Effects of pH on phosphorylation of the Ca^{2+}-ATPase of sarcoplasmic reticulum by inorganic phosphate

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The fluorescence intensity of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin has been shown to decrease on phosphorylation of the ATPase with P_i, this providing a convenient measure of the level of phosphorylation. Comparison of the fluorescence decrease observed with ATP and with high concentrations of P_i fix the value of the equilibrium constant for the phosphorylation reaction $E2PMg \rightleftharpoons E2P_iMg$ at pH 6.0 at about 2. Studies of the pH-dependence of phosphorylation show that $H_2PO_4^-$ and HPO_4^{2-} bind to the ATPase with equal affinity, but that only binding of $H_2PO_4^-$ leads to phosphorylation, described by an equilibrium constant of 2.3. Luminal Ca^{2+} can bind to a pair of sites on the ATPase, with affinities of 1.3×10^3 and 1.7×10^3 M^{-1} for the unphosphorylated and phosphorylated forms of the ATPase respectively, with stronger binding of Ca^{2+} to the phosphorylated form resulting in an increase in the effective equilibrium constant for phosphorylation.

INTRODUCTION

The mechanism of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) is usually described in terms of the E2–E1 model developed from the Post–Elbers scheme for (Na-K)-ATPase (Scheme 1) [1]. The scheme proposes that, in the E1 conformation, the ATPase has two outwardly facing Ca^{2+}-binding sites of high affinity. Following the binding of MgATP, the ATPase is phosphorylated and undergoes a change in conformation to a state in which the two Ca^{2+}-binding sites are of low affinity and inwardly facing ($E2PCa_2$). Following loss of Ca^{2+} to the lumen of the SR, the ATPase can be dephosphorylated and is recycled to E1. Binding of Ca^{2+} to high-affinity exterior-facing sites on the ATPase causes a change in chemical reactivity for the ATPase, from being reactive with P_i and water in the E2 state to being reactive with ATP and ADP in the E1 state.

Incubation of the ATPase with P_i in the absence of Ca^{2+} at acid pH values leads to its phosphorylation on Asp-351 [2–9]. Since acyl phosphates have larger negative free energies of hydrolysis than even ATP [10], spontaneous formation of an acyl phosphate, in the absence of any source of energy, is unusual. It implies stabilization of the acyl phosphate on the ATPase, presumably by interaction with Mg^{2+} (necessary for the reaction) and with (undefined) groups on the ATPase. The level of phosphorylation by P_i is increased in the presence of DMSO [11,12], whereas inhibitors of the ATPase such as thapsigargin and t-butylhydroquinone prevent phosphorylation by P_i [13,14]. Reconstitution of the Ca^{2+}-ATPase into bilayers of a phosphatidylcholine with short (C_{14}) fatty-acyl chains also prevents phosphorylation by P_i, whereas reconstitution into bilayers of a phosphatidylcholine with long (C_{24}) fatty-acyl chains has no effect [15].

The phosphorylation reaction is usually analysed in terms of Scheme 2, with random binding of Mg^{2+} and P_i [7,16]. The reaction scheme leads to the following equation for the equilibrium level of phosphoenzyme:

$$\frac{[MgEP]}{[E_t]} = \frac{[Mg][P_i]K_5}{(K_2K_3)^{-1} + ([Mg]/K_3) + ([P_i]/K_4) + (1+K_5)[Mg][P_i]} \quad (1)$$

where K_1–K_4 are association constants as defined in Scheme 2, K_5 is the equilibrium constant for the formation of MgEP, and $[E_t]$ is the concentration of active ATPase. Martin and Tanford [5] have shown that, at constant Mg^{2+}, the level of phosphoenzyme varies as a function of the concentration of P_i, according to the equation:

$$\frac{[MgEP]}{[E_t]} = \frac{n_{app}K_{app}[P_i]}{1 + K_{app}[P_i]} \quad (2)$$

where:

$$n_{app} = \frac{K_5[Mg]}{K_4^{-1} + (1+K_5)[Mg]} \quad (3)$$

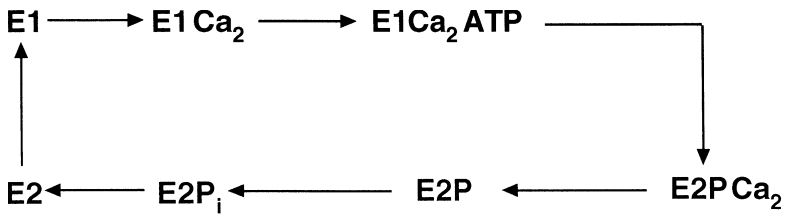
and the effective association constant for P_i is given by:

$$K_{app} = \frac{K_4^{-1} + (1+K_5)[Mg]}{(K_2K_3)^{-1} + K_3^{-1}[Mg]} \quad (4)$$

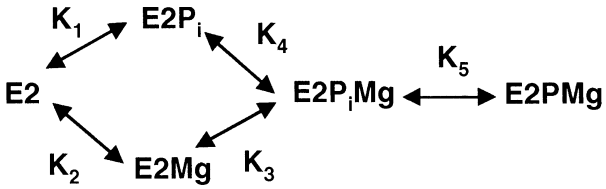
The maximum level of phosphoenzyme formation, observed at infinite concentrations of P_i and Mg^{2+}, is $[E_t]K_5/(1+K_5)$. Unfortunately, $[E_t]$ is generally not known; maximal levels of phosphoenzyme formation on incubation of the ATPase with ATP are typically about half that expected if all the ATPase were active (see [17]). The reason for the lower than expected level of phosphorylation is unclear (see [18,19]). Fitting experimental data on phosphorylation by P_i to the above equations gives different values for K_5 depending on whether or not it is assumed that all of the ATPase can be phosphorylated by P_i. Estimates for the value of K_5 vary from about 1 [5,7,20,21] to 16 or greater [4,9,22]. These different values lead to very different estimates of the free energy change for the phosphorylation reaction.

There is also very considerable experimental uncertainty about the effects of pH on the phosphorylation reaction. Beil et al. [23] have reported that phosphorylation is a function of the concentration of the $H_2PO_4^{2-}$ ion and that levels of phosphorylation at pH 6 and 7 become equal at high concentrations of P_i. Inesi et al. [22] reported a very different pH-dependence, with the level of phosphorylation of the ATPase at 10 mM P_i decreasing by only

Abbreviations used: Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; SR, sarcoplasmic reticulum.



Scheme 1 E1–E2 reaction scheme for the Ca^{2+}-ATPase



Scheme 2 Phosphorylation of the Ca^{2+}-ATPase by P_i

a factor of 3 between pH 6.0 and pH 8.0. The phosphorylation experiments involve incubation of low (μM) concentrations of the ATPase with high (mM) concentrations of $[^{32}P]P_i$, followed by precipitation of the ATPase with trichloroacetic acid, filtration and thorough washing to remove unreacted P_i. The low levels of phosphorylated ATPase that need to be detected against the high background of unreacted P_i limits the accuracy of the determinations and probably accounts for the different published results.

We have shown that the fluorescence intensity of the Ca^{2+}-ATPase labelled at Cys-344 with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) is sensitive to phosphorylation by either ATP or P_i [24]. Measurements of fluorescence changes do not suffer from the problem of non-specific binding experienced with measurements utilizing $[^{32}P]P_i$. Here we relate measurements of fluorescence changes to measurements of phosphorylation using $[^{32}P]P_i$ and derive values for the equilibrium constant for phosphorylation, and examine the effects of pH and luminal Ca^{2+} on this constant.

