Binding of Ca^{2+} to the $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{-ATPase}$ of sarcoplasmic reticulum: kinetic studies

Ian M. J. HENDERSON, Anthony P. STARLING, Matthew WICTOME, J. Malcolm EAST and Anthony G. LEE

Department of Biochemistry, University of Southampton, Southampton SP9 3TY, Hants., U.K.

Stop-flow fluorescence and rapid-filtration methods have been used to establish the kinetics of Ca^{2+} binding to, and dissociation from, the $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{-ATPase}$ of skeletal-muscle sarcoplasmic reticulum and to define the effects of H^+ and Mg^{2+} on Ca^{2+} binding and dissociation rates. The kinetics have been interpreted in terms of the scheme: $\text{E2} \rightleftharpoons \text{E1} \rightleftharpoons \text{E1Ca} \rightleftharpoons \text{E1}'\text{Ca} \rightleftharpoons \text{E1}'\text{Ca}_2$. The kinetics of the $\text{E2} \rightleftharpoons \text{E1}$ transition have been determined by measuring the rate of change of the fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole after a pH jump or the addition of Ca^{2+} to the labelled ATPase in the presence of thapsigargin or thapsivillosin A. It has been shown that Mg^{2+} has a marked effect on Ca^{2+} dissociation at pH 7.2 and that changes in the tryptophan fluorescence of the ATPase follow the same time course as the dissociation of $^{45}\text{Ca}^{2+}$. It is proposed that the effect of Mg^{2+} follows from binding to a 'gating' site, as detected

by changes in the fluorescence of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin. The rate of dissociation of Ca^{2+} from the ATPase increases with increasing pH. The rate of dissociation of Ca^{2+} decreases with increasing Ca^{2+} concentration in the medium, with an apparent affinity for Ca^{2+} greater than that seen for the change in fluorescence amplitude. It is shown that this follows if the first, inner, Ca^{2+} -binding site on the ATPase has a lower affinity for Ca^{2+} than the second, outer, site. Effects of H^+ and Mg^{2+} on Ca^{2+} dissociation can be treated by the quasiequilibrium approach. Mg^{2+} and H^+ also affect the rate of Ca^{2+} binding to the ATPase, and effects of H^+ and Mg^{2+} on the $\text{E2} \rightleftharpoons \text{E1}$ equilibrium explain the results of experiments in which the concentrations of H^+ and Mg^{2+} are jumped.

INTRODUCTION

In stopped-flow experiments with the $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{-ATPase}$ it has been shown that the fluorescence changes following Ca^{2+} binding or dissociation are rather slow (Dupont and Leigh, 1978). Initial studies suggested that the changes in tryptophan fluorescence observed on mixing the Ca^{2+} -bound ATPase with EGTA could be described by single exponential decays (Guillain et al., 1980, 1981) but subsequent studies showed that, although this was true at pH 6.0 (Orlowski and Champeil, 1991), decays observed at pH 7.2 in the presence of Mg^{2+} were biphasic (Moutin and Dupont, 1991). Fluorescence changes on binding of Ca^{2+} to the ATPase at pH 7 were also found to be biphasic (Champeil et al., 1983; Fernandez-Belda et al., 1984). The subsequent development of rapid-filtration methods made it possible to measure the rate of dissociation of Ca^{2+} directly using radioisotopes (Dupont, 1984). Dissociation of $^{45}\text{Ca}^{2+}$ from the Ca^{2+} -bound ATPase was monoexponential at pH 6.0 on washing with EGTA. However, on washing with $^{40}\text{Ca}^{2+}$, dissociation of the inner Ca^{2+} occurred at a rate half that of the outer Ca^{2+} and was inhibited by the presence of $^{40}\text{Ca}^{2+}$ in the medium (Dupont, 1982; Inesi, 1987; Orlowski and Champeil, 1991), consistent with a sequential binding mechanism (Scheme 1). A study of Ca^{2+} dissociation from the ATPase using an assay based on phosphorylation of the ATPase by ATP was also interpreted in

terms of dissociation of the inner Ca^{2+} at half the rate of the outer Ca^{2+} at pH 7.0 in the presence of Mg^{2+} (Petithory and Jencks, 1988). Direct studies of $^{45}\text{Ca}^{2+}$ dissociation from the ATPase, however, suggested a more complex relationship under these conditions (Starling et al., 1993).

Here we have reinvestigated the kinetics of Ca^{2+} binding and dissociation, largely using stopped-flow fluorescence methods. In combination with the equilibrium measurements reported in the preceding paper (Henderson et al., 1994), they allow the development of a fairly simple model for the Ca^{2+} -binding process.

MATERIALS AND METHODS

Sarcoplasmic reticulum from rabbit skeletal muscle and $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{-ATPase}$ were prepared as described in the preceding paper (Henderson et al., 1994). ATPase was labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) also as described (Henderson et al., 1994). Thapsigargin was obtained from Calbiochem and trilobolide was a generous gift from Dr. M. Holub.

Rapid-kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorimeter with BioKine V3.20a software for data acquisition and analysis. The instrument uses stepper motors to drive three mixing syringes arranged to feed into two mixers followed by the observation cuvette. The instrument has no stop syringe, but a high-speed electrovalve at the exit of the cuvette closes with the stopping of the motors to halt any liquid flow. Tryptophan fluorescence was excited through a monochromator set at 290 nm, and emitted light was collected at 90° and filtered by a high-pass cut-off filter with 50 % transmission at 320 nm (UV 32, Andover Corporation). Fluorescence of the NBD-labelled ATPase was excited at 430 nm, and emission was filtered through a high-pass cut-off filter with

Scheme 1

50% transmission at 455 nm (GG 455, Andover Corporation). Experiments were performed at 25 °C unless otherwise stated.

The time-dependence of $^{45}\text{Ca}^{2+}$ release from the ATPase was determined using a Biologic RFS-04 rapid-filtration system, at room temperature (typically 20 °C). A suspension of the ATPase (0.2 mg) in buffer containing 100 μM $^{45}\text{Ca}^{2+}$ and 500 μM [^3H]sucrose was loaded on to a Millipore HAWP filter (0.45 μm) and then rapidly perfused with buffer. The filter was dried overnight in air and 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 this volume was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound to the ATPase.

Simulations of Ca^{2+} dissociation were performed on an IBM-compatible PC using the program M30 of Zhang et al. (1989), which uses the method of Laplace transforms to solve differential equations, or on an IBM 3090 using FACSIMILE (Chance et al., 1977). In all simulations it was assumed that H^+ binding was fast compared with the rates of Ca^{2+} binding or dissociation so that H^+ binding can be treated by the quasiequilibrium approach (Cha, 1968). This assumption is justified below. 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

Comparison of $^{45}\text{Ca}^{2+}$ dissociation and tryptophan-fluorescence changes

Figure 1(a) shows an experiment in which the ATPase incubated with $^{45}\text{Ca}^{2+}$ was adsorbed on a cellulose filter and was perfused for various times with a medium containing 2 mM EGTA at pH 7.2. Plotted on the same Figure is the decrease in tryptophan fluorescence observed when the ATPase incubated in 100 μM Ca^{2+} at pH 7.2 is mixed in a stopped-flow fluorimeter with an equal volume of 2 mM EGTA to give a final pCa value of 7.5; the fluorescence change is plotted on a scale of 1 to zero to allow comparison with the $^{45}\text{Ca}^{2+}$ dissociation data. The $^{45}\text{Ca}^{2+}$ and tryptophan data fit well to single exponentials with rates of 10.3 and 8.5 s^{-1} respectively, but comparison of the data (Figure 1a) shows that, within the limited accuracy of the $^{45}\text{Ca}^{2+}$ data, both sets of data fit to the same curve. Figure 1(b) shows the same experiment but in the presence of 20 mM Mg^{2+} . It is clear that the data no longer fit to a single exponential but again the loss of $^{45}\text{Ca}^{2+}$ and the decrease in tryptophan fluorescence follow the same curve within experimental error.

