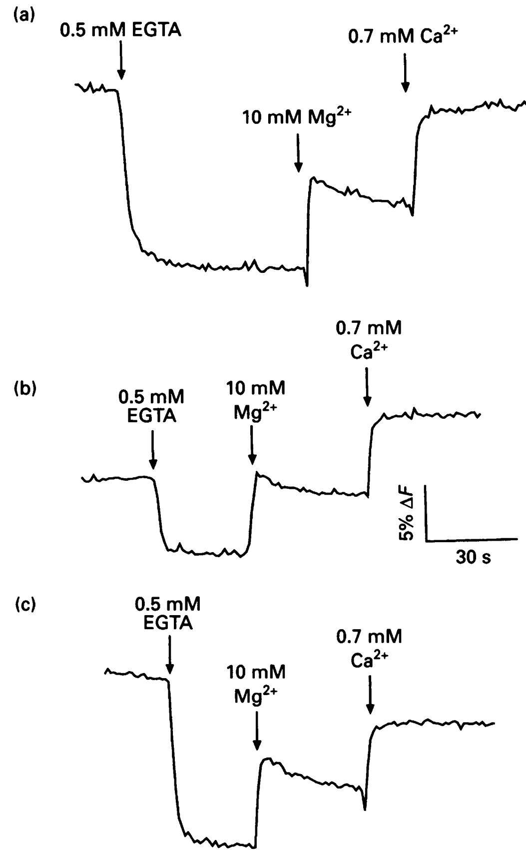


Figure 2 Effects of Mg^{2+} and Ca^{2+} on the fluorescence of NBD-labelled SR

In all experiments, labelled SR (80 μg/ml) was incubated in buffer (50 mM Mops/KOH, pH 7.0) and the NBD fluorescence monitored after sequential additions to give final concentrations of 0.5 mM EGTA, 10.0 mM Mg^{2+} and 0.7 mM Ca^{2+}. (**a**) Deoxycholate-treated SR; (**b**) SR; (**c**) Ca^{2+}-loaded SR.

RESULTS

NBD fluorescence

Wakayabashi et al. (1990b) have studied the fluorescence properties of NBD-labelled ATPase and have suggested that NBD fluorescence is sensitive to the E2–E1 conformation change, with a higher fluorescence intensity in the E1 conformation than in the E2 conformation. As shown in Figure 1 and by Wakayabashi et al. (1990b), the fluorescence intensity of NBD-labelled ATPase increases with increasing pH. The pH-dependence can be shown to fit well to the simple scheme E⇄EH where E is a high-fluorescence form (equivalent to E1 in Scheme 1) and EH is a low-fluorescent form (equivalent to E2 in Scheme 1) with a pK of 8.0. However, as reported by Wakabayashi et al. (1990b) and in the following paper (Henderson et al., 1994), the finding that the rate of the change of NBD-labelled ATPase from low- to high-fluorescent forms is pH-dependent is inconsistent with this scheme. The simplest scheme giving a pH-dependent rate for the change is Scheme 4. In this scheme, the pH-dependence of the E1/E2 equilibrium will be largely defined by the proton-binding constant for E2, K_{H7}. The value of K_{H7} also has to be consistent with measurements of the pH-dependence of the rate of the E2–E1 transition (Henderson et al., 1994). The value of K_1 (the equilibrium constant for the unprotonated forms E1/E2) largely determines the ratio E1/E2 at high pH and the value of the proton-binding constant for E1, K_{H6}, determines the proportion

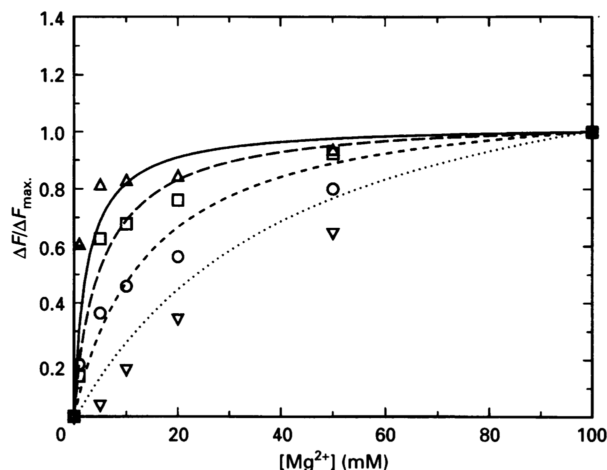
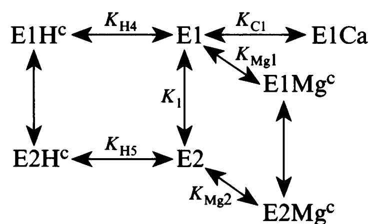


Figure 3 Effects of pH and Mg^{2+} on the fluorescence intensity of NBD-labelled ATPase

The data from Figure 1 are plotted as $\Delta F/F_{max.}$ against Mg^{2+} concentration, with $F_{max.}$ being the fluorescence value observed in 100 mM Mg^{2+}, for pH values of: 6.5 (▽); 7.0 (○); 7.5 (□); 8.0 (△). The curves are simulations calculated as described in the legend to Figure 1.



