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Conformational transitions in the $Ca^{2+}+Mg^{2+}$-activated ATPase and the binding of Ca^{2+} ions

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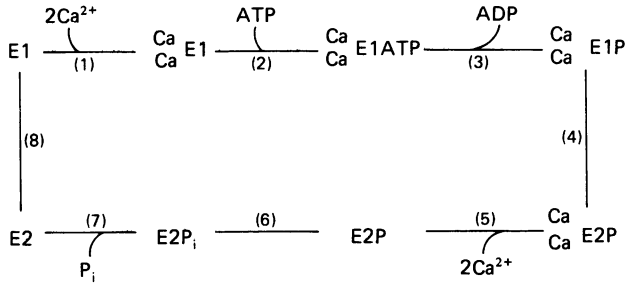
We have studied the fluorescence of the $Ca^{2+}+Mg^{2+}$-activated ATPase of sarcoplasmic reticulum labelled with fluorescein isothiocyanate. The change in intensity of fluorescein fluorescence caused by addition of Ca^{2+} to the labelled ATPase can be interpreted in terms of a two-conformation model for the ATPase, one conformation (E1) having a high affinity for Ca^{2+}, the other (E2) a low affinity. Effects of Ca^{2+} as a function of pH allow an estimate of the effect of pH on the E1/E2 ratio, consistent with kinetic studies. A model is presented for binding of Ca^{2+} to the ATPase as a function of pH that is consistent both with the data on the E1/E2 equilibrium and with literature data on Ca^{2+} binding.

INTRODUCTION

We have shown that the activity of the $Ca^{2+}+Mg^{2+}$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) purified from rabbit muscle SR and reconstituted into model membrane systems is sensitive to the chemical structure of the surrounding phospholipids when measured at saturating Ca^{2+} and high ATP concentrations (Warren *et al.*, 1974*a*; East & Lee, 1982; East *et al.*, 1984). The phospholipid supporting optimal activity is dioleoyl phosphatidylcholine, and either increasing or decreasing the fatty acyl chain length or changing the lipid head group leads to a decrease in ATPase activity (Lee *et al.*, 1986). Before these effects of lipids can be properly understood, it is necessary to develop a complete kinetic model for the ATPase. Fortunately, considerable information is available about the kinetics of the ATPase, particularly concerning the effects of substrate concentration, pH and ions (particularly K^+) on steady-state kinetics, the steady-state concentrations of reaction intermediates (particularly phosphorylated states of the ATPase) and time-dependent changes in the concentrations of reaction intermediates (de Meis, 1981). There have been a number of attempts to incorporate these data into partial kinetic models for the ATPase, but no attempt at a complete model has yet been reported (Tanford, 1982; Inesi & Hill, 1983; Pickart & Jencks, 1984; Fernandez-Belda *et al.*, 1984).

The most commonly used reaction scheme (Scheme 1) is that proposed by de Meis & Vianna (1979). The first two steps involve binding of Ca^{2+} and ATP to high-affinity sites exposed on the outer (cytoplasmic) side of the SR membrane. After phosphorylation and loss of ADP (step 3), the ATPase undergoes a conformation change in which the Ca^{2+}-binding sites become of low affinity and exposed to the inside of the SR (step 4). Ca^{2+} is then lost from these sites, and, after hydrolytic cleavage of the phosphorylated intermediate (step 6), the enzyme recycles (step 8). The basic feature of this model is the existence of two distinct conformations of the ATPase, one conformation (E1) having high affinity for Ca^{2+} and ATP and the other (E2) having low affinity for Ca^{2+} and



Scheme 1. Reaction scheme for the ATPase proposed by de Meis & Vianna (1979)

ATP but able to react with phosphate to give a phosphorylated enzyme (steps 7 and 6).

An important parameter in any kinetic model for the ATPase based on this scheme is the equilibrium constant E1/E2 for step 8. In a number of papers Pick's group has presented a fluorescence method for measuring this constant with the use of fluorescein-labelled ATPase (Pick, 1981*a*,*b*, 1982; Pick & Karlish, 1980, 1982; Pick & Bassilian, 1981). Reaction of the ATPase with FITC leads to inhibition of ATPase activity as the result of selective modification of the ATP-binding site, total inhibition following from binding of one fluorescein molecule per ATPase molecule (Clore *et al.*, 1982; Andersen *et al.*, 1982), and not one fluorescein molecule per two ATPase molecules as originally suggested (Pick & Karlish, 1980). Addition of Ca^{2+} to the modified ATPase has been shown to lead to a small decrease in fluorescein fluorescence (Pick & Karlish, 1980; Pick, 1981*b*), whereas addition of vanadate ions leads to a small increase in fluorescence (Pick, 1982). In the presence of Ca^{2+}, the ATPase will be totally in the form Ca_2E1. Since it is believed that vanadate binds to the ATP-binding site to produce an analogue of the phosphoenzyme (Pick, 1982; Highsmith *et al.*, 1985) in the presence of vanadate and absence of Ca^{2+}, the enzyme will be in a form analogous to E2P in Scheme 1. If it is assumed that the changes of fluorescein fluorescence follow only from the E2–E1 conformation

Abbreviations used: ATPase, $Ca^{2+}+Mg^{2+}$-activated ATPase; $C_{12}E_8$, dodecyl octaethylene glycol monoether; FITC, fluorescein 5′-isothiocyanate; SR, sarcoplasmic reticulum.

change (i.e. that the fluorescences of all E1 forms are equal, as are those of all E2 forms), then the ratio E1/E2 in the absence of Ca^{2+} or vanadate is given by

$$E1/E2 = b/a$$

where a is the Ca^{2+}-induced fluorescence quenching in the absence of vanadate and b is the vanadate-induced fluorescence enhancement (Pick, 1982).

As is discussed below, the results obtained by Pick (1982) appear to be inconsistent with results of kinetic studies on the E1–E2 change. We therefore decided to re-investigate the fluorescence response of FITC-labelled ATPase.

MATERIALS AND METHODS

FITC (isomer 1) was obtained from Aldrich Chemical Co. The SR was prepared from female rabbit (New Zealand White) hindleg muscle by homogenization in the presence of dithiothreitol and phenylmethanesulphonyl fluoride followed by centrifugation and treatment with 0.6 M-KCl to precipitate actinomyosin as described in East & Lee (1982). Typically, on polyacrylamide-gel electrophoresis the ATPase was 80% of the total protein present. Pure ATPase was isolated by treating sarcoplasmic reticulum with potassium cholate and loading on to a discontinuous sucrose gradient, as described in East & Lee (1982). Polyacrylamide-gel electrophoresis showed the presence of essentially pure ATPase (>95%), and the protein/phospholipid molar ratio of 1:30 was in agreement with previous reports (Warren *et al.*, 1974*b*).