The close agreement between the $^{45}\text{Ca}^{2+}$ and tryptophan data is shown in more dramatic form in Figure 2. As reported by Squier et al. (1990), displacement of $^{45}\text{Ca}^{2+}$ from the ATPase by Pr^{3+} is biphasic, with one-half of the Ca^{2+} dissociating with a rate comparable with that seen in perfusion with EGTA, the other half dissociating over a long time course of minutes, not shown in Figure 2. The important observation is that even under these extreme conditions, the change in tryptophan-fluorescence intensity accurately monitors the Ca^{2+} occupancy of the ATPase. An explanation was presented in the preceding paper (Henderson et al., 1994).

Effects of pH and Mg^{2+} on the tryptophan fluorescence changes accompanying Ca^{2+} dissociation

As shown in Figures 1 and 3, the rate of decrease in tryptophan fluorescence produced by mixing the ATPase in 100 μM Ca^{2+} with 2 mM EGTA increases with increasing pH. Further, whereas

curves obtained at pH 6 and 7.2 in the absence of Mg^{2+} fit well to single exponentials with rates of 4.0 ± 0.1 and $8.5 \pm 0.1 \text{ s}^{-1}$ respectively, that at pH 8.5 fits to the sum of two exponentials of equal amplitude and rates of 6.7 and 64.7 s^{-1} . Addition of 20 mM Mg^{2+} has no significant effects on rate at pH 6.0 (Figure 3). In contrast, Mg^{2+} increases the rate of the fluorescence change at pH 7.2, the curves no longer fitting to a single exponential (Figure 1). When the data at low concentrations of Mg^{2+} are fitted without restraint to the sum of two exponentials, good fits can be obtained with a wide range of combinations of amplitudes and rates. However, at high Mg^{2+} (50 mM) stable fits were obtained to two exponentials with rates of 46 s^{-1} and 6 s^{-1} and relative amplitudes of 0.52 and 0.48 respectively (Figure 4). With the rate of the fast component fixed at 46 s^{-1} , good fits could then be obtained to the data at lower Mg^{2+} concentrations, with a rate for the slow component between 5 and 7 s^{-1} and relative amplitudes for the two components shown in Figure 4.

The effects of Mg^{2+} on the rate of the fluorescence decrease at pH 8.5 are more complex (Figure 3), with low concentrations increasing the rate of the fluorescence change and high concentrations decreasing the rate. Whereas rates recorded at 0, 0.5 and 1 mM Mg^{2+} were biexponential, at 2 mM and above they became

Figure 1 Comparison of $^{45}\text{Ca}^{2+}$ dissociation from the $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{--ATPase}$ with the change in tryptophan-fluorescence intensity

The symbols ($\square$) show the dissociation of $^{45}\text{Ca}^{2+}$ when the ATPase incubated in buffer (20 mM Mops/Tris, pH 7.2) containing 50 μM $^{45}\text{Ca}^{2+}$ was perfused with the same buffer containing 2 mM EGTA in the absence of Mg^{2+} (a) or in the presence of 20 mM Mg^{2+} (b). The dotted line shows the decrease in tryptophan-fluorescence intensity plotted on a scale from 1 to zero when the ATPase in buffer (150 mM Mops, 80 mM Tris, pH 7.2, 100 μM Ca^{2+}) was mixed in a 1:1 ratio with the same buffer containing 2 mM EGTA, in the absence of Mg^{2+} (a) or in the presence of 20 mM Mg^{2+} (b). The solid lines show simulations calculated by using the parameters given in Table 1.

Table 1 The reaction steps, rate constants and equilibrium constants of the kinetic model for Ca²⁺ dissociation

The superscript C denotes binding to a Ca²⁺-binding site. Parameters describing binding to the 'gating' site are given in Table 1 of Henderson et al. (1994).

Reaction step	Off rate		Equilibrium	
	Symbol*	Value (s ⁻¹)	Symbol	Value
E1'Ca ₂ HMg → E1'CaHMg	rE1HMg ⁷	12.9	K _{C2x}	1.3 × 10 ¹⁰ M ⁻¹
E1'Ca ₂ H → E1'CaH	rE1H ⁷	19.1	K _{C2x}	1.3 × 10 ¹⁰ M ⁻¹
E1'Ca ₂ → E1'Ca	rE1 ⁷	200	K _{C2x}	1.3 × 10 ¹⁰ M ⁻¹
E1'Ca ₂ Mg → E1'CaMg	rE1Mg ⁷	2700	K _{C2x}	1.3 × 10 ¹⁰ M ⁻¹
E1'CaHMg → E1CaHMg	rE1HMg ⁶	60	K _{C3}	1.0
E1'CaH → E1CaH	rE1H ⁶	150	K _{C3}	1.0
E1'Ca → E1Ca	rE1 ⁶	150	K _{C3}	1.0
E1'CaMg → E1CaMg	rE1Mg ⁶	0	K _{C3}	1.0
E1CaHMg → E1HMg	rE1HMg ⁵	12.9	K _{C1c}	3.5 × 10 ⁸ M ⁻¹
E1CaH → E1H	rE1H ⁵	12.9	K _{C1b}	3.5 × 10 ⁸ M ⁻¹
E1Ca → E1	rE1 ⁵	100.0	K _{C1a}	5.8 × 10 ⁶ M ⁻¹
E1CaMg → E1Mg	rE1Mg ⁵	675	K _{C1d}	5.8 × 10 ⁶ M ⁻¹
E1Mg ^C → E1	k _{3b}	1000†	K _{Mg1}	[K _{C1} /2000]‡, K _{C1} /667§
E2Mg ^C → E1	k _{4b}	[0.1]‡, 1000§	K _{Mg2}	K _{C1} /1.0 × 10 ⁴
E2 → E1	k _{11a}	80.0	K ₁	4.0
E2H → E1H	k _{11b}	2.0	K ₂	6.67 × 10 ⁻³
E2Mg ^C → E1Mg ^C	k ₂₁	k ₁₁	K ₃	K ₁ K _{Mg1} /K _{Mg2}

* Superscript 7 refers to the E1'Ca₂ → E1'Ca step (Scheme 4), superscript 6 refers to the E1'Ca → E1Ca step (Scheme 5) and superscript 5 refers to the E1Ca → E1 step (Scheme 6).

† Assumed fast and set to 1000 s⁻¹.

‡ Using the binding constant for Mg²⁺ estimated for NBD-labelled ATPase (Henderson et al., 1994).

§ Using the alternative binding constant for Mg²⁺ (see the text).