Scheme 5

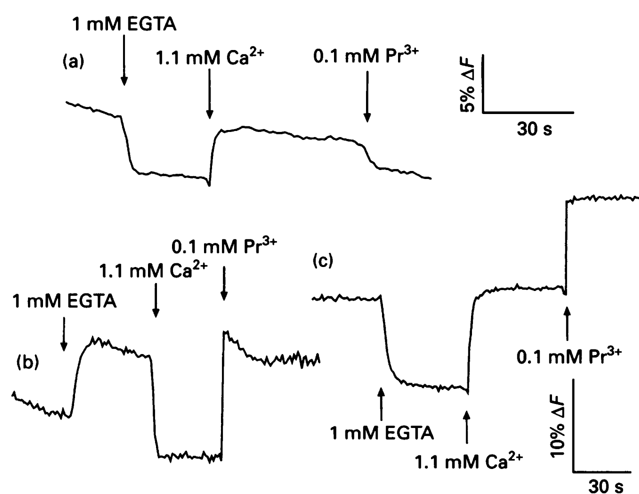


Figure 4 Effects of Ca^{2+} and Pr^{3+} on tryptophan and NBD fluorescence

(**a**) Change in tryptophan fluorescence intensity for the (Ca^{2+}–Mg^{2+})-ATPase (0.7 μM) in buffer (150 mM Mops, 80 mM Tris, pH 7.2) on addition of 1 mM EGTA, 1.1 mM Ca^{2+} and 0.1 mM Pr^{3+} (final concentrations). (**b**) and (**c**) Changes in NBD fluorescence intensity for NBD-labelled ATPase (0.7 μM) in (**b**) 100 mM Tris/27 mM Mes, pH 8.5 or (**c**) 150 mM Tris/130 mM Mes, pH 6.0, on addition of 1 mM EGTA, 1.1 mM Ca^{2+} and 0.1 mM Pr^{3+} (final concentrations).

of E1 forms (E1 and E1H) present at acid pH. Wakabayashi et al. (1990b) have shown that addition of vanadate to NBD-labelled ATPase at pH 6.0 results in only a very small decrease in fluorescence intensity, and we have shown that addition of thapsigargin (Wictome et al., 1992a,b) to the NBD-labelled ATPase at pH 6.0 results in an approx. 2% decrease in fluorescence intensity. As shown in Figure 1, the data fit well to Scheme 4 with pK values of 8.5 and 5.7 for E2 and E1 respectively, with a value for K_1 of 4.0 (Table 1). These parameters give the proportion of E1 forms at pH 6.0 of 2%.

As shown in Figure 2, addition of Mg^{2+} to NBD-labelled ATPase in SR vesicles results in an increase in fluorescence intensity, attributable to a shift in the E2–E1 equilibrium towards E1. If Mg^{2+} bound only to the E1 form of the ATPase then, at high Mg^{2+}, all the ATPase would be present as E1Mg. However, as shown in Figure 3, plots of fluorescence intensity against Mg^{2+} concentration reach limiting values at high (100 mM) Mg^{2+}, and the fluorescence intensity values observed at each pH at 100 mM Mg^{2+} do not correspond to the maximum fluorescence value observed at pH 8.5 in the presence of 100 mM Mg^{2+} (Figure 1). Mg^{2+} must therefore be able to bind to both E1 and E2 forms of the ATPase so that the ATPase is not all in E1 at high Mg^{2+}. A higher affinity for Mg^{2+} for E1 explains the shift to E1 at high Mg^{2+} concentrations. Plotting the data at each pH as the fractional change in fluorescence makes it clear that Mg^{2+} binding is competitive with H^+ binding (Figure 3) with a pK of 9.7. With the values of the binding constants for Mg^{2+} to E1 and E2 given in Table 1, good fits to the data can be obtained (Figures 1 and 3). As shown below, the pK derived for this site also fits studies of the effect of pH on the binding of Ca^{2+} to the first Ca^{2+}-binding site on the ATPase, suggesting that the binding site for Mg^{2+} affecting the E1/E2 equilibrium is the first Ca^{2+}-binding site, as in Scheme 5.

Comparing the effects of Mg^{2+} on the fluorescence of NBD-labelled ATPase in sealed and leaky vesicles gives information about the transmembrane location of the Mg^{2+}-binding sites. Effects of Mg^{2+} on sealed SR vesicles (Figure 2b) and on vesicles made leaky by treatment with deoxycholate (Figure 2a) are identical, indicating that the Mg^{2+}-binding sites on both E1 and E2 forms of the ATPase must be accessible from the cytoplasm. Figure 2 also shows the response of NBD-labelled ATPase in sealed SR vesicles passively loaded with Ca^{2+}. The identical response to Mg^{2+} for the loaded vesicles argues that Ca^{2+} cannot bind to internal sites affecting the E1–E2 equilibrium.

Tryptophan fluorescence

Binding of Ca^{2+} to the ATPase results in an increase in tryptophan fluorescence intensity (Figure 4). Plots of changes in tryptophan fluorescence against pCa for NBD-labelled ATPase are shifted by about 0.2 pCa units relative to those of the unlabelled ATPase (results not shown). Plots of tryptophan and NBD fluorescence intensities on addition of Ca^{2+} are very similar for the labelled ATPase (results not shown). However, tryptophan and NBD fluorescence are monitoring different events on the ATPase. This is clear in experiments showing the response to Pr^{3+}. It has been shown that addition of Pr^{3+} to the Ca^{2+}-bound ATPase results in biphasic displacement of Ca^{2+}, with Pr^{3+} being able to bind to sites on the ATPase other than the Ca^{2+}-binding sites (Squier et al., 1990; Henao et al., 1992). As shown in Figure 4(a), addition of Pr^{3+} to the Ca^{2+}-bound ATPase results in a biphasic decrease in tryptophan fluorescence intensity, with an initial fast phase being followed by a much slower phase, decreasing at long times to the fluorescence level observed in EGTA (results not shown). In contrast, addition of Pr^{2+} to the NBD-labelled ATPase in the