MATERIALS AND METHODS

SR was purified from skeletal muscle as described by East and Lee [25]. Br-DMC was obtained from Molecular Probes. Ammonium vanadate was dissolved in 100 mM KOH to give a 100 mM stock solution. Thapsivillosin A was purified from roots of *Thapsia vellosa* as described by Wictome et al. [26].

To label the ATPase, SR was suspended to 8 mg of protein/ml in buffer (50 mM Tris, pH 7.0, 200 mM sucrose) at room temperature and incubated with a 10:1 molar ratio of Br-DMC/ATPase in the dark for 1 h; Br-DMC was added from a stock solution (20 mM) in dimethyl formamide. Unbound Br-DMC was separated from the labelled SR by centrifugation in a bench-top centrifuge through two Sephadex G-50 columns pre-equilibrated with the above buffer. Fluorescence spectra were recorded by using an SLM 8000C spectrofluorimeter. DMC fluorescence was excited at 350 nm and observed at 425 nm.

To measure levels of phosphorylation, SR (0.2 mg of protein/ml) was incubated in buffer (150 mM Mes/Tris, pH 6.0) containing 5 mM EGTA, 10 mM Mg^{2+} and the required concentration of $[^{32}P]P_i$ at 25 °C for 15 s, followed by quenching with 5 ml of a mixture of 10% trichloroacetic acid and 200 mM phosphoric acid. The mixture was filtered through a Whatman GF/B glass-fibre filter and washed three times with the trichloroacetic acid/phosphoric acid mixture. Radioactivity on the filters was counted in Optiphase Hisafe 3. Non-specific binding was determined by quenching the ATPase before the addition of $[^{32}P]P_i$.

SR vesicles (10 mg of protein/ml) were passively loaded with Ca^{2+} by incubation in 50 mM Tris/HCl and 200 mM sucrose containing the required concentration of Ca^{2+} at pH 7.0 for 2 h.

Concentrations of free P_i and Mg^{2+} were calculated using association constants of 50 and 5.0×10^6 M^{-1} for the binding of Mg^{2+} and H^+ respectively to HPO_4^{2-} [27].

RESULTS

Addition of Ca^{2+} to DMC-labelled SR had no effect on the intensity of DMC fluorescence, but the subsequent addition of 20 mM Mg^{2+} resulted in a small (3%) decrease in fluorescence intensity at pH 6.0, after correction for dilution (Figure 1). Addition of 20 μM ATP then resulted in a further decrease in intensity of approx. 12%; addition of further ATP resulted in no additional change in fluorescence intensity. Addition of Mg^{2+} in

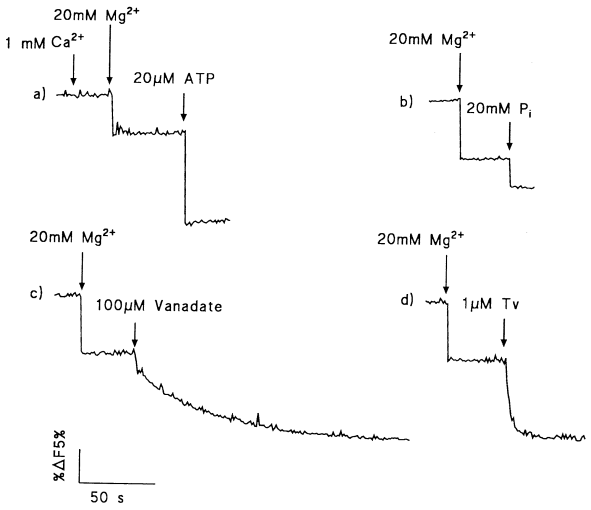


Figure 1 Ligand effects on the fluorescence intensity of DMC-labelled SR

DMC-labelled SR was incubated in buffer (40 mM Tris, 1 mM EGTA) at pH 6.0. (**a**), and the effects of the addition of 1.1 mM Ca^{2+}, 20 mM Mg^{2+} and 20 μM ATP on the fluorescence intensity were monitored. (**b**)–(**d**) Effects of 20 mM Mg^{2+} in the absence of Ca^{2+}, followed by the addition of 20 mM P_i (**b**), 100 μM vanadate (**c**) or 1 μM thapsivillosin A (Tv) (**d**). The traces shown are not corrected for dilution. '%ΔF5%' denotes a change in fluorescence intensity of 5%.