Figure 2 Effect of Pr³⁺ on the rate of dissociation of ⁴⁵Ca²⁺ and on the change in tryptophan-fluorescence intensity

The symbols (○, □) show the dissociation of ⁴⁵Ca²⁺ (nmol/mg of protein) when the ATPase incubated in buffer (150 mM Mops, 80 mM Tris, pH 7.2) containing 50 μM ⁴⁵Ca²⁺ was perfused with the same buffer containing either 2 mM EGTA (○) or 0.1 mM Pr³⁺ (□). The dotted lines show the decrease in tryptophan-fluorescence intensity (mV) when the ATPase in buffer (0.4 mg of protein/ml) containing 0.2 mM Ca²⁺ was mixed in a 1:1 ratio with the same buffer containing either 0.2 mM Pr³⁺ (curve A) or 2 mM EGTA (curve B).

monoexponential; Figure 5 shows the effect of Mg²⁺ on this monoexponential rate.

Effect of Ca²⁺ concentration on the tryptophan-fluorescence changes accompanying Ca²⁺ dissociation

Figure 6 shows data recorded at pH 6.0 as a function of final pCa value. It is clear that with increasing Ca²⁺ concentration both the rate and the amplitude of the change decrease. At high pCa

values, a small rapid initial decrease in fluorescence intensity is observed, followed by a larger and slower change. The slow phase fits well to a single exponential, and the presence of the small fast phase has little effect on the goodness of fit. The rates and amplitudes obtained by fitting the data to single exponentials are shown in Figure 7 for data obtained at pH 6.0 and 7.2 in the absence of Mg²⁺ and at pH 6.0 in the presence of 20 mM Mg²⁺.

Other effects on the tryptophan-fluorescence changes accompanying Ca²⁺ dissociation

The effect of temperature on the rate of the fluorescence change after the mixing of Ca²⁺-bound ATPase with EGTA at pH 7.2 was studied (Figure 8). In the absence of Mg²⁺, the data were fitted to a single exponential at all temperatures, and the rates gave a linear Arrhenius plot with an activation energy of 54.6 ± 2.5 kJ · mol⁻¹. In the presence of 20 mM Mg²⁺, the data at all temperatures fitted to the sum of two exponentials of equal amplitude, each component giving linear Arrhenius plots with activation energies of 57.3 ± 3.8 kJ · mol⁻¹ and 47.0 ± 1.2 kJ · mol⁻¹ respectively for the fast and slow components (Figure 8). Comparison of these activation energies with that expected for passive ion diffusion through a channel (16 kJ · mol⁻¹; Hille, 1984) suggests that access to the Ca²⁺-binding sites is controlled by conformational changes on the ATPase.

Figure 9 shows the results of a pH-jump experiment in which the pH was jumped simultaneously with mixing with 2 mM EGTA in the presence of Mg²⁺. The rate of the fluorescence change observed on jumping the pH from 8.5 to 6.0 was identical with that observed when the ATPase was incubated in pH 6.0 buffer and mixed with EGTA to give a final pCa value of 5.4 (Figure 7), and the rate of the fluorescence change observed on jumping the pH from 6.0 to 8.5 was identical with that observed when the ATPase was incubated in pH 8.5 buffer (see Figure 3). Similarly, in the absence of Mg²⁺, pH jumps from 8.5 to 6.0 or

Figure 3 Kinetics of the decrease in tryptophan fluorescence caused by Ca^{2+} dissociation from the ATPase

The decrease in tryptophan fluorescence was recorded at pH 6.0 (a) or 8.5 (b, c): (a) in the absence (A) or presence (B) of 20 mM Mg^{2+} ; (b, c) in the absence (A) or presence of 1.0 (B), 2.5 (C) or 40 (D) mM Mg^{2+} . In (a) curve B has been offset by 0.1 for clarity. In the stopped-flow experiment, the enzyme syringe contained 0.4 mg/ml ATPase in buffer at pH 6.0 (130 mM Mes, 50 mM Tris) or pH 8.5 (27 mM Mes, 100 mM Tris) containing 100 μM Ca^{2+} and the indicated concentration of Mg^{2+} . The substrate syringe contained 20 mM (pH 6.0) or 2 mM EGTA (pH 8.5) in the same buffer. The contents of the two syringes were mixed in a 1:1 ratio to give final pCa values of 6.4 and 10.3 at pH 6.0 and 8.5 respectively. The solid lines show simulations calculated by using the rate constants given in Table 1.

from 6.0 to 8.5 gave traces identical with those obtained when the ATPase was incubated in buffer at pH 6.0 or 8.5 (results not shown). The rates of binding and dissociation of H^+ are therefore fast compared with the rates of the other processes involved in Ca^{2+} dissociation, and thus effects due to H^+ can be treated by the quasiequilibrium approach.

Figure 4 Effect of Mg^{2+} concentration on the relative amplitudes of the two components of the decrease in tryptophan fluorescence caused by Ca^{2+} dissociation from the ATPase at pH 7.2

Measurements were performed as described in the legend to Figure 3. The experimental data were fitted to the sum of two exponentials with the rate constant of the fast component fixed at 50 s^{-1} . The relative amplitudes of the slow ($\square$) and fast ($\circ$) components are plotted against Mg^{2+} concentration (mM). The solid lines show the variation of the amplitudes of the slow and fast components obtained when theoretical simulations calculated by using the parameters in Table 1 are fitted in the same way to the sum of two exponentials.

Figure 5 Effect of Mg^{2+} on the rate of decrease in tryptophan fluorescence caused by Ca^{2+} dissociation from the ATPase at pH 8.5

Measurements were performed as described in the legend to Figure 3. The experimental data for Mg^{2+} concentrations of 2.0 mM or greater were fitted to a single exponential and the rate plotted ($\square$). The solid line shows the variation in rate obtained when theoretical simulations calculated by using the parameters in Table 1 are fitted in the same way.

Experiments in which the Mg^{2+} concentration was jumped suggest that the Mg^{2+} -binding steps affecting Ca^{2+} dissociation are also fast. Thus fluorescence changes observed when the Ca^{2+} -bound ATPase incubated in the absence of Mg^{2+} at pH 7.2 were mixed in a 1:1 ratio with buffer containing EGTA and 20 mM Mg^{2+} were identical with those obtained when the Ca^{2+} -bound ATPase was incubated in buffer containing 10 mM Mg^{2+} and mixed with buffer containing EGTA and 10 mM Mg^{2+} (results not shown).

Figure 6 Effect of Ca²⁺ concentration on the kinetics of the decrease in tryptophan fluorescence caused by Ca²⁺ dissociation from the ATPase at pH 6.0 in the presence of 20 mM Mg²⁺

In the stopped-flow experiment the enzyme syringe contained 0.4 mg/ml ATPase in buffer (130 mM Mes, 50 mM Tris, pH 7.2) containing 100 μ M Ca²⁺ and 20 mM Mg²⁺. The substrate syringe contained 20 mM Mg²⁺ in the same buffer and a concentration of EGTA to give after 1:1 mixing a final pCa of: A, 6.92; B, 6.22; C, 5.71; D, 5.48; E, 4.7. The dotted lines show the observed decreases in tryptophan fluorescence and the solid lines show simulations calculated by using the parameters in Table 1.