Concentrations of protein and fluorescein were assessed from absorption spectra for samples (150 μl) added to 1% SDS (800 μl) and 2 M-NaOH (50 μl), by using the absorption coefficient (1.2 litre·g^{-1}·cm^{-1}) for the ATPase at 280 nm given by Hardwicke & Green (1974) and a molar absorption coefficient of 80000 M^{-1}·cm^{-1} at 500 nm for fluorescein (Pick & Karlish, 1980).

A variety of procedures were used to label SR and ATPase. The first involved reaction with an excess of FITC followed by separation of free FITC. SR (1.2 mg in 40 μl of 100 mM-KCl/40 mM-Hepes/KOH buffer, pH 6.3) was diluted to a total volume of 300 μl with reaction buffer (0.2 M-sucrose/100 μM-EGTA/50 mM-Tris/50 mM-maleate buffer, pH 8.0). FITC was then added from a stock solution in dry dimethylformamide (6 mM) to give a 2:1 molar ratio of FITC to ATPase, assuming that 80% of the protein in SR was ATPase. The final concentration of dimethylformamide in the reaction mixture was typically 1% (v/v). The reaction mixture was then left to stand at room temperature for 1 h. Unbound FITC was then removed by passage through a 5 ml column of coarse-grade Sephadex G-50, equilibrated in 0.2 M-sucrose/50 mM-Tris/HCl buffer, pH 7.0, at 4 °C, and contained in 5 ml disposable plastic syringes plugged with a disc of porous polyethylene sheet (pore size 70 μM) (Belart).

The columns were pre-spun in a bench-top centrifuge at full speed for 2 × 1 min. Samples of reaction mixture (250 μl) were applied to the columns followed by 250 μl of 0.2 M-sucrose/50 mM-Tris/HCl buffer, pH 7.0, at 4 °C. The column was then spun for 1 min at full speed, and the eluate collected. The final protein concentrations were between 0.4 and 0.8 mg/ml, with an FITC/ATPase labelling ratio of between 0.7:1 and 1.1:1. Samples were kept on ice and used within 6 h of preparation. Purified ATPase was labelled by using the same procedure.

At FITC/ATPase labelling ratios below 1:1 at pH 8 we found that reaction was essentially complete, so that separation of bound and free FITC became unnecessary. To obtain an FITC/ATPase labelling ratio of approx. 0.5:1, SR (0.6 mg) in 35 μl of 1 M-KCl/0.25 M-sucrose/50 mM-potassium phosphate buffer, pH 8, was incubated with FITC (2.5 nmol) added from a stock solution in dry dimethylformamide (6 mM). The reaction mixture was left to stand at room temperature for 1 h and then diluted with 250 μl of 0.2 M-sucrose/50 mM-Tris/HCl buffer, pH 7, and stored on ice until use. Samples of pure ATPase were also labelled in the same way.

Fluorescence spectra were recorded on Spex Fluorolog and Perkin–Elmer MPF44A fluorimeters. Labelled protein (15–20 μg) was added to 2.5 ml of 5 mM-$MgSO_4$/100 mM-KCl/50 mM-Tris/50 mM-maleate buffer at the appropriate pH and temperature. EGTA and $CaCl_2$ were added from stock solutions to give total concentrations of 100 and 400 μM respectively. Ammonium vanadate was dissolved in 100 mM-KOH to give a 100 mM stock solution and was added to the fluorescence samples to a final concentration of 100 μM. Fluorescence spectra were recorded with excitation at 495 nm, fluorescence intensity being measured at 525 nm.

Kinetic simulations were carried out by using the FACSIMILE program (Chance *et al.*, 1977) running on an ICL 2976 computer.

RESULTS

Although they were not reported on by Pick's group (Pick, 1981*a*,*b*, 1982; Pick & Karlish, 1982), we find large time-dependent changes in the fluorescence of SR labelled with FITC. When SR was incubated with FITC at an FITC/ATPase molar ratio of 2:1, free FITC separated from the ATPase by passage through a Sephadex G-50 column and the fluorescence intensity determined immediately after dilution into buffer, we observed an initial low fluorescence intensity that increased by approx. 25% over 1 h. When the SR was labelled at an FITC/protein molar ratio of 0.5:1 we found that virtually all (>90%) of the FITC was covalently attached to protein, so that separation of free and bound FITC was no longer necessary. We found similar time-dependent changes in this system, and these are illustrated in Fig. 1. We found that, if samples were taken from the FITC/SR reaction mixture, diluted into buffer at pH 7 and their fluorescence intensities determined immediately, then the fluorescence intensities decreased over 10 min to 60% of that of an equivalent amount of FITC in the same buffer, but that samples taken over the next few hours showed gradually increasing fluorescence intensities. The fluorescence intensity of these samples, however, was not stable, and increased markedly over about 30 min following dilution into buffer at pH 7. We found that the rate of these time-dependent changes following dilution increased with increasing temperature. Thus we found that, if the samples were incubated for 30 min at 30 °C after dilution to pH 7, then stable and reproducible fluorescence intensities were obtained. The final fluorescence intensity obtained after approx. 1 h of reaction between FITC and SR at 20 °C was 80% of that of a corresponding sample of FITC.

When the same reaction protocol was followed with

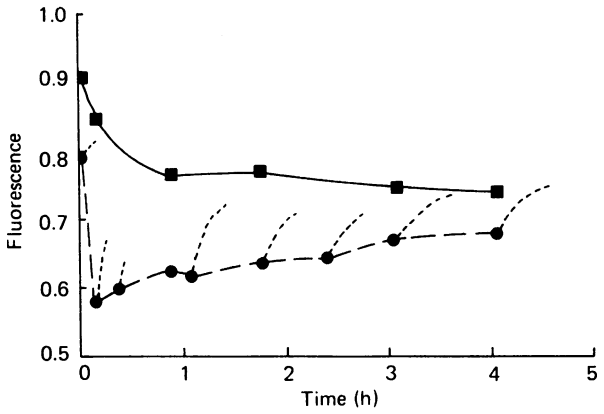


Fig. 1. Variation of fluorescence intensity for SR labelled with FITC as a function of time

SR (1.2 mg) in 70 μl of 0.25 M-sucrose/1 M-KCl/50 mM-potassium phosphate buffer, pH 8.0, was incubated with FITC at an FITC/protein molar ratio of 0.5:1. At the given times 1.2 μl samples were withdrawn, diluted into 3 ml of 100 mM-KCl/5 mM-$MgSO_4$/100 μM-EGTA/50 mM-Tris/50 mM-maleate buffer, pH 7.0, and fluorescence intensities determined immediately (●, ——). Fluorescence intensities of these samples were also determined as a function of time following dilution (------). At the same given times 1.2 μl samples were withdrawn, diluted into buffer, incubated for 30 min at 30 °C, cooled at 20 °C for 5 min and the fluorescence intensities measured (■, ——). All fluorescence intensities were measured at 20 °C, and are expressed relative to that for free FITC.