Table 1 Phosphorylation of the Ca^{2+}-ATPase by ATP

SR (0.1 mg of protein/ml) was incubated with the given concentrations of [γ-^{32}P]ATP and Ca^{2+} for 15 s in 40 mM Tris, pH 6.0, containing 100 mM KCl and 5 mM $MgSO_4$ at 25 °C, before quenching the reaction.

		Level of phosphorylation (nmol of EP/mg of protein)	
[ATP] (μM)	[Ca^{2+}] (mM)	SR	DMC-labelled SR
20	0.1	1.2	–
100	0.1	3.3	3.2
200	0.1	3.3	3.3
20	1.0	3.5	–
100	1.0	3.6	3.5
200	1.0	3.6	3.5

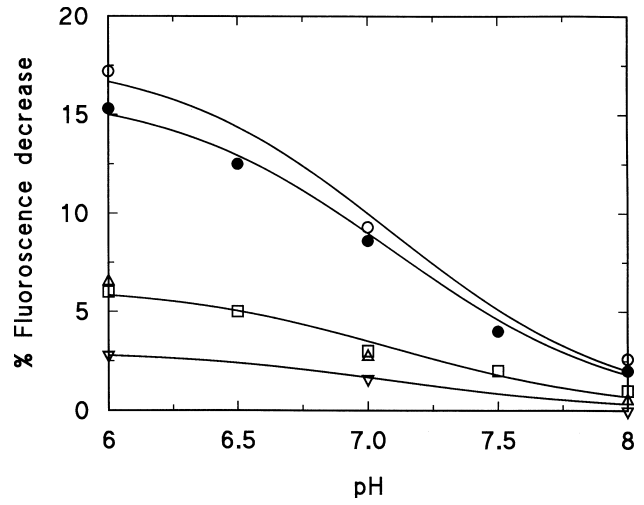


Figure 2 Effect of pH on the changes in fluorescence intensity observed on addition of ligands to DMC-labelled SR

DMC-labelled SR was incubated in buffer (40 mM Tris) at the appropriate pH in the presence of 1.0 mM EGTA, and the magnitude of the fluorescence responses to the addition of 20 mM Mg^{2+} (□), 100 μM ATP (△), 100 μM adenosine 5'-[β,γ-imido]triphosphate (▽) and 1 μM Tv (○) was monitored. Similarly, the total fluorescence response on addition of 20 mM Mg^{2+} and 100 μM ATP in the presence of 0.1 mM free Ca^{2+} was monitored (●). The fluorescence responses have been corrected for dilution. The solid lines show simulations to protonation of a single site with a binding constant for H^+ of 1.25×10^7, with the protonated form showing a fluorescence response on addition of ligand and the non-protonated form showing no response, as described in the text.