The rate of change of NBD fluorescence for NBD-labelled ATPase

The fluorescence intensity of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) is low in the E2 state (predominant at low pH) and high in the E1 state (predominant at high pH) (Wakabayashi et al., 1990; Henderson et al., 1994). Figure 10 shows the change in NBD fluorescence intensity observed when the pH of the ATPase incubated in EGTA is jumped from 6.0 to 8.5. The specific inhibitors, thapsigargin and trilobolide, shift the E2–E1 equilibrium strongly towards E2 at all pH values. As the pH-dependence of the rate of the fluorescence change (Figure 11) is identical with the pH-dependence of the E1/E2 equilibrium [see (Henderson et al., 1994)], we have suggested that the pH-dependence of the E2_{thapsigargin} → E1_{thapsigargin} transition is defined by the pH-dependence of the E2 → E1 transition (Wictome et al., 1992a,b). The rate of the response to Ca²⁺ is shown as a function of pH in Figure 11 for the ATPase incubated with a 1:1 molar ratio of thapsigargin or trilobolide.

The E1/E2 equilibrium of the ATPase can be described by Scheme 2. Treating H⁺ binding by the quasiequilibrium approach, the effective rate of the E2 → E1 transition, k_{1r}^{eff} , can be written as:

$$k_{1r}^{\text{eff}} = \frac{k_{1fa}}{(1 + K_{H7})[H^+]} + \frac{k_{1fb}K_{H7}[H^+]}{(1 + K_{H7})[H^+]}$$

where the values of K_{H7} and the equilibrium constants k_{1fa}/k_{1ba} (K_1) and k_{1fb}/k_{1bb} have been defined by the equilibrium experiments reported in the preceding paper (Henderson et al., 1994). The experiment shown in Figure 10 in the absence of Mg²⁺ defines the value of k_{1r}^{eff} at pH 8.5, and the experiments in Figure 11 define its pH-dependence. As shown, the data fit with values of 80.0 and 2.0 for k_{1fa} and k_{1fb} respectively, assuming decreases

Figure 7 Effect of pCa on the rates and relative amplitudes of the fluorescence changes caused by Ca²⁺ dissociation from the ATPase

Measurements were performed as described in the legend to Figure 3 at pH 6.0 (a, b) in buffer (130 mM Mes, 50 mM Tris) in the absence (a) or presence (b) of 20 mM Mg²⁺ or pH 7.2 (c) in buffer (130 mM Mes, 50 mM Tris, pH 7.2). Data were fitted to single exponentials and the calculated rates (○) and amplitudes (□) are plotted as a function of pCa. The solid lines show the variation of rates and amplitudes with pCa obtained when theoretical simulations calculated by using the parameters in Table 1 are fitted in the same way to single exponentials; for the data at pH 7.2, a binding constant K_{C2x} half that in Table 1 was assumed (see the Discussion section).

in k_{1r}^{eff} by factors of 700 and 160 in the presence of thapsigargin and trilobolide A respectively.

In the presence of Mg²⁺, the magnitude of the change in NBD fluorescence on jumping the pH from 6.0 to 8.5 is greater than in the absence of Mg²⁺ (Figure 10) because binding of Mg²⁺ shifts the E1/E2 equilibrium towards E1 (Henderson et al., 1994). The effect of Mg²⁺ on the rate of the E2 → E1 transition is best defined by its effect on the rate of Ca²⁺ binding at pH 7.2 (see below).

Figure 8 Arrhenius plots of the rates of the fluorescence change caused by Ca^{2+} dissociation at pH 7.2

Measurements were performed as described in the legend to Figure 1: (a) data in the absence of Mg^{2+} were fitted to a single exponential and the rates plotted ($\circ$); (b) data in the presence of 20 mM Mg^{2+} were fitted to the sum of two exponentials with equal amplitudes and the rates of the fast ($\square$) and slow ($\circ$) components plotted.

Figure 9 Effect of a pH jump on the kinetics of the decrease in tryptophan fluorescence caused by Ca^{2+} dissociation from the ATPase in the presence of 20 mM Mg^{2+}

The traces show the change in fluorescence intensity when the ATPase (0.4 mg/ml) in buffer (3 mM Mes/Tris) containing $100 \mu\text{M}$ Ca^{2+} at pH 8.5 (A) or 6.0 (B) is mixed in a 1:1 ratio with buffer containing 2 mM EGTA: (A) 130 mM Mes, 50 mM Tris, pH 6.0; (B) 100 mM Tris, 27 mM Mes, pH 8.5. All buffers contained 20 mM Mg^{2+} and final pCa values were (A) 5.4 and (B) 8.4.

Figure 10 Effect of a pH jump on the fluorescence intensity of NBD-labelled ATPase

The Figure shows the change in NBD fluorescence when NBD-labelled ATPase (0.4 mg/ml) in pH 6.0 buffer (3 mM Mes/Tris, 2 mM EGTA) is mixed in a 1:1 molar ratio with pH 8.5 buffer (100 mM Tris, 27 mM Mes, 2 mM EGTA). In experiment A, both buffers contained 20 mM Mg^{2+} and in experiment B no Mg^{2+} was present. The solid lines are simulations of the change in the fraction of E1 forms of the ATPase calculated with the parameters given in Table 1. The data have been plotted so that a change in fluorescence intensity of 30 mV corresponds to a calculated change in the E1 fraction of 1.0.

Figure 11 Effect of pH on the rate of increase in fluorescence intensity of NBD-labelled ATPase in the presence of inhibitors observed on addition of Ca^{2+}

The increase in fluorescence intensity of NBD-labelled ATPase in buffer (50 mM Mes/KOH, pH 6.0–6.8; 50 mM HEPES/KOH, pH 7.0–7.8; 50 mM Tris/HCl, pH 8.0–8.5) containing 0.3 mM EGTA was observed after the addition of Ca^{2+} to a final concentration of 0.7 mM in the presence of a 1:1 molar ratio of inhibitor/ATPase: $\circ$, thapsigargin; $\square$, trilobolide. The experimental curves were fitted to single exponentials and the rates are plotted as a function of pH. The curves are the rates calculated by using the parameters in Table 1, assuming decreases in the rate of the $\text{E2} \rightarrow \text{E1}$ transition by factors of 700 and 160 for thapsigargin and trilobolide respectively.

Effect of pH and Mg^{2+} on the tryptophan-fluorescence changes accompanying Ca^{2+} binding

As shown in Figure 12, the rate of the tryptophan-fluorescence change on the binding of Ca^{2+} to the ATPase increases markedly with increasing pH. Also as shown, the presence of 20 mM Mg^{2+}

Scheme 2

Figure 12 Effect of pH and Mg²⁺ on the kinetics of the increase in tryptophan fluorescence caused by Ca²⁺ binding

The Figure shows the increase in tryptophan fluorescence when ATPase (0.4 mg/ml) in buffer at (a) pH 6.0 (130 mM Mes, 50 mM Tris), (b) pH 7.2 (150 mM Mops, 80 mM Tris) or (c) pH 8.5 (100 mM Tris, 27 mM Mes) containing 0.2 mM EGTA was mixed in a 1:1 ratio with the same buffer but containing 0.3 mM Ca²⁺. The dotted traces show experimental data in the absence (A) or presence (B) of 20 mM Mg²⁺. The solid and broken lines show simulations calculated using the parameters in Table 1, in the presence or absence of 20 mM Mg²⁺ respectively.