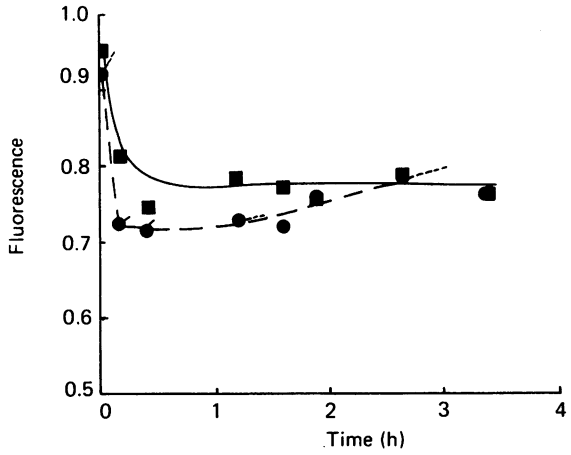


Fig. 2. Variation of fluorescence intensity for purified ATPase labelled with FITC as a function of time

ATPase (1.2 mg) was labelled with FITC, and fluorescence intensities were determined immediately after dilution into buffer at pH 7 (●, ——), as described in Fig. 1 legend. Fluorescence intensities were also determined as a function of time following dilution (------). ■ (——), Fluorescence intensities determined after a 30 min incubation at 30 °C, as described in Fig. 1 legend. Fluorescence intensities are expressed relative to that for free FITC.

purified ATPase, the fluorescence intensity was also observed to fall to approx. 80% of that of free FITC after 10 min of reaction, but now little change of fluorescence intensity occurred with time after dilution into buffer at pH 7, the level of fluorescence observed immediately after dilution being very close to that observed after a 30 min incubation at 30 °C (Fig. 2).

The final fluorescence intensity after equilibration was independent of the FITC/ATPase labelling ratio between 0.01:1 and 0.9:1 (results not shown). The fluorescence intensity was, however, markedly dependent on pH, as shown in Fig. 3. The fluorescence intensity–pH plots fitted well to a simple Henderson–Hasselbach equation, assuming that the fluorescence intensity of the monoanion species formed at low pH was zero. The curves shown in Fig. 3 were calculated assuming a fluorescence intensity ratio for the dianion form of free FITC/bound FITC of 1.25:1 with pK values of 6.4 and 5.8 for free and bound FITC respectively. The value obtained for free FITC compares well with the literature value of 6.3 for fluorescein (Stanton *et al.*, 1984).

As previously reported by Pick's group (Pick, 1981*a*,*b*, 1982; Pick & Karlish, 1982), we found that addition of Ca^{2+} and vanadate to FITC-labelled SR cause a small decrease and increase in fluorescence intensity respectively. We observed similar changes for the FITC-labelled ATPase (Fig. 4 and Table 1). Although the most reproducible results were obtained with samples equilibrated to a constant fluorescence level, similar responses to Ca^{2+} and vanadate were also observed for samples whose fluorescence intensity was still changing with time.

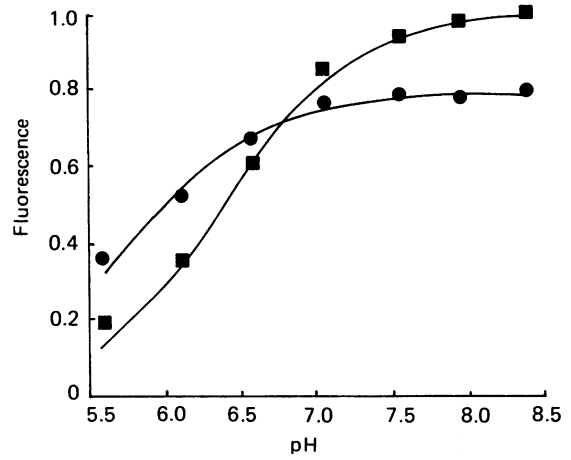


Fig. 3. Fluorescence intensity of FITC-labelled ATPase as a function of pH

Fluorescence intensities were determined as a function of pH for FITC (■) and FITC-labelled ATPase (●) at an FITC/protein molar ratio of 0.5:1. The continuous lines show fits to the Henderson–Hasselbach equation (see the text).

DISCUSSION

It has been established that FITC specifically labels the ATPase at the ATP-binding site (Pick & Karlish, 1982; Clore *et al.*, 1982; Andersen *et al.*, 1982). In principle, this modification could significantly alter the E1/E2 equilibrium, but for the following reasons this seems not to be the case. Although reaction with ATP is blocked by FITC labelling, acetyl phosphate can still act as substrate, resulting in accumulation of Ca^{2+}, suggesting that FITC

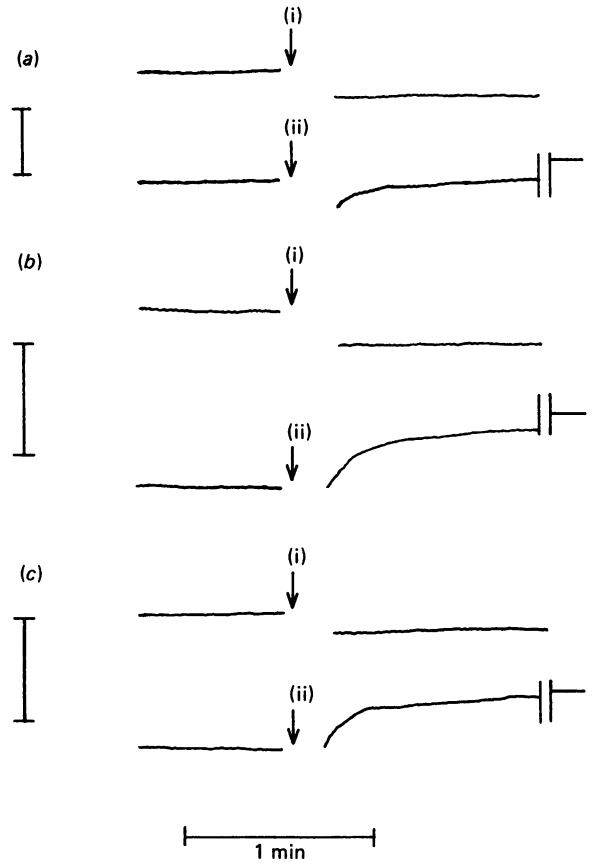


Fig. 4. Changes in fluorescence intensity for FITC-labelled ATPase caused by addition of Ca^{2+} and vanadate

The Figure shows the fluorescence intensity of FITC-labelled ATPase as a function of time and the response to the addition of (i) Ca^{2+} to a final concentration of 300 μM and (ii) vanadate to a final concentration of 100 μM at (*a*) pH 6, (*b*) pH 7 and (*c*) pH 8, all at 20 °C. The vertical bars represent a 20% change of fluorescence intensity at each pH.

blocks only ATP binding and not any other step in the reaction cycle (Pick & Karlish, 1982). Secondly, binding of vanadate to native and that to FITC-labelled ATPase are very similar (Pick, 1982; Highsmith *et al.*, 1985). Thirdly, the same response to Ca^{2+} binding is observed by measuring the tryptophan and fluorescein fluorescence for the unmodified and modified ATPases (Pick, 1982; Andersen *et al.*, 1982).