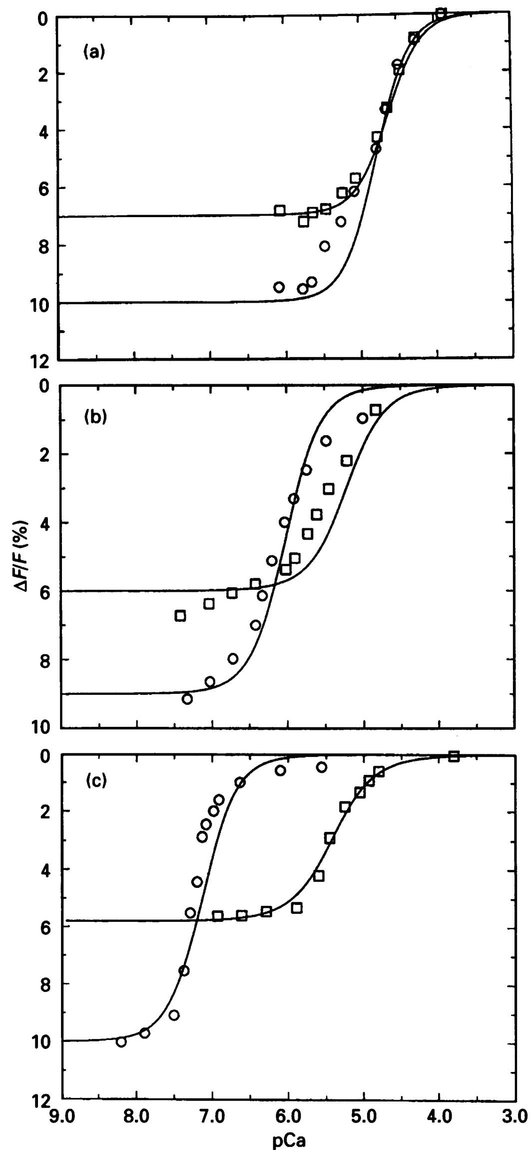


Figure 5 Ca^{2+}-dependence of tryptophan fluorescence intensity in the presence and absence of Mg^{2+}

The figure shows the decrease in tryptophan fluorescence intensity of the ATPase (0.7 μM) 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 8.5 (in 27 mM Mes/100 mM Tris). ○, Absence of Mg^{2+}; □, presence of 20 mM Mg^{2+}. The lines are simulations of Ca^{2+} occupancy of the ATPase calculated using the parameters in Table 1, and scaled to match the observed change in fluorescence intensity.

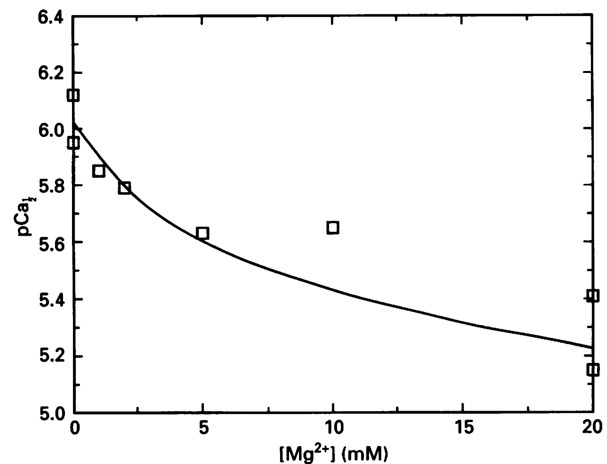


Figure 6 Effect of Mg^{2+} on Ca^{2+}-binding

Shown are the concentrations of Ca^{2+} ($pCa_{\frac{1}{2}}$) required to produce half the fluorescence response to Ca^{2+} at pH 7.2 at the given Mg^{2+} concentrations. The line is a simulation calculated by using parameters in Table 1.

Table 2 Effect of Mg^{2+} on the tryptophan fluorescence intensity of the ATPase

The Mg^{2+}-dependence of the change in tryptophan fluorescence intensity for the ATPase (0.7 μM) was determined with excitation and emission wavelengths of 290 and 315 respectively in 130 mM Mes/50 mM Tris (pH 6.0), 150 mM Mops/80 mM Tris (pH 7.2) or 27 mM Mes/100 mM Tris, pH 8.5. All buffers contained 0.3 mM EGTA.

pH	K_d (observed) (mM)	Observed percentage fluorescence change	Calculated K_d (mM)	
			$K_{Mg3} = 7.8 \times 10^5$	$K_{Mg3} = 5.2 \times 10^{5*}$
6	> 10	−0.8	25	37
7	3.5 ± 0.9	5.5	2.5	3.9
8.5	0.25 ± 0.15	3.8	0.1	0.13

* Alternative value, as described in the text.

presence of Ca^{2+} at either pH 6.0 or 8.5 results in an increase in fluorescence intensity, whereas addition of Ca^{2+} results in an increase in NBD fluorescence at pH 6.0 but a decrease at pH 8.5 (Figure 4). As described above, NBD fluorescence is believed to respond to the E1–E2 transition but many studies have shown that the increase in tryptophan fluorescence intensity observed on addition of Ca^{2+} parallels Ca^{2+} binding to the ATPase measured directly using $^{45}Ca^{2+}$ (Dupont, 1982; Fernandez-Belda et al., 1984; Scofano et al., 1985; Orlowski and Champeil, 1991).