Table 2 Rates describing tryptophan-fluorescence changes on Ca²⁺ binding

Rates were obtained by fitting the tryptophan-fluorescence curves and the simulations of Ca²⁺ binding at pH 7.2 at the given Mg²⁺ concentrations to the sum of two exponentials.

[Mg ²⁺] (mM)	Experimental				Simulation			
	<i>k</i> (s ⁻¹)		Amplitude		<i>k</i> (s ⁻¹)		Amplitude	
	Fast	Slow	Fast	Slow	Fast	Slow	Fast	Slow
2.5	6.3	49.0	0.67	0.31	6.2	57.0	0.68	0.31
4.0	7.8	44.0	0.58	0.42	6.2	47.5	0.63	0.36
6.0	8.0	53.0	0.57	0.43	6.2	41.0	0.59	0.41
8.0	7.1	34.0	0.54	0.46	6.2	37.5	0.56	0.43
10.0	6.7	37.2	0.52	0.48	6.2	35.0	0.53	0.46

Table 3 Rates and amplitudes derived from fitting the tryptophan fluorescence changes on Ca²⁺ binding at pH 8.5 and the indicated Mg²⁺ concentrations

[Mg ²⁺] (mM)	Experimental		Simulation <i>k</i> (s ⁻¹)
	<i>k</i> (s ⁻¹)	Amplitude (mV)	
0	161, 11.2*	100, 100*	—
0.1	138, 10.6*	110, 30*	—
0.5	106	110	102
1.0	80	140	77.9
2.0	74	160	72.6
2.5	71	175	69.8
5.0	55	200	58.7
10.0	39	220	47.0
20.0	35	200	37.3

* Data fitted to a double exponential.

has little effect on the rate at pH 6.0, increases it at pH 7.2 and decreases it at pH 8.5. The fluorescence curves obtained at pH 6.0 in the presence or absence of Mg²⁺ fit to a single exponential with a rate constant of 1 s⁻¹. At pH 7.2 in the presence or absence of Mg²⁺, the data fit to the sum of two exponentials with the rate constants and amplitudes given in Table 2. The major effect of Mg²⁺ is to increase the amplitude of the fast component with a slight decrease in the rate of the fast component. At pH 8.5 the effects of Mg²⁺ are more complex. In the absence of Mg²⁺ or at a Mg²⁺ concentration of 0.1 mM, the data fit to a sum of two exponentials but at higher Mg²⁺ concentrations the fluorescence changes become monoexponential (Table 3).

Figure 13 shows the effect of a pH jump on the rate of the tryptophan-fluorescence change on Ca²⁺ binding. When the pH jump is performed from pH 8.5 to 6.0, an initial rapid phase is observed before the slower change typical of pH 6.0 (Figure 13a). In contrast, when the pH is jumped from 6.0 to 8.5 in the presence of 20 mM Mg²⁺, an initial lag phase is observed (Figure 13b); a similar lag phase is observed in the absence of Mg²⁺ (results not shown).

Figure 14 shows the results of experiments in which the Mg²⁺ concentration is jumped. At pH 7.2, the rate of change of tryptophan fluorescence on binding Ca²⁺ after a jump in Mg²⁺ concentration from 0 to 20 mM is slower than that observed for the ATPase preincubated with 20 mM Mg²⁺ (Figure 14a). At pH 8.5, the rate of change of tryptophan fluorescence is the same

Figure 13 Effect of a pH jump on the kinetics of the increase in tryptophan fluorescence caused by Ca^{2+} binding to the ATPase

(a) Effect of a pH jump from 8.5 to 6.0. In the control experiment (A) ATPase in pH 6.0 buffer (130 mM Mes, 50 mM Tris) containing 0.2 mM EGTA was mixed in a 1:1 ratio with the same buffer containing 0.3 mM Ca^{2+} . In the pH-jump experiment (B) ATPase in pH 8.5 buffer (3 mM Tris/Mes, 50 mM Tris) containing 0.2 mM EGTA was mixed in a 1:1 ratio with pH 6.0 buffer (130 mM Mes, 50 mM Tris) containing 0.3 mM Ca^{2+} . (b) Effect of a pH jump from 6.0 to 8.5. In the control experiment (A) ATPase in pH 8.5 buffer (100 mM Tris, 27 mM Mes, 20 mM Mg^{2+}) containing 0.2 mM EGTA was mixed in a 1:1 ratio with the same buffer containing 0.3 mM Ca^{2+} . In the pH-jump experiment (B) ATPase in pH 6.0 buffer (3 mM Mes/Tris, 20 mM Mg^{2+}) was mixed in a 1:1 ratio with pH 8.5 buffer (100 mM Tris, 27 mM Mes, 20 mM Mg^{2+}) containing 0.3 mM Ca^{2+} . The dotted lines show the experimental data and the solid lines show simulations calculated using the parameters in Table 1.

for a jump in Mg^{2+} concentration from 0 to 20 mM or after preincubation of the ATPase with 20 mM Mg^{2+} . Similarly, at pH 6.0, identical responses are obtained on binding Ca^{2+} either with a jump in Mg^{2+} concentration or after preincubation with Mg^{2+} (results not shown).

DISCUSSION

In the preceding paper (Henderson et al., 1994), a model was presented for equilibrium binding of Ca^{2+} to the $(\text{Ca}^{2+}\text{-Mg}^{2+})$ -ATPase. This model is now used to interpret studies of the kinetics of Ca^{2+} binding and dissociation. The model proposes binding of Ca^{2+} to two sites on the ATPase, in competition with binding of H^{+} and Mg^{2+} at both sites. A third site, referred to as the 'gating' site, binds H^{+} and Mg^{2+} , affecting Ca^{2+} binding; binding constants for H^{+} and Mg^{2+} at the 'gating' site were obtained by analysis of fluorescence experiments with the ATPase

Figure 14 Effect of a jump in Mg^{2+} concentration from 0 to 20 mM on the kinetics of the increase in tryptophan fluorescence caused by Ca^{2+} binding to the ATPase

Experiments were performed at pH 7.2 (a) or 8.5 (b). In the control experiments (A) ATPase in buffer (150 mM Mops, 80 mM Tris, pH 7.2 or 100 mM Tris, 27 mM Mes, pH 8.5) containing 0.2 mM EGTA and 20 mM Mg^{2+} was mixed in a 1:1 ratio with the same buffer containing 0.3 mM Ca^{2+} . In the Mg^{2+} jump experiments (B) the ATPase incubated in buffer containing 0.2 mM EGTA in the absence of Mg^{2+} was mixed in a 1:1 ratio with the same buffer containing 0.3 mM Ca^{2+} and 40 mM Mg^{2+} . In (b) the upper trace has been offset by 0.2 for clarity. The dotted lines show experimental traces and the solid lines show simulations calculated using the parameters in Table 1.

labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin. Finally, it was proposed that the ATPase could exist in two conformational states: E1 with high-affinity binding sites for Ca^{2+} exposed to the cytoplasm and E2 unable to bind Ca^{2+} to cytoplasmic sites, with the equilibrium between E1 and E2 being dependent on protonation of a single residue on the ATPase [see Figure 9 in Henderson et al. (1994)].