The ATPase can be labelled with excess FITC, the excess FITC being removed by passage through a Sephadex G-50 column. Alternatively, the ATPase can be labelled at FITC/protein molar ratios lower than 1:1 when, at pH 8, essentially all the FITC reacts, making the separation step unnecessary. We observe that the intensity of fluorescein fluorescence at pH 7 is approx. 80% of that of the corresponding concentration of free FITC (Figs. 1 and 2), independently of the degree of protein labelling. As well as this fluorescence quenching on the ATPase, there is a shift in pK_a value for the fluorescein from 6.4 in buffer to 5.8 on the ATPase (Fig. 3), indicating a stabilization of the ionized form of the carboxy group of the fluorescein on the protein surface. These pK_a values measured by fluorescence spectroscopy are weighted averages of ground-state and excited-state pK_a values. The ground-state pK_a value of sodium fluorescein (6.0) is slightly less than the value obtained by fluorescence (6.3) (Stanton *et al.*, 1984).

For FITC-labelled SR we observe a large slow increase in fluorescence intensity on diluting into buffer at pH 7. We have no explanation for this observation. Andersen *et al.* (1982) observed a large increase in fluorescence intensity with time for FITC-labelled ATPase in the presence of the detergent $C_{12}E_8$ in the absence of Ca^{2+}. This was, however, correlated with a gradual loss of ATPase activity. Our systems show no such loss of activity with time. However, the observations that these time-dependent changes are not seen with FITC-labelled ATPase, and that the final equilibrium fluorescence values of FITC-labelled ATPase and SR are the same, suggest that it is these final fluorescence values that are important.

Table 1. Experimental E1/E2 ratios for ATPase

Values of the E1/E2 ratio calculated from the responses to Ca^{2+} and vanadate of FITC-labelled ATPase at 20 °C and FITC-labelled SR at 32 °C

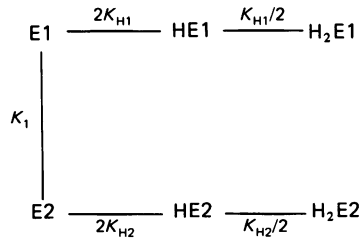
System	pH	Fluorescence response		E1/E2 ratio		
		To Ca^{2+}	To vanadate	From ratio of Ca^{2+} response to vanadate response	From Ca^{2+} response	Theoretical E1/E2 ratio*
ATPase (20 °C)	6	−7.6	7.5	1.0	0.14†	0.14
	7	−5.6	11.0	2.0	0.5	0.54
	8	−3.0	10.5	3.5	1.9	1.88
SR (32 °C)	6	−9.9	1.7	0.2	0.14†	
	7	−8.8	2.5	0.3	0.3	
	8	−3.6	6.8	1.9	2.0	

* Calculated according to Scheme 2 with $K_{H1} = 4.0 \times 10^6$, $K_{H2} = 2.0 \times 10^7$ and $K_1 = 2.5$.
† Fixed (see the text).

As reported by Pick's group (Pick, 1982; Pick & Karlish, 1982), addition of Ca^{2+} and vanadate to both FITC-labelled SR and FITC-labelled ATPase leads to decreases and increases in fluorescence intensity respectively. If, as suggested by Pick (1982), fluorescence is sensitive only to the E1–E2 equilibrium, then the equilibrium constant E1/E2 can be put equal to b/a, where b and a are the vanadate and Ca^{2+} responses respectively. Such an analysis gives values for the equilibrium constant at pH 6 of 1 and 0.2 at 20 °C and 32 °C respectively (see Table 1). These values compare reasonably well with the estimates of 1 at 15 °C, 0.5 at 20 °C and 0.1 at 32 °C published by Pick & Karlish (1982). However, they do not agree well with estimates for the equilibrium constant derived from kinetic measurements. The rate of the transition E1–E2 can be estimated from measurements of the rate of phosphorylation of the enzyme after addition of EGTA and phosphate to the enzyme initially in the Ca_2E1 form (reversal of steps 2–6) (Chaloub *et al.*, 1979; Chaloub & de Meis, 1980). The rate constant for this transition is approx. 23 s^{-1} at pH 6 and temperatures somewhere between 22 and 28 °C (Froud & Lee, 1986). The rate of the transition E2–E1 has been estimated from changes in tryptophan fluorescence of the ATPase on addition of Ca^{2+} to be between 1.0 and 4 s^{-1} at pH 6 and 20 °C (Dupont & Leigh, 1978; Guillain *et al.*, 1980, 1981). Because of the differences in temperature and because of the inherent inaccuracies in many of these determinations, exact comparisons are difficult. However, it is shown below that the data are fitted reasonably well assuming rate constants of 3.3 and 23 s^{-1} for the E2–E1 and E1–E2 transitions respectively at pH 6, giving an equilibrium constant E1/E2 of 0.14. This value is significantly different from that estimated from fluorescein fluorescence. One possible explanation for the difference is that the fluorescence change on addition of vanadate does not simply reflect the transition to the E2 conformation but also includes contributions from changes at the ATP-binding site caused directly by vanadate binding. This seems likely, since phosphorylation of FITC-labelled SR by acetyl phosphate leads to very large changes (approx. 60%) in fluorescence intensity (Pick, 1981*b*). The fluorescence response to Ca^{2+}, however, is more likely to reflect only the E2–E1 conformation change, since Ca^{2+}-binding and ATP-binding sites are thought to be distant on the ATPase (Highsmith & Murphy, 1984), so that binding of Ca^{2+} at the Ca^{2+}-binding sites is unlikely to affect directly a probe at the ATP-binding site. If it is assumed that the response to Ca^{2+} does indeed reflect the E2–E1 equilibrium, then it is possible to determine the E1/E2 ratio as a function of pH from the Ca^{2+} response alone, as long as a value for the E1/E2 ratio is known at at least one pH value. It can be shown readily that, if at one pH the Ca^{2+} response and ratio E1/E2 are a_1 and x_1 respectively and at another pH are a_2 and x_2 respectively, then:

$$x_2 = [x_1 a_1 + (a_1 - a_2)]/a_2$$

Assuming a value for E1/E2 of 0.14 at pH 6, values at other pH values can be calculated (Table 1). Values obtained for SR at 20 °C are very similar to those for ATPase (results not shown). For SR at 32 °C, the E1/E2 ratios at pH 7 and 8 are also very close to those at 20 °C, again assuming a value of 0.14 for the ratio at pH 6 (Table 1). Pick & Karlish (1982) report a large temperature-dependence for the E1/E2 ratio, but it is clear from the data in Table 1 that this comes about from the large change in vanadate response with temperature.