Figure 5 shows tryptophan fluorescence intensity as a function of Ca^{2+} concentration in the presence and absence of Mg^{2+} at pH 6.0, 7.2 and 8.5. The presence of Mg^{2+} results in a shift in the Ca^{2+} response curves to higher concentrations of Ca^{2+}, the effect being particularly marked at pH 8.5. Figure 6 shows the shift in the Ca^{2+} concentration giving 50% fluorescence response as a function of Mg^{2+} concentration at pH 7.2.

Guillain et al. (1982) have shown that the effects of Mg^{2+} on the tryptophan fluorescence of the ATPase are distinct from those of Ca^{2+}. At pH 6.0, a small decrease in fluorescence intensity at 315 nm is observed on addition of Mg^{2+}, whereas increases in intensity are observed at higher pH values (Table 2). The small magnitudes of the changes observed, together with the necessity to correct for sample dilution, limits the accuracy of the determinations, but binding constants for Mg^{2+} obtained at pH 7.0 and 8.5 are given in Table 2.

DMC fluorescence

It has been shown that the DMC fluorescence of the ATPase labelled with Br-DMC decreases on addition of Mg^{2+} (Stefanova et al., 1992). Figure 7 shows the effect of Mg^{2+} as a function of pH in the presence or absence of Ca^{2+}; in the absence of Ca^{2+}, a

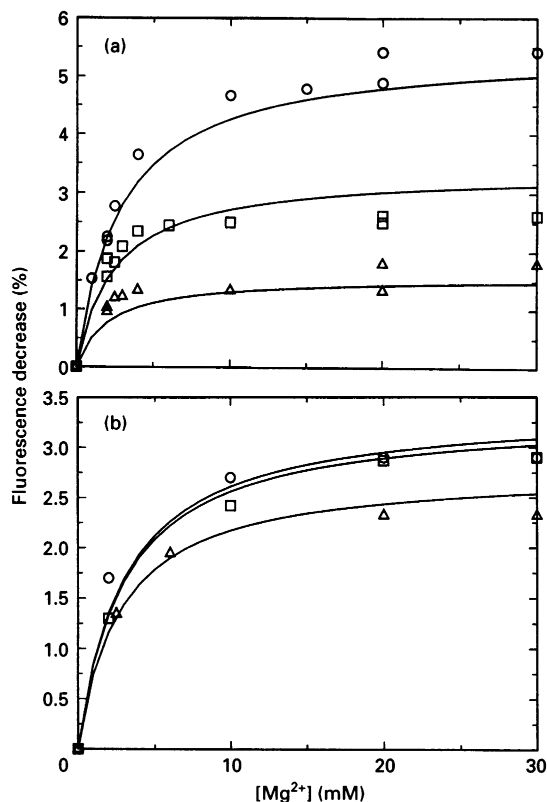
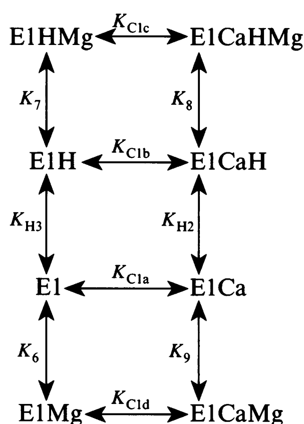


Figure 7 Effect of Mg^{2+} on the fluorescence intensity of DMC-labelled ATPase

DMC-labelled ATPase was incubated in 40 mM Tris/maleate at pH 6.0 (○), 7.0 (□) or 8.0 (△) in 1 mM EGTA (**a**) or 1 mM Ca^{2+} (**b**). Lines are simulations calculated using the parameters in Table 1, as described in the text.



Scheme 6

large decrease in the magnitude of the response to Mg^{2+} is observed, but with the K_d for Mg^{2+} remaining constant at about 2 mM. In the presence of Ca^{2+}, the effect of pH is less marked, and again the K_d for Mg^{2+} is about 2 mM. The observation of a pH-independent K_d for Mg^{2+} with a pH-dependent magnitude of change (different in the presence and absence of Ca^{2+}) is consistent with Scheme 6.

The assignment of the Ca^{2+}-free and Ca^{2+}-bound forms in Scheme 6 as E1 and E1Ca respectively will be justified in the Discussion. If the fluorescence decreases resulting from binding of Mg^{2+} to the protonated forms E1H and E1CaH are greater than those on binding of Mg^{2+} to the unprotonated forms E1 or E1Ca, then binding of Mg^{2+} will result in a larger decrease in fluorescence intensity at low pH than at high pH, as observed. If the proton-binding constant K_{H2} for E1Ca is greater than that for E1 (K_{H3}), then the effect of decreasing pH will be less in the presence of Ca^{2+} than in its absence, also as observed. With equal Mg^{2+}-binding constants for all forms of the ATPase, the K_d for Mg^{2+} will be unaltered by addition of Ca^{2+} or by changing pH. As shown in Figure 7, the experimental data fit to the observed data in terms of this scheme with a value for the Mg^{2+}-binding constant ($K_6 = K_7 = K_8 = K_9$) of 500.0 and values of the proton-binding constants K_{H2} and K_{H3} of 5.0×10^8 and 1.25×10^7 respectively, assuming a decrease in fluorescence intensity after Mg^{2+} binding to the unprotonated form that is 20% of that resulting from binding to the protonated form. In terms of the full model for Ca^{2+} binding described later, H^+ and Mg^{2+} binding at this site is assumed to be equal for E1 and E2 and for E1Ca, E1′Ca and E1′Ca_2.