Nature of the tryptophan-fluorescence change

At pH 7.2 in the absence of Mg^{2+} , dissociation of $^{45}\text{Ca}^{2+}$ from the Ca^{2+} -bound ATPase fits well to a single exponential, but in the presence of 20 mM Mg^{2+} , although it is clear that dissociation of Ca^{2+} no longer fits to a single exponential, the precision of the data is insufficient to obtain a unique and reliable fit to the sum of two exponentials (Figure 1). More precise data can be obtained by stopped-flow fluorescence, measuring the change in tryptophan fluorescence that occurs on Ca^{2+} binding or dissociation. It has been suggested that the tryptophan fluorescence change corresponds to the $\text{E2} \rightarrow \text{E1}$ (Moutin and Dupont, 1991; Guillaud et al., 1981) or $\text{E1Ca} \rightarrow \text{E1'Ca}$ (Fernandez-Belda et al., 1984; Froud and Lee, 1986) steps in Scheme 1, or directly reflects the

Scheme 3

Scheme 4

occupancy of the Ca^{2+} sites on the ATPase (Orlowski and Champeil, 1991). The data shown in Figure 2 argue strongly that it does not reflect the $\text{E2} \rightarrow \text{E1}$ transition. Perfusion of the $^{45}\text{Ca}^{2+}$ -bound ATPase with Pr^{3+} leads to a biphasic release of $^{45}\text{Ca}^{2+}$ with one-half of the Ca^{2+} dissociating with a rate comparable with that seen on perfusion with EGTA, the other half dissociating over a time course of minutes. The decrease in tryptophan fluorescence observed on mixing the Ca^{2+} -bound ATPase with Pr^{3+} follows the same time course as the dissociation of $^{45}\text{Ca}^{2+}$. In the preceding paper (Henderson et al., 1994), it was shown that measurements of the fluorescence of NBD-labelled ATPase indicated that the ATPase was in the E1 form in the presence of Pr^{3+} .

The results presented in Figure 2 suggest that the tryptophan-fluorescence change reflects changes in the Ca^{2+} occupancy of the ATPase. This is also suggested by the close parallels observed between binding of $^{45}\text{Ca}^{2+}$ and tryptophan-fluorescence changes in equilibrium experiments (Dupont, 1982; Fernandez-Belda et al., 1984; Scofano et al., 1985; Orlowski and Champeil, 1991). This could come about if the change in tryptophan fluorescence occurred at the $\text{E1Ca} \rightarrow \text{E1'Ca}$ transition, with the equilibrium constant for this step being equal to 1, and the rate of this step being fast, under most conditions, relative to the rates of the other steps involved in Ca^{2+} binding and dissociation (Henderson et al., 1994). It will be shown here that this same model can account for changes in tryptophan fluorescence observed by stopped flow on Ca^{2+} binding or dissociation.

Ca^{2+} dissociation from the ATPase

The basic features of the model for Ca^{2+} binding and dissociation have been largely determined by the equilibrium binding studies detailed in the preceding paper (Henderson et al., 1994). At very low final free Ca^{2+} concentrations, there will be no rebinding of Ca^{2+} , so that the rate of the fluorescence decrease caused by Ca^{2+}

Scheme 5

Scheme 6

dissociation will depend on the off rates for the $\text{E1'Ca}_2 \rightarrow \text{E1'Ca}$ and $\text{E1Ca} \rightarrow \text{E1}$ transitions, and on the forward and back rates for the $\text{E1'Ca} \rightarrow \text{E1Ca}$ step (at pH 6.0 and 7.2 this step is fast and may be treated by the quasiequilibrium method, but at pH 8.5, as will be shown, this step becomes rate-limiting as the calculated rates of Ca^{2+} dissociation from E1'Ca_2 and E1Ca become very fast). The $\text{E1}-\text{E2}$ equilibrium will have no effect, as there is no rebinding of Ca^{2+} to E1.

As shown in Figures 1 and 3, the rates of the tryptophan-fluorescence changes observed when the Ca^{2+} -bound ATPase is mixed with EGTA increase with increasing pH. The rate of the tryptophan-fluorescence change at pH 7.2 increases on addition of Mg^{2+} (Figure 1), as previously reported by Moutin and Dupont (1991), whereas Mg^{2+} has little effect at pH 6.0, and has a complex effect at pH 8.5 (Figure 3). The observation that H^+ and Mg^{2+} affect the rates of the tryptophan-fluorescence changes indicates that H^+ and Mg^{2+} must be able to bind to E1'Ca_2 , at a site referred to in the preceding paper (Henderson et al., 1994) as the 'gating' site. Effects on dissociation of Ca^{2+} from E1'Ca_2 and from E1Ca and effects on the $\text{E1'Ca} \rightarrow \text{E1Ca}$ transition are all described in Scheme 3 where X and Y represent E1'Ca_2 and E1'Ca respectively.

If the binding of H^+ and Mg^{2+} are fast so that they can be treated by the quasiequilibrium approach (this will be justified later), then the effective rate k_{7b} for dissociation of the first Ca^{2+} from E1'Ca_2 (Scheme 4) can readily be calculated as a function of pH and Mg^{2+} concentration.

As described in the preceding paper (Henderson et al., 1994), the equilibrium constant for the $\text{E1'Ca} \rightarrow \text{E1Ca}$ transition has been fixed at 1. As described below, the rate of this step and its dependence on pH and Mg^{2+} concentration is fixed by the data obtained at pH 8.5 and fits Scheme 3 with X and Y representing E1'Ca and E1Ca respectively. Again under quasiequilibrium conditions, the effective rate constants k_{6b} and k_{6f} (Scheme 5) can be calculated. The rate of the $\text{E1Ca} \rightarrow \text{E1}$ step (Scheme 6) is also assumed to be dependent on occupation of the 'gating' site by H^+ and Mg^{2+} as described by Scheme 3 with X and Y representing E1Ca and E1 .

Rate constants giving good fits to the data are presented in Table 1. The observed small effect of Mg^{2+} on the rate of tryptophan-fluorescence change at pH 6.0 (Figure 3) indicates similar rates of Ca^{2+} dissociation from $\text{E1'Ca}_2\text{HMg}$ and $\text{E1'Ca}_2\text{H}$ and from E1CaHMg and E1CaH . The faster rate observed at higher pH indicates faster rates of Ca^{2+} dissociation from unprotonated than from protonated species. The single exponential fit observed for the pH 6.0 data defines the rate of dissociation of Ca^{2+} from $\text{E1'Ca}_2\text{H}$ as being twice that from E1Ca , as described in the Appendix. The observed biexponential change observed at pH 8.5 in the absence of Mg^{2+} and the

decrease in rate observed at pH 8.5 at high Mg^{2+} can be simulated assuming that the rate of the $E1'Ca \rightarrow E1Ca$ step becomes slower than the rates of the $E1'Ca_2 \rightarrow E1'Ca$ and $E1Ca \rightarrow E1$ steps at this pH (Figures 3 and 5).