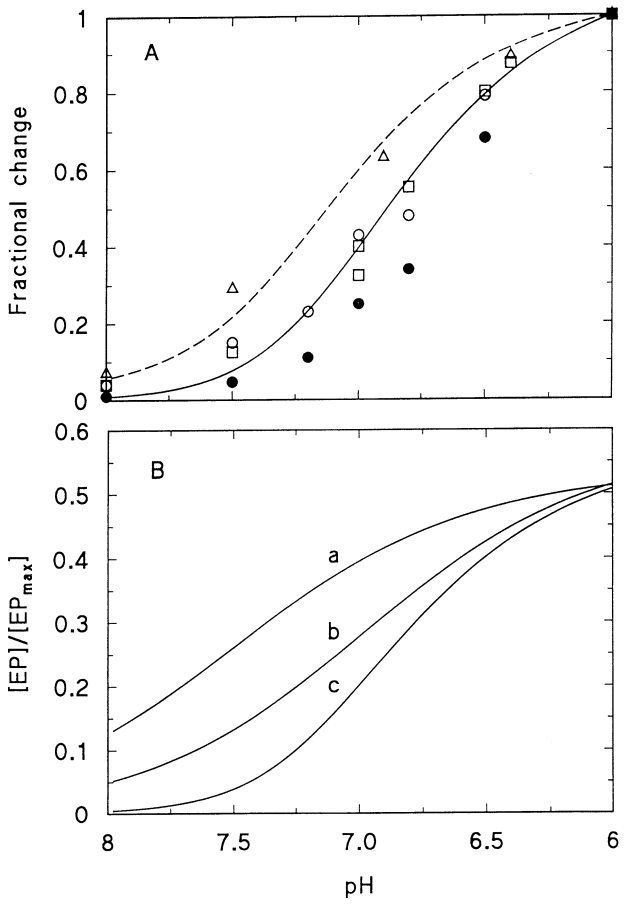


Figure 3 Effect of pH on the phosphorylation of the Ca^{2+}-ATPase by P_i

(**A**) The SR was incubated in 40 mM Tris at the appropriate pH, containing 5 mM EGTA, 10 mM Mg^{2+} and 10 mM [^{32}P]P_i, and the level of phosphoenzyme formation was determined (□); this is expressed as a fraction of the level determined at pH 6.0. The total fluorescence response of DMC-labelled SR to the addition of 10 mM Mg^{2+} and 10 mM P_i was also determined (●), and again expressed as a fraction of the response obtained at pH 6.0. ○ shows the fluorescence responses corrected for the intrinsic pH-dependence of fluorescence, as described in the text. Corrected fluorescence responses are also shown for vesicles loaded with 20 mM Ca^{2+} (△). The levels of phosphoenzyme formation as a function of pH, calculated as described in the text using the parameters in Table 2, are shown for unloaded (solid line) and Ca^{2+}-loaded (broken line) vesicles. (**B**) Simulations of the level of phosphorylation of the ATPase at total concentrations of 10 mM Mg^{2+} and 10 mM [^{32}P]P_i, as a function of pH. Curve a shows the effect of pH on phosphorylation if only $H_2PO_4^-$ can bind to the ATPase. Curve b shows the expected levels of phosphorylation if both $H_2PO_4^-$ and HPO_4^{2-} can bind to the ATPase, but only $H_2PO_4^-$ can phosphorylate it. Curve c also includes the effects of pH and Mg^{2+} on the E1–E2 equilibrium for the ATPase, calculated using the parameters given in Lee et al. [31]. Binding constants K_1–K_4 are given in Table 2. For curve a, $K_5 = 1.9$. For curves b and c, the values of K_{5a} and K_{5b} (see Scheme 3) are 2.3 and 0 respectively.

the absence of Ca^{2+} resulted in a fluorescence drop of 5%. Subsequent addition of either 100 μM vanadate or 1 μM thapsivillosin A resulted in a further decrease in fluorescence intensity, so that the total change in intensity was equal to that observed on addition of Mg^{2+} and ATP in the presence of Ca^{2+} (Figure 1). Addition of 20 mM P_i in the presence of 20 mM Mg^{2+} resulted in a decrease in fluorescence intensity smaller than that seen on addition of vanadate or thapsivillosin A (Figure 1).