DISCUSSION

The basis of the observed fluorescence changes

Different fluorescence probes monitor different conformational changes on the ATPase. Many studies have shown that tryptophan fluorescence changes parallel Ca^{2+} binding to the ATPase (Dupont, 1982; Fernandez-Belda et al., 1984; Scofano et al., 1985; Orlowski and Champeil, 1991) whereas NBD fluorescence appears to monitor the E2–E1 transition (Wakabayashi et al., 1990a,b; Wictome et al., 1992a,b). This difference is clear in experiments with Pr^{3+}. Pr^{3+} has been shown to displace Ca^{2+} from the Ca^{2+}-bound ATPase, probably as a result of binding to sites on the ATPase other than the Ca^{2+}-binding sites (Squier et al., 1990; Henao et al., 1992). As shown in Figure 4, and as reported previously (Squier et al., 1990; Henao et al., 1992), addition of Pr^{2+} to the Ca^{2+}-bound ATPase results in biphasic decrease in tryptophan fluorescence intensity, with an initial fast phase being followed by a much slower phase [this is clearer in stop-flow measurements (Henderson et al., 1994)]. However, NBD fluorescence responds differently to the addition of Pr^{3+}. At both pH 6 and 8.5, addition of Pr^{3+} to the Ca^{2+}-bound ATPase results in an increase in fluorescence intensity, in contrast with Ca^{2+}, the addition of which results in an increase in NBD fluorescence at pH 6.0 but a decrease at pH 8.5 (Figure 4). The effects of Ca^{2+} on NBD fluorescence can be understood in terms of a scheme in which E2 and E1 are low- and high-fluorescence states respectively and E1′Ca_2 is a state of intermediate fluorescence intensity (Wakabayashi et al., 1990b). The effects of Pr^{3+} on NBD fluorescence suggest that Pr^{3+} binds to the ATPase and shifts the equilibrium strongly to E1 with displacement of Ca^{2+}. Thus the fluorescence intensity is the same for the ATPase at pH 8.5 in the presence of EGTA, where it will be largely E1 (Figure 1) and in the presence of Pr^{3+}. At pH 6, in EGTA the ATPase is predominantly in the E2 state (Figure 1) and addition of Pr^{3+} after addition of Ca^{2+} therefore results in an increase in fluorescence intensity.

The change in tryptophan fluorescence intensity on binding Ca^{2+} is unlikely to follow directly from occupation of the two Ca^{2+}-binding sites on the ATPase; this would require equal changes in fluorescence for binding at the two Ca^{2+}-binding sites and there is no reason to expect an equal distribution of the 13

tryptophan residues in the ATPase about the two binding sites. Figure 8 illustrates a model which could, however, account for a tryptophan fluorescence change that reflects Ca^{2+} occupancy. The model envisages a conformation change on the ATPase after binding of the first Ca^{2+} ion. In the absence of Ca^{2+}, only a single, inner site is available for Ca^{2+} binding. After binding of Ca^{2+} to this initial site to give E1Ca, the ATPase undergoes a conformational change to give E1′Ca with the appearance of the second Ca^{2+}-binding site. Binding of Ca^{2+} to this second, outer, site then gives E1′Ca_2. In this model, it is proposed that the states E1′Ca and E1′Ca_2 are states of high fluorescence, and, with the equilibrium constant for the E1Ca–E1′Ca step equal to one, the relative fluorescence changes on binding one and two Ca^{2+} ions will be in the proportion 0.5:1, as required. An alternative would be binding of the two Ca^{2+} ions in a channel-like structure with the change in tryptophan fluorescence occurring after occupancy of the outer of the two Ca^{2+}-binding sites again with an equilibrium constant of 1 for the E1Ca–E1′Ca step. However, as shown in the following paper (Henderson et al., 1994), such a model is not consistent with rapid kinetic experiments.

The fluorescence of the ATPase labelled with Br-DMC at Cys-344 is insensitive to the binding of Ca^{2+} and is therefore also insensitive to the E2–E1 transition. However, it is sensitive to the binding of Mg^{2+} and to phosphorylation (Stefanova et al., 1992).

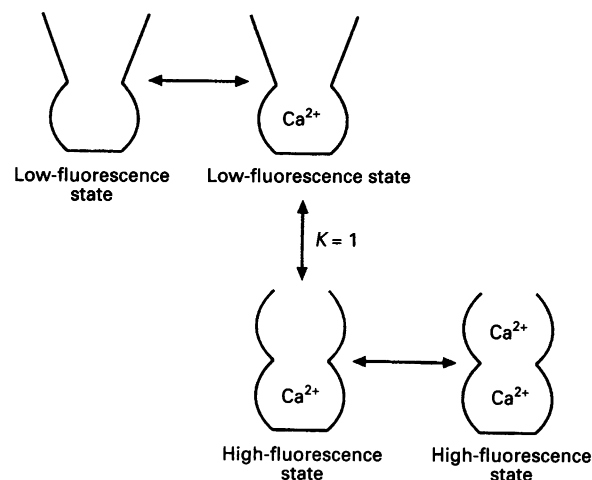


Figure 8 Model for Ca^{2+} binding to the ATPase

Binding is proposed to involve a conformational change after binding of the first Ca^{2+}ion to the ATPase.