Decreases in tryptophan-fluorescence intensity on Ca^{2+} dissociation at higher pCa values show an initial small rapid phase followed by a larger and slower change (Figure 6). As the larger slow phase fits to a single exponential, and as the presence of the small initial fast phase has little effect on the goodness of fit, it is convenient to describe these curves by the amplitude and rate parameters obtained from a single exponential fit. Figure 7 shows the variation of amplitude and rate with pCa at pH 6.0 in the absence and presence of 20 mM Mg^{2+} . The variation of amplitude with pCa is identical with the variation of tryptophan-fluorescence intensity with pCa observed in equilibrium binding experiments (Henderson et al., 1994). Importantly, however, as the Ca^{2+} concentration increases, the rate of the tryptophan-fluorescence change decreases, with an apparent high affinity for Ca^{2+} . As shown in the Appendix the variation of rates and amplitudes with pCa depends on the relative affinities of E1 and $E1'Ca$ for Ca^{2+} ; the effective affinity of E1 for Ca^{2+} must be much lower than that of $E1'Ca$ to obtain the observed dependence on pCa (Appendix). The apparent affinity of E1 for Ca^{2+} is defined by the intrinsic affinity of the site for Ca^{2+} , by binding of H^+ and Mg^{2+} at the Ca^{2+} -binding site and by the $E2 \rightarrow E1$ equilibrium. All of these constants except that defining the intrinsic affinity for Ca^{2+} have been defined by experiments with NBD-labelled ATPase (Henderson et al., 1994). The value of the intrinsic affinity for the first Ca^{2+} site was therefore chosen to fit the data in Figures 6 and 7, with the value for the intrinsic affinity of Ca^{2+} for the second site then being chosen to fit the equilibrium Ca^{2+} -binding data (Henderson et al., 1994). The binding constants describing these processes are given in the preceding paper (Henderson et al., 1994) (see also Table 1).

At pH 7.2 differences between the Ca^{2+} -dependencies of rate and amplitude are considerably smaller than observed at pH 6.0 (Figure 7). For the experiment shown in Figure 7, the Ca^{2+} -dependence of the change in amplitude of the fluorescence change was different from that observed in equilibrium binding experiments at pH 7.2 (Henderson et al., 1994). However, the affinity of the ATPase for Ca^{2+} has been observed to vary between preparations (Lee et al., 1983; Orlowski and Champeil, 1991), and the data shown in Figure 7 can be reproduced with a binding constant for the second Ca^{2+} a factor of two smaller than that given in Table 1.

It is assumed in all the simulations that binding of H^+ to all sites and binding of Mg^{2+} to the second Ca^{2+} -binding site are fast and can be treated by the quasiequilibrium approach. This assumption is justified by pH-jump and Mg^{2+} -jump experiments. As shown in Figure 9, the rate of change of fluorescence observed in Ca^{2+} -dissociation experiments in which the pH was jumped from 8.5 to 6.0 was the same as that observed when the ATPase was incubated at pH 6.0 (see Figure 7) and similarly the rate observed with a pH jump from 6.0 to 8.5 was the same as that observed after preincubation at pH 8.5 (Figure 3b). Similarly, experiments at pH 7.2 in which the ATPase was either preincubated in 10 mM Mg^{2+} or incubated in the absence of Mg^{2+} then mixed in a 1:1 ratio with 20 mM Mg^{2+} gave identical kinetics for the change in tryptophan fluorescence on Ca^{2+} dissociation (results not shown).

Ca^{2+} binding to the ATPase

The rate of Ca^{2+} binding to the ATPase is affected by the rate of

the $E2 \rightarrow E1$ transition. Analysis of the rate of the NBD fluorescence change for NBD-labelled ATPase on jumping the pH from 6.0 to 8.5 and of the rate of reversal of the effects of inhibitors of the ATPase due to Ca^{2+} binding (Figures 10 and 11) define this rate (Table 1), as described in the Results section. The Ca^{2+} -binding rates are defined by the dissociation rates and the equilibrium constants (Table 1). The only undefined rate parameters are then those describing Mg^{2+} binding to the first Ca^{2+} -binding site. As shown in Figure 12, Mg^{2+} has little effect on the rate of the tryptophan-fluorescence change after Ca^{2+} binding at pH 6.0 but markedly increases the rate observed at pH 7.2. At pH 7.2 in the presence of Mg^{2+} the fluorescence curves fit to sums of two exponentials, with the rate varying relatively little with Mg^{2+} concentration but with a marked increase in the relative amplitude of the fast component with increasing Mg^{2+} (Table 2). In terms of Scheme 1, the effect of Mg^{2+} follows from a Mg^{2+} -induced shift in the $E2 \rightarrow E1$ equilibrium towards E1. With the binding constants for Mg^{2+} estimated in Henderson et al. (1994) for NBD-labelled ATPase, it was only found possible to simulate the effect of Mg^{2+} assuming a low rate of dissociation of Mg^{2+} from the first Ca^{2+} -binding site in the E2 conformation [Table 1; Scheme 5 in (Henderson et al., 1994)]. However, it is possible that NBD labelling reduces the affinity of the ATPase for Mg^{2+} (Henderson et al., 1994), and the data in Table 2 can also be simulated with fast dissociation of Mg^{2+} if the binding constant for Mg^{2+} for the unlabelled ATPase is three times that of the labelled ATPase (Table 1).

As shown, with the parameters in Table 1, it is possible to simulate the rate of tryptophan change on Ca^{2+} binding at pH 6.0, 7.2 and 8.5 (Figure 12), the effect of Mg^{2+} on the rates and amplitudes of the two exponentials required to fit the data at pH 7.2 (Table 2) and the inhibitory effect of Mg^{2+} at pH 8.5 (Figure 12, Table 3) which, in the proposed model, occurs because of Mg^{2+} inhibition of the $E1Ca \rightarrow E1'Ca$ step.

Effects of pH and Mg^{2+} jumps are more complex on Ca^{2+} binding than on Ca^{2+} dissociation. Thus Figure 13(a) shows that when the pH is jumped from 8.5 to 6.0, an initial rapid phase is observed before the slower change typical of pH 6.0. This effect does not indicate slow binding of H^+ to a site on the ATPase, but rather follows from the high proportion of the ATPase in the E1 state at high pH; the magnitude of the initial fast phase depends on the relative rates of Ca^{2+} binding and of the $E1 \rightarrow E2$ transition, and is reproduced well in the simulations. In pH jumps from 6.0 to 8.5, a lag phase is observed. Again, this does not represent slow dissociation of H^+ from some site on the ATPase but rather reflects the rate of the $E2 \rightarrow E1$ transition at pH 8.5; the lag phase is also reproduced well in the simulations.

At pH 6.0, jumps in Mg^{2+} concentration have no effect on the time course of the tryptophan-fluorescence change on binding Ca^{2+} (results not shown), consistent with the lack of effect of Mg^{2+} on the rate of change of tryptophan fluorescence at pH 6.0 (Figure 12). However, at pH 7.2, the rate of change of tryptophan fluorescence on binding Ca^{2+} after a jump in Mg^{2+} concentration from 0 to 20 mM is slower than that observed when the ATPase is preincubated with 20 mM Mg^{2+} (Figure 14a). Simulations (Figure 14a) show that this effect arises because of the increased proportion of E1 forms of the ATPase present at pH 7.2 in the presence of Mg^{2+} (Henderson et al., 1994). At pH 8.5, the rate of change of tryptophan fluorescence is the same for a jump in Mg^{2+} concentration from 0 to 20 mM or after preincubation with 20 mM Mg^{2+} (Figure 14b). This is also reproduced in the simulations (Figure 14b) and follows partly from the suggestion that the ATPase is largely in the E1 form at pH 8.5, even in the absence of Mg^{2+} (Henderson et al., 1994) and partly from the suggestion that, at pH 8.5, the rate of the tryptophan-fluorescence

change is largely controlled by the rate of the E1Ca → E1'Ca step.