Scheme 2. Effect of H^+ binding on the E1/E2 equilibrium of ATPase

It is now necessary to fit the observed variation in E1/E2 ratio to a specific model for proton binding to the ATPase. It can be shown that the variation in ratio with pH is too steep to involve a single ionization, and that at least two ionizable groups must be involved. The simplest model is then to assume two identical ionizing groups, with different pK values in the E1 form and the E2 form (Scheme 2). The ratio of total E1 to total E2 forms of the ATPase can then be written as:

$$\frac{E1_{total}}{E2_{total}} = \frac{K_1(1+2K_{H1}[H]+K_{H1}{}^2[H]^2)}{(1+2K_{H2}[H]+K_{H2}{}^2[H]^2)}$$

where K_{H1} and K_{H2} are the intrinsic proton association constants for E1 and E2 respectively, the factors 2 account for statistical factors (Roberts, 1977) and K_1 = E1/E2. The data can be fitted well to this model (Table 1) with the parameters given in Table 2.

Table 2. Kinetic parameters for ATPase at 25 °C

Parameters were obtained by simulations as described in the text.

Reaction	Equilibrium constant: Symbol	Equilibrium constant: Value	Forward rate constant (s^{-1})
$E1+H^+ \rightleftharpoons HE1$	K_{H1}	4.0×10^6	
$E2+H^+ \rightleftharpoons HE2$	K_{H2}	2.0×10^7	
$E1+H^+ \rightleftharpoons E1H$	K_{H3}	1.5×10^8	
$E1'Ca_2+H^+ \rightleftharpoons E1'HCa_2$	K_{H8}	1.67×10^7	
$E1_0^{Ca}+H^+ \rightleftharpoons E1_0^{HCa*}$	K_{H9}	1.5×10^8	
$E1'^{Ca}_0+H^+ \rightleftharpoons E1'^{HCa*}_0$	K_{H10}	1.0×10^9	
$E2 \rightleftharpoons E1$	K_1	2.5	140.0
$HE2 \rightleftharpoons HE1$		†	15.5
$H_2E2 \rightleftharpoons H_2E1$		†	1.7
$E1+Ca^{2+} \rightleftharpoons E1Ca$	K_{C1}	5.0×10^5	1.0×10^8
$E1H+Ca^{2+} \rightleftharpoons E1HCa$		‡	1.0×10^8
$E1H_2+Ca^{2+} \rightleftharpoons E1H_2Ca$		‡	1.0×10^8
$E1Ca \rightleftharpoons E1'Ca$	K_{C2}	1.68	12.0
$E1HCa \rightleftharpoons E1'HCa$		‡	12.0
$E1H_2Ca \rightleftharpoons E1'H_2Ca$		‡	12.0
$E1'Ca+Ca^{2+} \rightleftharpoons E1'Ca_2$	K_{C3}	1.44×10^8	1.0×10^8
$E1'HCa+Ca^{2+} \rightleftharpoons E1'HCa_2$		‡	1.0×10^8
$E1'H_2Ca+Ca^{2+} \rightleftharpoons E1'H_2Ca_2$		‡	1.0×10^8

* 0 represents an unoccupied Ca^{2+}-binding site (see Scheme 4).
† Defined by K_{H1}, K_{H2} and K_1.
‡ Defined by K_{H3}, K_{H8}, K_{H9} and K_{H10}.

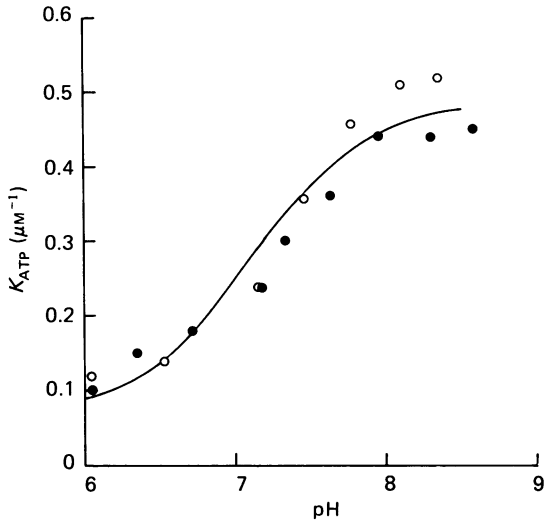


Fig. 5. Binding of ATP to SR or ATPase

The experimental data from Meissner (1973) show the variation of the association constant for the binding of MgATP to SR (○) or ATPase (●) at 0 °C. The line is the simulation assuming binding to the E1 form with $K_{ATP} = 7.0 \times 10^5$.

The calculated variation of E1/E2 ratio with pH provides a ready explanation for the pH-dependence of the binding of MgATP reported by Meissner (1973). As shown in Fig. 5, the pH-dependence of binding can be simulated by assuming that the MgATP binds only to the E1 conformation with an association constant of 7.0×10^5. The alternative explanation would be that binding was affected by an ionizable group of pK 7.3 at the ATP-binding site. Although this possibility cannot be ruled out, the fluorescence data for FITC-labelled ATPase give no evidence for an ionization in the ATP-binding site in this pH range (Fig. 3).

Since it is known that there are two Ca^{2+}-binding sites per ATPase molecule (Hill & Inesi, 1982; Barrabin *et al.*, 1984), that over the pH range 5–7 one proton is released for each Ca^{2+} ion bound (Watanabe *et al.*, 1981) and that the Ca^{2+} affinities in the E1 and E2 forms are markedly different, it is tempting to propose that the ionizable groups responsible for the shift in the E1/E2 equilibrium are those that must exist at the Ca^{2+}-binding site. However, there must be at least one other ionizable group per Ca^{2+}-binding site to explain the observed increase in co-operativity of Ca^{2+} binding with increasing pH (Hill & Inesi, 1982; Fig. 6), as described below.

An apparent co-operativity of close to 2 in Ca^{2+} binding would be produced by the sequence of reactions:

$$E2 \rightarrow E1 \rightarrow E1Ca \rightarrow E1Ca_2$$

if the E1/E2 ratio were 10^{-2}:1 or less, but this is not consistent with the fluorescence and kinetic data. The observed increase in co-operativity with increasing pH is also unexpected. In a sequence of reactions of the type:

$$P + Ca^{2+} \rightarrow PCa$$

$$PCa + Ca^{2+} \rightarrow PCa_2$$

$$P + H^+ \rightarrow PH$$

where binding of H^+ is competitive with the binding of Ca^{2+}, high proton concentrations will make the binding of Ca^{2+} appear more co-operative by competing with the binding of the first Ca^{2+} ion.