Binding of H^+ and Mg^{2+} to the ATPase

As shown in Figures 1 and 3, pH and Mg^{2+} affect the NBD fluorescence of the NBD-labelled ATPase, indicating effects on the E2–E1 equilibrium of the ATPase. As shown in Figures 5 and 6, and as previously reported (Fernandez-Belda et al., 1984; Guillian et al., 1982; Champeil et al, 1983), pH and Mg^{2+} also alter the dependence of tryptophan fluorescence on Ca^{2+} concentration. Mg^{2+} binding to the ATPase in the absence of Ca^{2+} is indicated by the observed changes in tryptophan fluorescence (Table 2). In the presence of Ca^{2+}, Mg^{2+} has no effect on the tryptophan fluorescence of the ATPase (results not shown), suggesting that the effect of Mg^{2+} on tryptophan fluorescence could follow from binding at one or both of the Ca^{2+}-binding sites on the ATPase. The two Ca^{2+}-binding sites on the ATPase are thought to be located in the transmembrane region of the ATPase, involving four acidic residues, one each in the predicted α-helices 4, 5, 6 and 8 (Clarke et al., 1990), and binding of Mg^{2+} and H^+ is likely at such acidic sites.

The fluorescence of DMC-labelled ATPase is sensitive to the binding of Mg^{2+} in both the absence and presence of Ca^{2+} (Figure 7), so that the responsible Mg^{2+}-binding site cannot be one of the two Ca^{2+}-binding sites. Moutin and Dupont (1991) have reported that binding of Mg^{2+} to the Ca^{2+}-bound ATPase increases the rate of dissociation of Ca^{2+}, also implying that Mg^{2+} must be able to bind to the ATPase at sites other than at the Ca^{2+}-binding sites. Finally, phosphorylation of the ATPase by P_i in the absence of Ca^{2+} requires the presence of Mg^{2+}, with a reported K_d value of 9 mM (Punzengruber et al., 1978; de Meis et al., 1982; Froud and Lee, 1986b).

The simplest model that we have been able to develop consistent with the effects of pH and Mg^{2+} on Ca^{2+} binding as monitored by tryptophan fluorescence, on the rates of Ca^{2+} binding and dissociation described in the following paper (Henderson et al., 1994) and on the fluorescence of the NBD- and DMC-labelled ATPase is shown in Figure 9. It is proposed that H^+, Mg^{2+} and Ca^{2+} are in competition for binding at the two Ca^{2+}-binding sites. Binding of H^+ and Mg^{2+} at a third site leads to the observed changes in fluorescence for the DMC-labelled ATPase; this site is referred to as the 'gating' site as it is

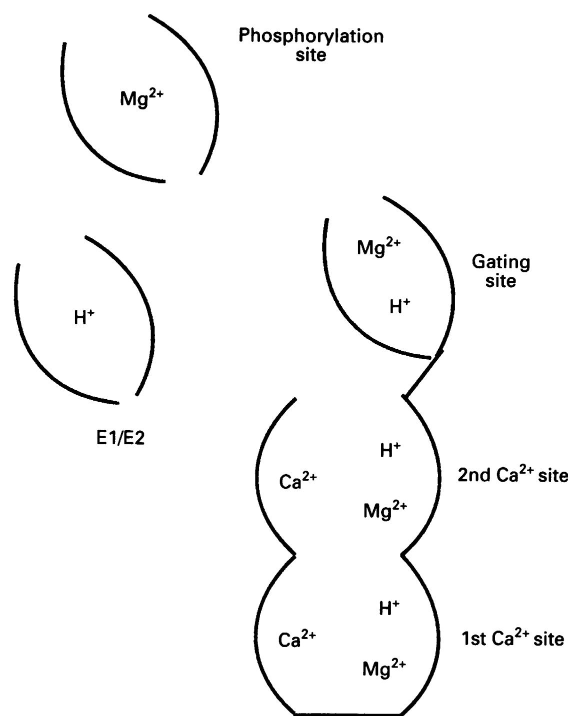


Figure 9 Schematic diagram of the Ca^{2+}- and Mg^{2+}-binding sites on the $(Ca^{2+}–Mg^{2+})$-ATPase

It is proposed that H^+, Mg^{2+} and Ca^{2+} are in competition for binding at the two Ca^{2+}-binding sites on the ATPase. Binding of H^+ and Mg^{2+} at the 'gating' site affects the affinity of the ATPase for Ca^{2+} and the rate of binding and dissociation of Ca^{2+}. Binding of Mg^{2+} at a fourth site is involved in phosphorylation by P_i and could be a Mg^{2+} subsite of the binding site for MgATP. Protonation of a further site on the ATPase affects the E1–E2 equilibrium.

proposed to affect the affinity of the ATPase for Ca^{2+} and the rate of binding and dissociation of Ca^{2+} (Moutin and Dupont, 1991; Henderson et al., 1994). It is proposed that binding of Mg^{2+} at a fourth site is involved in phosphorylation by P_i, but it remains a possibility to be tested that this site and the 'gating'

site could be the same site. Protonation of a further site on the ATPase affects the E1–E2 equilibrium as reflected by changes in the fluorescence of the NBD-labelled ATPase. The model is similar to that presented previously also following Scheme 3 (Froud and Lee, 1986a) in that it is proposed that binding of Ca^{2+} is competitive with H^+ binding at the two Ca^{2+}-binding sites, but is also affected by protonation of two other residues. However, use of the NBD-labelled ATPase allows a more accurate determination of the pH-dependence of the E2–E1 equilibrium than was possible using FITC-labelled ATPase (Froud and Lee, 1986a), requiring a significant change in this part of the model. Incorporation of the effects of H^+ and Mg^{2+} on the rates of Ca^{2+} dissociation from the ATPase (Moutin and Dupont, 1991; Henderson et al., 1994) and on the fluorescence of DMC-labelled ATPase (Stefanova et al., 1992) have also led to significant changes.