Vanadate binds to the ATPase as an analogue of phosphate, with micromolar affinity [28], so that under the conditions used in Figure 1, the ATPase will be fully vanadate-bound. Thapsivillosin A also binds to the ATPase with high affinity, to give a modified E2 state of the ATPase [26]. In the presence of Ca^{2+} and ATP at pH 6.0, the ATPase was maximally phosphorylated (Table 1; [29]); labelling the ATPase with DMC had no effect on the level of phosphoenzyme formed with [γ-^{32}P]ATP (Table 1) or [^{32}P]P_i (results not shown). It has been shown elsewhere that the effects of Mg^{2+} and ATP on fluorescence intensity are additive, so that the magnitude of the total decrease in fluorescence observed on sequential addition of Mg^{2+} and ATP is constant, and independent of the concentration of Mg^{2+} [24]. We therefore conclude that phosphorylation of all of the active ATPase in the preparation, or binding of ligands that shift the E1–E2 equilibrium totally towards E2, results in a 15% decrease in fluorescence intensity. The 9% decrease in intensity observed with 20 mM Mg^{2+} and 20 mM P_i would then correspond to 60% phosphorylation of the active ATPase. As shown below (Figure

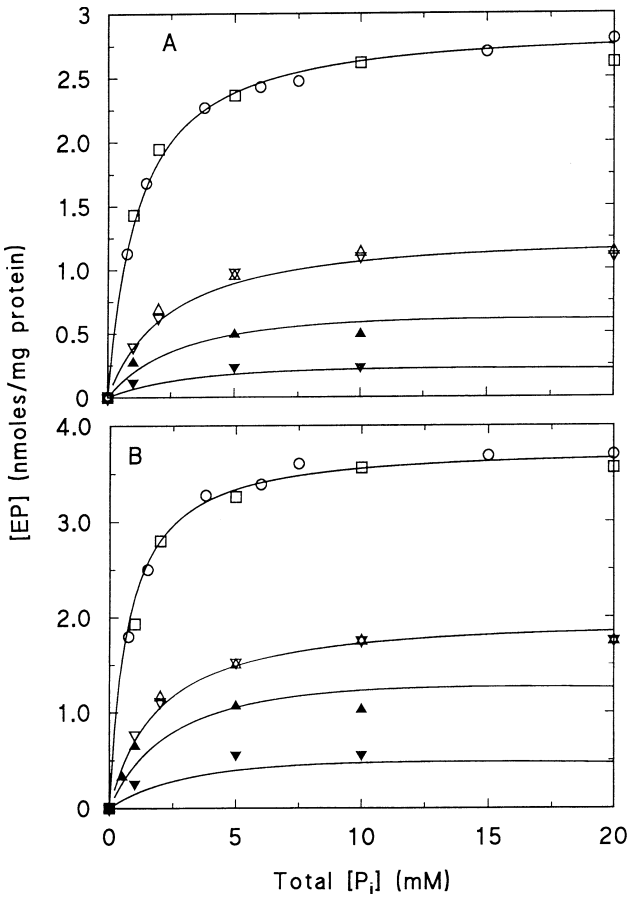


Figure 4 Phosphorylation of the Ca^{2+}-ATPase as a function of P_i concentration

SR was phosphorylated with [^{32}P]P_i in 150 mM Mes/Tris containing 10 mM Mg^{2+} and either 20 mM EGTA at pH 6.0 (○) or 5 mM EGTA at pH 7.0 (△), and the level of phosphoenzyme formation was determined. The total fluorescence response to the addition of 10 mM Mg^{2+} and the given concentration of P_i was also determined under the same conditions for DMC-labelled SR at pH 6.0 (□) and pH 7.0 (▽), and with 2 mM (▲) or 0.5 mM (▼) Mg^{2+} at pH 7.0. (**A**) Unloaded vesicles; (**B**) vesicles loaded with 20 mM Ca^{2+}. The fluorescence data at pH 7.0 were scaled to those at pH 6.0 to account for the intrinsic pH-dependence of fluorescence, as described in the text. All the fluorescence data were then scaled such that a 3.7% change in fluorescence intensity was equivalent to the formation of 1 nmol of EP/mg of protein, to allow for easy comparison between the fluorescence and ^{32}P data. The solid lines show simulations using the parameters in Table 2 and a value for [E_t] of 4.7 nmol/mg of protein.