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APPENDIX

Dissociation of Ca²⁺ from the (Ca²⁺–Mg²⁺)-ATPase

Dissociation of Ca²⁺ from two independent identical sites on the ATPase can be described as two independent reactions of the form:

The concentration of the Ca²⁺-bound ATPase at any time t , [E1Ca] _{t} , is then given as a function of the concentration at time 0, [E1Ca]₀ [see Benson (1960)] as:

$$\frac{[\text{E1Ca}]_t}{[\text{E1Ca}]_0} = 1 - \alpha(1 - e^{-\lambda t}) \quad (\text{A2})$$

where

$$\alpha = \frac{1}{1 + K\text{Ca}} \quad (\text{A3})$$

and

$$\lambda = k_{12}(1 + K\text{Ca}) \quad (\text{A4})$$

and the equilibrium constant for Ca²⁺ binding $K = k_{21}/k_{12}$.

This equation predicts that the amplitude of the tryptophan fluorescence change will decrease with increasing Ca²⁺, but that the rate of the change will increase with increasing Ca²⁺. Both will have the same dependence on the concentration of Ca²⁺, given by K . This is not what is observed experimentally, so that Ca²⁺ dissociation cannot occur from two independent sites.

Under conditions where the E1'Ca → E1Ca step is fast and at low Ca²⁺ concentrations so that no back reactions occur, Scheme 1 for Ca²⁺ dissociation simplifies to:

This is a special case of that considered by Lowry and John (1910):

Under the conditions that at time $t = 0$, $A_1^t = A_1^0$ and $A_2^t = A_3^t = 0$, the concentrations A_1^t , A_2^t and A_3^t of the three species at time t are given by:

$$A_1^t = A_1^0 \left\{ \frac{k_{21}k_{32}}{\lambda_2\lambda_3} + \left[\frac{k_{12}(\lambda_2 - k_{23} - k_{32})}{\lambda_2(\lambda_2 - \lambda_3)} \right] e^{-\lambda_2 t} + \left[\frac{k_{12}(k_{23} + k_{32} - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)} \right] e^{-\lambda_3 t} \right\} \quad (\text{A7})$$

$$A_2^t = A_1^0 \left\{ \frac{k_{12}k_{32}}{\lambda_2\lambda_3} + \left[\frac{k_{12}(k_{32} - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)} \right] e^{-\lambda_2 t} + \left[\frac{k_{12}(\lambda_3 - k_{32})}{\lambda_3(\lambda_2 - \lambda_3)} \right] e^{-\lambda_3 t} \right\} \quad (\text{A8})$$

$$A_3^t = A_1^0 \left\{ \frac{k_{12}k_{23}}{\lambda_2\lambda_3} + \left[\frac{k_{12}k_{23}}{\lambda_2(\lambda_2 - \lambda_3)} \right] e^{-\lambda_2 t} - \left[\frac{k_{12}k_{23}}{\lambda_3(\lambda_2 - \lambda_3)} \right] e^{-\lambda_3 t} \right\} \quad (\text{A9})$$

where

$$\lambda_2 = \frac{1}{2}(p + q) \quad (\text{A10})$$

$$\lambda_3 = \frac{1}{2}(p - q)$$

and

$$p = k_{12} + k_{21} + k_{23} + k_{32} \quad (\text{A11})$$

$$q = \{p^2 - 4(k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})\}^{\frac{1}{2}}$$

Thus the behaviour of the system is described by just two rate constants λ_2 and λ_3 .

The fractional occupancy of the Ca²⁺ sites, F is given by:

$$F = A_1^t + 0.5A_2^t \quad (\text{A12})$$

Table A1 Rate constants describing Ca^{2+} dissociation from the ATPase

The calculated rate constants λ_2 and λ_3 at various final pCa values derived from the equations describing two sequential reactions, assuming that $K_1 = 10^6 \text{ M}^{-1}$ and $k_{12} = k_{23} = 10.0 \text{ s}^{-1}$, together with the rate (k) and amplitude obtained when the calculated lines are fitted to a single exponential.

	pCa	$\lambda_2 \text{ (s}^{-1}\text{)}$	$\lambda_3 \text{ (s}^{-1}\text{)}$	$k \text{ (s}^{-1}\text{)}$	Amplitude
$K_2 = 10^6$	7.0	142	7.8	6.7	0.98
	6.5	18.8	7.6	7.0	0.83
	6.0	7.0	10.0	10.0	0.50
	5.5	59.4	23.8	25.5	0.18
	5.0	141.6	78.4	86.7	0.05
$K_2 = 10^4$	7.0	13.7	7.3	6.3	1.03
	6.5	17.4	5.76	5.5	1.01
	6.0	26.2	3.89	4.0	0.96
	5.5	49.7	2.28	2.35	0.85
	5.0	119	1.76	1.81	0.47

Equilibrium constants for Ca^{2+} binding are defined by:

$$K_1 = \frac{k_{32}}{k_{23}} \quad K_2 = \frac{k_{21}}{k_{12}} \quad (\text{A13})$$

Under conditions where back reactions can be ignored (low Ca^{2+} concentrations), the equations simplify to:

$$F = \frac{(2k_{23} - k_{12})e^{-k_{12}t} - k_{12}e^{-k_{23}t}}{k_{23} - k_{12}} \quad (\text{A14})$$

and if $k_{23} = k_{12}/2$, then the equation simplifies further to a single exponential with rate k_{23} .

When the back reactions become significant, simplification is not possible. Typical simulations under these conditions are shown in Figure A1. K_2 , k_{12} and k_{23} were set at 10^6 , 10 s^{-1} and 10 s^{-1} respectively for all simulations. In the first set of calculations, K_1 was fixed at 10^6 , equal to K_2 and the calcium occupancy (F) against time calculated according to the above equations for various final Ca^{2+} concentrations. Values of the calculated rates λ_2 and λ_3 are given in Table A1. At lower pCa values, an initial fast phase is clearly present in the simulations (as in the experimental data, see Figure 6). Nevertheless, the simulated curves fit reasonably well to single exponentials. The rate derived by the curve fitting is close to the value of the slow component λ_3 (Table A1). The rate of Ca^{2+} dissociation increases with increasing Ca^{2+} concentration, whereas the experimental data show a decrease in rate with increasing Ca^{2+} concentration.

Figure A1 Simulation of the effect of Ca^{2+} concentration on Ca^{2+} dissociation from the ATPase

The figure shows simulations of Ca^{2+} dissociation according to the above equations, with $K_1 = 10^6$, $k_{12} = k_{23} = 10 \text{ s}^{-1}$ and $K_2 = 10^6$ (a) or 10^4 (b) and pCa values of: A, 7.0; B, 6.5; C, 6.0; D, 5.5; E, 5.0.

The simulations were reported with $K_1 = 10^4$ (Figure A1) with the calculated rates λ_2 and λ_3 at each pCa value given in Table A1. Again the simulated curves fitted well to a single exponential with a rate close to the value of λ_3 . In this case, however, the rate of decrease in Ca^{2+} occupancy decreases with increasing Ca^{2+} concentration, as is the case with the experimental data.

Simulations therefore show that K_1 must be less than K_2 for the rate of dissociation to decrease with increasing Ca^{2+} concentration.

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