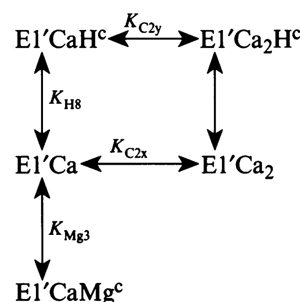
The E2–E1 transition

The effects of pH on the fluorescence of the NBD-labelled ATPase (Figure 1) can be described in terms of Scheme 4 with the parameters given in Table 1. As shown in Figures 1 and 3, the effect of Mg^{2+} on the fluorescence of the NBD-ATPase is pH-dependent and can be described in terms of Scheme 5 with Mg^{2+} binding with different affinities to E1 and E2 (Table 1). In terms of the E2–E1 scheme, the E1 state has two high-affinity binding sites for Ca^{2+} exposed on the cytoplasmic side of the membrane, whereas E2 cannot bind Ca^{2+} cytoplasmically. It is known that binding of Ca^{2+} to the ATPase is competitive with binding of H^+ (Hill and Inesi, 1982; Fernandez-Belda et al., 1984) (see also Figure 5). It is therefore proposed that the Mg^{2+}-binding site affecting the E2–E1 equilibrium is one of the two Ca^{2+}-binding sites; as will be shown, the pH effect on Ca^{2+} binding can be simulated assuming that it is the first of the two sites (Scheme 5; Table 1). As effects of Mg^{2+} on the NBD-labelled ATPase are the same in sealed and leaky vesicles (Figure 2), the Mg^{2+}-binding site on both the E2 and the E1 states must be exposed to the cytoplasm, thus implying the cytoplasmic exposure of the first Ca^{2+}-binding site on both E2 and E1. However, as shown in the following paper (Henderson et al., 1994), the rate of dissociation of Mg^{2+} from the site in E2 appears to be very slow, suggesting partial occlusion of the site in E2. Ca^{2+} in the lumen of the SR is unable to bind to E2 and modify the E2–E1 equilibrium as shown by the identical fluorescence responses recorded for Ca^{2+}-loaded and empty vesicles (Figure 2). The inability of Ca^{2+} to bind to luminal sites on the unphosphorylated ATPase has been previously deduced from a variety of phosphorylation experiments (Petithory and Jencks, 1988).

Wakabayashi et al. (1990a) have reported that the effective binding constant of the ATPase for Ca^{2+} is reduced by a factor of about 2 on labelling with NBD and the shift in the Ca^{2+}-dependence of tryptophan fluorescence on labelling with NBD is consistent with such a reduction in affinity for Ca^{2+} (results not shown). It is therefore possible that the affinity of the first Ca^{2+}-binding site for Mg^{2+} is also reduced on labelling with NBD. As shown in Henderson et al. (1994), a value for the K_{Mg1} three times that in Table 1 is in better agreement with the kinetic data; effects of Mg^{2+} on Ca^{2+} binding can then be fitted by reducing K_{Mg3} by a factor of 1.5, giving a slightly better fit to the Mg^{2+} titration data (Table 2).

The Ca^{2+}-binding sites

Ca^{2+} binding is assumed to follow Scheme 3. Binding at the first Ca^{2+}-binding site on the ATPase has been shown in Scheme 5.



Scheme 7

The equilibrium constant for the E1Ca–E1′Ca step (K_{C3} in Table 1) is fixed at 1 at all H^+ and Mg^{2+} concentrations so that, as described above, the change in tryptophan fluorescence intensity will monitor Ca^{2+} occupancy.

Binding of Ca^{2+} to the second Ca^{2+}-binding site on the ATPase is also assumed to be in competition with binding of Mg^{2+} and H^+. However, simulations show a too strong effect of H^+ if binding of H^+ and Ca^{2+} are strictly competitive, but the data can be matched if weak binding of Ca^{2+} to the protonated site is allowed as in Scheme 7. The values of K_{C2x} and K_{C2y} were chosen to be consistent with the effects of Ca^{2+} concentration on the rate of Ca^{2+} dissociation from the ATPase, as described in the following paper (Henderson et al., 1994).

To maintain the equilibrium constant for the E1Ca–E1′Ca step equal to 1 at all H^+ and Mg^{2+} concentrations, it is necessary to assume that the second Ca^{2+}-binding site binds H^+ and Mg^{2+} in all the conformational states E2, E1, E1Ca and E1′Ca with equal affinity.

Effects of Mg^{2+} and Ca^{2+} on the tryptophan fluorescence of the (Ca^{2+}–Mg^{2+})-ATPase differ in that, whereas Mg^{2+} causes a shift of the emission spectrum to shorter wavelengths, the latter causes an increase in intensity with no shift (Guillain et al., 1982; results not shown). The change in tryptophan fluorescence intensity measured at 315 nm on addition of Mg^{2+} resulting from this shift is pH-dependent (Table 2). The small change in intensity observed at pH 6.0 makes any interpretation unreliable at this pH, but comparing the data at pH 7.0 and 8.5 makes it clear that the affinity for Mg^{2+} increases with increasing pH. Further, the observation that Mg^{2+} has no effect on tryptophan fluorescence emission in the presence of Ca^{2+} suggests that the effect could follow from binding of Mg^{2+} at one of the two Ca^{2+}-binding sites on the ATPase. As shown in Table 2, reasonable fits to the experimental data can be obtained if it is assumed that the Mg^{2+} response follows from binding at the second of the two Ca^{2+}-binding sites, with the binding parameters given in Table 1. This would also be consistent with experiments studying the binding of Ca^{2+} to the ATPase reconstituted with the short-chain phospholipid dimyristoleoylphosphatidylcholine [di($C_{14:1}$)PC]. When the ATPase is reconstituted with di($C_{14:1}$)PC, the stoichiometry of Ca^{2+}-binding changes from the usual two Ca^{2+} ions bound per ATPase molecule to one per ATPase molecule (Michelangeli et al., 1990; Starling et al., 1993). The affinity of the ATPase for Ca^{2+} is, however, slightly increased, suggesting that binding occurs to the second, higher-affinity, Ca^{2+}-binding site on the ATPase (Michelangeli et al., 1990). Further, it is observed that, in the absence of Mg^{2+}, binding of Ca^{2+} to the ATPase reconstituted with di($C_{14:1}$)PC no longer affects tryptophan fluorescence intensity. As binding of Mg^{2+} results in an increase in intensity, as observed for the native ATPase, addition