Studies by Dupont (1982) and Fernandez-Belda *et al.* (1984) of Ca^{2+} binding have been interpreted in terms of sequential binding of the type:

$$E \rightarrow ECa \rightarrow E'Ca \rightarrow E'Ca_2$$

where the ECa–E'Ca transition is relatively slow. If this slow step is pH-sensitive, then it can define the co-operativity of Ca^{2+} binding (Scheme 3).

In this scheme we envisage two identical independent Ca^{2+}-binding sites, each containing two ionizable residues 1 and 2. Binding of Ca^{2+} is competitive with protonation of residue 1 with proton association constant K_{H1}. Binding of Ca^{2+} is, however, not competitive with protonation of residue 2, although the binding constants for protons at this site do depend on the occupancy of the site by Ca^{2+}. If it is assumed that the two Ca^{2+}-binding

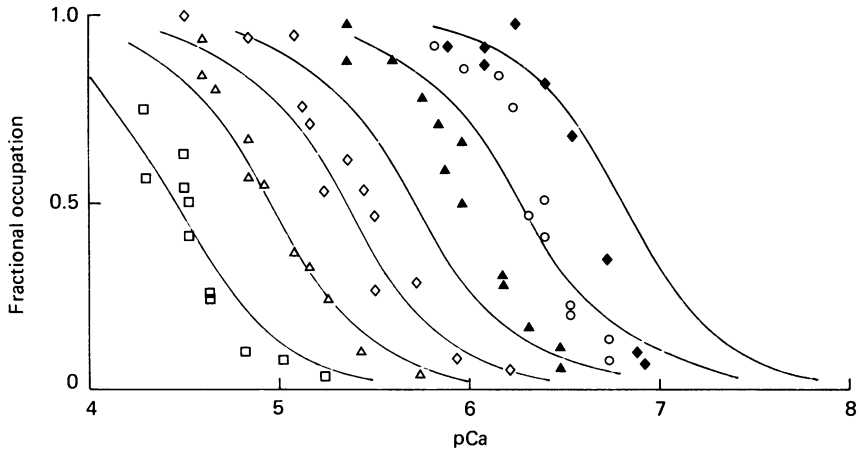
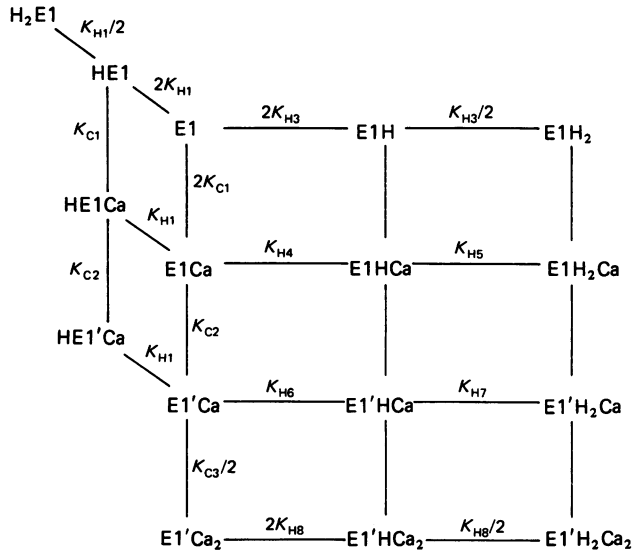


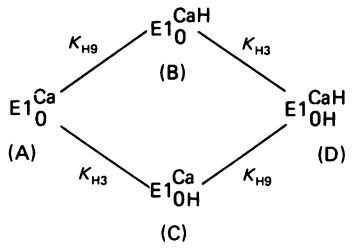
Fig. 6. Binding of Ca^{2+} to ATPase

The experimental data from Hill & Inesi (1982) show the fractional occupation of the Ca^{2+}-binding sites as a function of pCa at pH 5.5 (□), pH 6.0 (△), pH 6.4 (◇), pH 6.8 (▲), pH 7.5 (○) and pH 8.5 (◆). The lines are simulations in terms of Scheme 3 with the parameters listed in Table 2.



Scheme 3. Binding of H^+ and Ca^{2+} to the E1 conformation of ATPase

Binding of Ca^{2+} is competitive with protonation of residue 1 with proton association constant K_{H1}. Binding of Ca^{2+} is non-competitive with protonation of residue 2 with proton association constants K_{H3}–K_{H8}, proton affinities being determined by the state of association with Ca^{2+}. For clarity, binding of protons to residue 1 is only indicated for the unprotonated state of residue 2.



Scheme 4. Protonation of sites of ATPase in the presence of Ca^{2+}

Possible protonations are indicated in the case where only one Ca^{2+} site on the ATPase is occupied. 0 represents a Ca^{2+}-binding site unoccupied by Ca^{2+}.

sites are identical and independent, then a further simplification of the model is possible. Consider (Scheme 4) the case where only a single Ca^{2+}-binding site is occupied, E1Ca in Scheme 3. Protonation of the ionizable residue 2 is possible in either the occupied or the unoccupied site. The association constant for protonation of residue 2 in the site unoccupied by Ca^{2+} is K_{H3}. The association constant for protonation of residue 2 in the site occupied by Ca^{2+} will, in general, be different, and has been written as K_{H9}. Thus:

$$K_{H4} = K_{H3} + K_{H9}$$

Protonation at the second site will then occur to give $E1CaH_2$ (D in Scheme 4), and it is readily shown that:

$$K_{H5} = K_{H3} \cdot K_{H9}/(K_{H3} + K_{H9})$$

Thus the three variables K_{H3}, K_{H4} and K_{H5} have been reduced to two, K_{H3} and K_{H9}. Similarly, we can write for protonation of residue 2 in E1'Ca:

$$K_{H6} = K_{H8} + K_{H10}$$

and

$$K_{H7} = K_{H8} \cdot K_{H10}/(K_{H8} + K_{H10})$$

where K_{H10} is the association constant for binding of protons to group 2 in the site unoccupied by Ca^{2+} in the E1' conformation of the ATPase. If we then assume for simplicity that binding of protons to residue 2 is the same in the E1 and E2 conformations [this is justified in an accompanying paper (Gould *et al.*, 1986)] and that binding of Ca^{2+} to the E2 conformation is weak, then a set of equations can be derived giving the proportion of the ATPase in the various forms [E2], [E1], [E1Ca], [E1'Ca] and [E1'Ca_2]. Putting:

$$F^{E1} = (1 + 2K_{H3}[H] + K_{H3}^2[H]^2)(1 + 2K_{H1}[H] + K_{H1}^2[H]^2)$$

$$F^{E1Ca} = (1 + K_{H4}[H] + K_{H4}K_{H5}[H]^2)(2K_{C1}[Ca] + 2K_{H1}[H]K_{C1}[Ca])$$

$$F^{E1'Ca} = (1 + K_{H6}[H] + K_{H6}K_{H7}[H]^2)(2K_{C1}K_{C2}[Ca] + 2K_{H1}[H]K_{C1}K_{C2}[Ca])$$

$$F^{E1'Ca_2} = (1 + 2K_{H8}[H] + K_{H8}^2[H]^2)K_{C1}K_{C2}K_{C3}[Ca]^2$$

and

$$F^{E2} = (1 + 2K_{H2}[H] + K_{H2}^2[H]^2)(1 + 2K_{H3}[H] + K_{H3}^2[H]^2)/K_1$$

then the concentration of enzyme in, for example, the E1 form is:

$$[E1] = [E_{tot.}]F^{E1}/(F^{E1} + F^{E1Ca} + F^{E1'Ca} + F^{E1'Ca_2} + F^{E2})$$

where $[E_{tot.}]$ is the total ATPase concentration and where square brackets denote all protonated forms of the given species, so that, for example:

$$[E1] = E1 + E1H + E1H_2 + HE1 + HE1H + HE1H_2 + H_2E1 + H_2E1H + H_2E1H_2$$

Corresponding equations give the concentrations of all other forms of the ATPase.

The most complete set of data for Ca^{2+} binding as a function of pH is that of Hill & Inesi (1982). Fig. 6 shows a set of simulations obtained by using the above equations, chosen to give the best visual match to the experimental data. The values of K_{H1} and K_{H2} were fixed on the basis of the E1/E2 equilibrium data, and K_{H8} was fixed at 1.67×10^7 to fit the pH-dependence of the Ca_2E1P–Ca_2E2P transition (see Gould *et al.*, 1986), leaving three variables describing proton binding, K_{H3}, K_{H9} and K_{H10}, and three describing Ca^{2+} binding, K_{C1}, K_{C2} and K_{C3}; all other variables could be described in terms of these. As shown below, the value of K_{C2} can be fixed independently from kinetic data. These parameters each have distinctive effects on the calculated binding curves. The values of K_{C1}, K_{C2} and K_{C3} define Ca^{2+} binding at pH 8.5 and its co-operativity. With these fixed, K_{H3} then defines Ca^{2+} binding at pH 5.5, with K_{C2} having a large effect on co-operativity. K_{H10} also affects the co-operativity at pH 8.5, with values less than 1×10^8 giving a low co-operativity, and the co-operativity no longer increasing for values beyond 1×10^9. The value of K_{H9} has very little effect on the simulations, and so for simplicity has been put equal to K_{H3}.

It can be seen that at low pH agreement between theory and experiment is good, but not at high pH (Fig. 6). First,

at high pH, the pCa value giving half occupancy of sites is significantly shifted from the calculated value. Hill & Inesi (1982) presented a model for Ca^{2+} binding to the ATPase in terms of a four-site model with co-operative interactions between the four sites. They also found significant differences between experimental and theoretical pCa values giving half occupancy at high pH values, and introduced an arbitrary shift to the pCa values in their theoretical profiles of +0.06, −0.12 and −0.25 at pH values of 8.5, 7.5 and 6.8 respectively (Fig. 1 in Hill & Inesi, 1982). Similar shifts applied here would also give excellent agreement between the experimental and theoretical pCa values for half-saturation. It is also clear, however, that, although our model matches the experimental co-operativities in Ca^{2+} binding at low pH values, the calculated co-operativities are too low at high pH. Watanabe *et al.* (1981) estimated that the Hill coefficient h for these Ca^{2+}-binding plots varied from 1.46 at pH 6 to 3.3 at pH 8.5. A two-site model of the type that we propose cannot give h values greater than 2. However, these h values are subject to much experimental error, particularly since the free Ca^{2+} concentrations calculated for EGTA-containing buffer depend very markedly upon the assumed EGTA-binding constants for H^+ and Ca^{2+}. Indeed, Silva & Verjovski-Almeida (1983) have published Ca^{2+}-binding plots at pH 7.5 with an h value of 2, compared with the value of 2.95 reported by Watanabe *et al.* (1981). As shown in Fig. 7, the data reported by Silva & Verjovski-Almeida (1983) fit well to the theoretically calculated plot, after shifting of the experimental pCa values by −0.2 because of the different EGTA–Ca^{2+} binding constants assumed by these authors.

One feature of the model presented here is that the co-operativity of Ca^{2+} binding and the pCa value giving half-saturation depend on the value of K_{C2} describing the E1Ca–E1′Ca conformation change. It has been shown that addition of the detergent $C_{12}E_8$ both decreases Ca^{2+} affinity and decreases the co-operativity of Ca^{2+} binding (Silva & Verjovski-Almeida, 1983). These effects are easily mimicked in terms of Scheme 3. With all other parameters constant but changing K_{C2} from 1.68 to 0.12, it is possible to get good agreement with the experimental data for Ca^{2+} binding in the presence of $C_{12}E_8$ (Fig. 7).

Rates can be assigned to these processes by comparison with Ca^{2+}-binding data (Dupont, 1984) and fluorescence data (Guillain *et al.*, 1980, 1981; Fernandez-Belda *et al.*, 1984; Dupont, 1984). For simplicity, all protonations are assumed to be fast, so that they can be treated by using the quasi-equilibrium approach (Cha, 1968). It is then necessary to assign rates to the Ca^{2+}-binding steps and to the conformational transitions E2–E1. Since the proton affinities of residue 1 at the Ca^{2+}-binding site are different in the E1 and E2 conformations (Table 2), the forward rates for the E2–E1, HE2–HE1 and H_2E2–H_2E1 transitions (k_{+1}, k_{+2} and k_{+3} respectively) will, in general, be different. Since the proton affinity of residue 2 at the Ca^{2+}-binding site has been assumed to be equal for E1 and E2, it will also be assumed that protonation of this residue has no effect on the rate of the E1–E2 transition. For simplicity we have put:

$$k_{+2} = (k_{+1}k_{+3})^{\frac{1}{2}}$$

The rate of the E1Ca–E1′Ca transition will also, in general, be pH-dependent, because of the different proton affinities of E1Ca and E1′Ca, but effects will probably be small since the equilibrium constant for the transition changes only from 1.68 to 1 from the unprotonated to doubly protonated forms.

Fig. 8 shows a simulation of the Ca^{2+}-binding data of Dupont (1984), with the rate parameters listed in Table 2. The initial fast increase in Ca^{2+} binding is defined by the rate constant for the [E1Ca]–[E1′Ca] transition, and the later part of the curve by the rate of the [E2]–[E1] transition. We have assumed that the forward rate of the [E1Ca]–[E1′Ca] transition is unaffected by pH, so that the back rate becomes pH-dependent. As shown in Gould *et al.* (1986), only this assumption is consistent with data on the rate of reaction of the ATPase with ATP. We have also assumed that the forward rate of the [E2]–[E1]

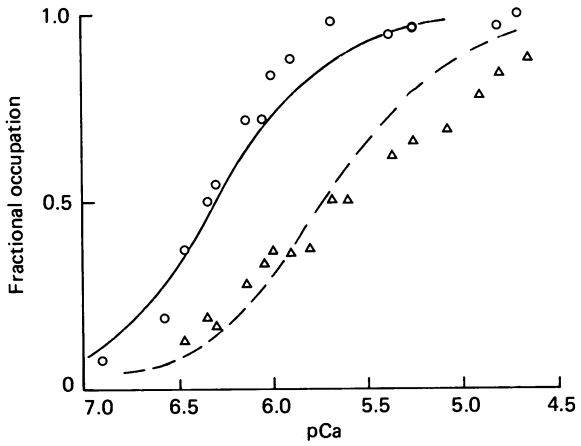


Fig. 7. Effect of $C_{12}E_8$ on Ca^{2+} binding to ATPase

The experimental data from Silva and Verjovski-Almeida (1983) show the fractional occupation of the Ca^{2+}-binding sites as a function of pCa at pH 7.5 in the absence (○) and in the presence (△) of $C_{12}E_8$. The experimental data points have been shifted by −0.2 pCa unit. The lines are simulations in terms of Scheme 3 with the parameters listed in Table 2 and $K_{C2} = 1.68$ (——) or $K_{C2} = 0.12$ (– – –).

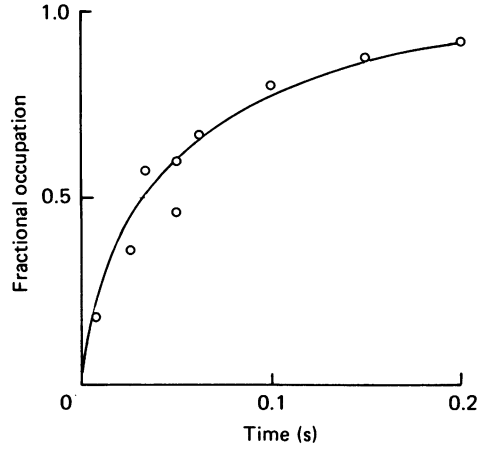


Fig. 8. Ca^{2+} binding to ATPase as a function of time

The experimental data (○) from Dupont (1984) show the fractional occupation of the Ca^{2+}-binding sites as a function of time following the addition of 50 μM-Ca^{2+} to the ATPase at pH 7.2 at 20 °C. The continuous line is a simulation in terms of Scheme 3 with the parameters listed in Table 2.

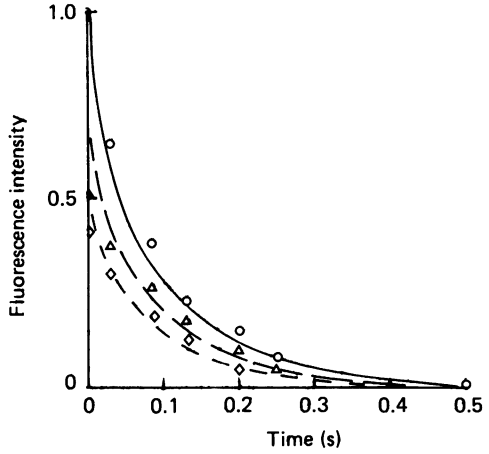


Fig. 9. Rate of Ca^{2+} dissociation from the ATPase

The experimental data from Fernandez-Belda *et al.* (1984) show the fractional changes in fluorescence (F/F_{total}) as a function of time following the addition of EGTA at pH 6.8 to ATPase incubated with Ca^{2+} at 100 μM (○), 1.9 μM (△) and 1.0 μM (◇). The lines are simulations to these three cases (——, 100 μM-Ca^{2+}; — —, 1.9 μM-Ca^{2+}; – – – –, 1.0 μM-Ca^{2+}), with the parameters listed in Table 2.

transition is more pH-dependent than is the back rate; again, this is required to interpret data on the rate of phosphorylation by phosphate (Froud & Lee, 1986) and by ATP (Gould *et al.*, 1986).

Further information about the rates of Ca^{2+} binding has come from studies of the changes in tryptophan fluorescence following Ca^{2+} binding. Fernandez-Belda *et al.* (1984) assumed that the observed changes in fluorescence reflected only transitions between the E1 and E1′ conformations. However, Dupont (1984) has shown that the time course of the fluorescence change on addition of Ca^{2+} exactly matches Ca^{2+} binding. If we assume, therefore, that the fluorescence change exactly follows the occupancy of the Ca^{2+}-binding sites, then we can simulate the change in fluorescence on addition of EGTA to the ATPase in the presence of Ca^{2+} (Fig. 9). The initial fast decrease in fluorescence defines the rate of release of Ca^{2+} from [E1′Ca_2], and the later lower rate of fluorescence decrease defines the rate of the [E1′Ca]–[E1Ca] transition. Assuming that the rate of Ca^{2+} binding is independent of pH, simulations are achieved closely matching the experimental data (Fig. 9). The simulations show that the forward and back rate constants for the [E1Ca]–[E1′Ca] transition must be about equal at neutral pH. The parameters listed in Table 2 also provide good simulations (not shown) for the rate of change of fluorescence on Ca^{2+} binding at pH 6.8 presented by Fernandez-Belda *et al.* (1984).

Guillain *et al.* (1980, 1981) reported simple-exponential time courses for the change in tryptophan fluorescence following addition of Ca^{2+} to the ATPase at pH 6 and pH 7. This is in disagreement with the studies by Fernandez-Belda *et al.* (1984) at pH 6.8 and by Dupont (1984) at pH 7.2, and with the simulations described above. However, simulations of the fluorescence changes following Ca^{2+} addition at pH 6 show a more nearly exponential form with a rate defined by that of the [E2]–[E1] transition because at pH 6 the ATPase is largely in the E2 form. The simulations give a time to half-maximal fluorescence change of 0.22 s, independent of added Ca^{2+} concentration between pCa 5 and pCa 2, corresponding to a rate constant of 3.1 s^{-1}. This can be compared with the values reported by Guillain *et al.* (1980, 1981) at 20 °C, which vary between 1 and 4 s^{-1}.

Finally, although the model for Ca^{2+} binding presented here may seem over-elaborate, it includes only two identical sites each containing two H^+-binding sites and one Ca^{2+}-binding site, and we have been unable to develop any simpler model consistent with the available data. In the following papers we show that an exactly analogous model for the binding of Ca^{2+} to the E2 and E2P states of the ATPase can account for the effect of Ca^{2+} on phosphorylation by phosphate and for the inhibitory effects of high Ca^{2+} concentrations on ATPase activity.

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