of Ca^{2+} in the presence of Mg^{2+} therefore results in a decrease in fluorescence intensity (A. P. Starling, J. M. East and A. G. Lee, unpublished work). These observations are consistent with the model proposed above. The increase in tryptophan fluorescence intensity observed for the native ATPase on addition of Ca^{2+} follows from the E1Ca–E1′Ca transition, which does not occur on binding of the single Ca^{2+} ion to the ATPase reconstituted with di($C_{14:1}$)PC. However, if the Mg^{2+} response follows from binding to the second Ca^{2+}-binding site, then this response should be observed for the ATPase reconstituted with di($C_{14:1}$)PC, as observed experimentally.

The 'gating' site

The final binding site that needs to be considered is that for Mg^{2+} affecting the fluorescence of DMC-labelled ATPase in the presence or absence of Ca^{2+}. The observation that pH does not affect the K_d for Mg^{2+} but does affect the magnitude of the fluorescence change observed on binding Mg^{2+} (Figure 7) has been interpreted in terms of Scheme 6. The observed smaller effect of pH in the presence of Ca^{2+} than in its absence is consistent with stronger binding of H^+ to this site in the presence of Ca^{2+}. Binding of Mg^{2+} to the ATPase increases the rate of dissociation of Ca^{2+} from the ATPase in a pH-dependent manner, with an apparent affinity for Mg^{2+} identical with that required to fit the DMC-labelled ATPase data (Henderson et al., 1994). It is therefore proposed that one Mg^{2+}-binding site is responsible for both effects. Further, as Mg^{2+} affects the rate of dissociation of both the first and the second Ca^{2+} ions with equal apparent affinities for Mg^{2+} (Henderson et al., 1994), Mg^{2+} must bind with equal affinity to E1Ca and E1′Ca_2 and thus the change in H^+ affinity in the presence of Ca^{2+} must occur on binding of the first Ca^{2+}, as in Scheme 6. Binding to this site, in the absence of Ca^{2+}, must be identical in E2 and E1, or binding would alter the E2–E1 equilibrium.

The differences in H^+ affinity between E1 and E1Ca mean that binding of H^+ and Mg^{2+} to this site will affect the affinity of the first Ca^{2+}-binding site for Ca^{2+}. The value of the effective binding constant K_{C1} for Ca^{2+} in Scheme 5 can be calculated as a function of pH and Mg^{2+} according to Scheme 6. The equal affinities of E1, E1H, E1Ca and E1CaH for Mg^{2+} imply that $K_{C1c} = K_{C1b}$ and $K_{C1a} = K_{C1d}$ (Scheme 6, Table 1). The higher value for K_{H2} than K_{H3} implies, however, that K_{C1b} is greater than K_{C1a}. In this scheme, just one of the Ca^{2+}-binding parameters K_{C1b} or K_{C1a} is free and has to be chosen to fit the Ca^{2+}-binding data and the rapid kinetic data described in the following paper (Henderson et al., 1994). As H^+ and Mg^{2+} binding to the 'gating' site on all the Ca^{2+}-bound forms of the ATPase (E1Ca, E1′Ca and E1′Ca_2) are assumed equal (defined by K_{H2}, K_8 and K_9 in Scheme 6), binding to this site will have no effect on the E1Ca–E1′Ca equilibrium or on the affinity of the second Ca^{2+}-binding site for Ca^{2+}.

Final comments

The final set of values chosen to fit the data is given in Table 1, and Figures 5 and 6 show that a good fit to the tryptophan fluorescence data is obtained. It should, however, be noted that some variation in Ca^{2+} affinity of the ATPase is observed between preparations (Lee et al., 1983; Orlowski and Champeil, 1991) and thus minor changes in Ca^{2+}-binding parameters might be required on comparing different sets of experimental data.

The location of the H^+-binding sites affecting Ca^{2+} binding are unknown. Experiments using site-directed mutagenesis have suggested that four negatively charged residues, one each in four of the transmembrane α-helices, are involved in Ca^{2+} binding (Clarke et al., 1990). It is likely that the helices are organized with each of the two Ca^{2+}-binding site containing two negatively charged residues (Lee et al., 1993). It is possible that close proximity of the carboxyl groups leads to strong electrostatic interaction between the anionic forms of these groups and anomalously high pK_a values for H^+ dissociation. It is possible therefore that the H^+-binding sites with pK_a values of 9.7 and 10.3 (Table 1) postulated at the first and second Ca^{2+}-binding sites respectively correspond to carboxyl groups. More likely, H^+ binding could occur at other residues, with the carboxyl groups being fully ionized. Direct titration of the ATPase at pH 6.0 with Ca^{2+} leads to the liberation of 1 H^+ per Ca^{2+} bound (Chiesi and Inesi, 1980) in agreement with the model presented here.

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