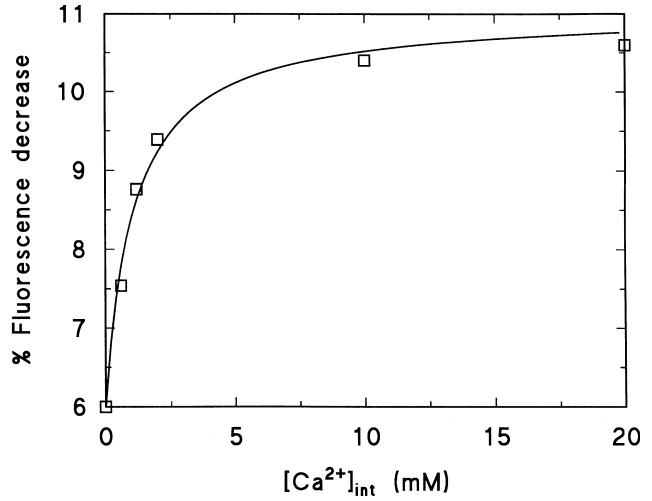


Figure 5 Effect of Ca^{2+} loading on phosphorylation of the Ca^{2+}-ATPase at pH 7.0

DMC-labelled SR (5–10 of mg protein/ml) was loaded with Ca^{2+} by incubation with the given concentrations of Ca^{2+} ([Ca^{2+}]$_{int}$). Ca^{2+}-loaded SR was then diluted 100-fold into 40 mM Tris, pH 7.0, containing 6.0 mM EGTA, and the total fluorescence response to the addition of 20 mM Mg^{2+} and 10 mM P_i was recorded within 30 s. The solid line shows a simulation assuming two identical luminal binding sites for Ca^{2+} on the ATPase, with association constants of 1.3×10^3 M^{-1} and 1.7×10^3 M^{-1} for the non-phosphorylated and phosphorylated forms of the ATPase respectively.

4), phosphoenzyme formation from P_i is almost maximal at 20 mM P_i in the presence of high Mg^{2+} concentrations, so that a maximum level of 60% phosphorylation of the active ATPase defines K_5 as being about 2 at pH 6.0.

The effects of ligand binding and of phosphorylation on the fluorescence intensity of DMC-labelled ATPase were pH-sensitive: changes decreased in magnitude with increasing pH (Figure 2). The pH-dependence of the decrease in fluorescence intensity observed on addition of Mg^{2+} to DMC-labelled ATPase in the absence of Ca^{2+} is consistent with protonation of a single site with a binding constant for H^+ of 1.25×10^7, with the fluorescence of the protonated form being sensitive to the binding of Mg^{2+} and that of the non-protonated form showing no effect [30]. All of the fluorescence responses studied here show this same pH-dependence (Figure 2). Therefore, in studies of pH-dependence in which fluorescence changes were compared with levels of formation of [^{32}P]phosphoenzyme, the fluorescence changes were corrected for this intrinsic pH-dependence by multiplying by a factor of $(1.0+[H^+] \times 1.25 \times 10^7)/([H^+] \times 1.25 \times 10^7)$.

The level of phosphorylation of the ATPase by 10 mM [^{32}P]P_i in the presence of 10 mM Mg^{2+} decreased with increasing pH; for ease of comparison with the fluorescence data, the phosphorylation data are expressed as a fraction of the phosphorylation level observed at pH 6.0 (Figure 3A). The magnitude of the total fluorescence decrease seen on addition of 10 mM Mg^{2+} and 10 mM P_i decreased more steeply with increasing pH than was observed for the level of phosphorylation (Figure 3A). However, if the fluorescence decrease was corrected for the intrinsic pH-dependence of the fluorescence response as described above, then the pH-dependencies of phosphorylation and of the fluorescence response matched very closely (Figure 3A); this gives extra confidence in the data obtained using [^{32}P]P_i, despite the low levels of phosphoenzyme detected.

Figure 4(A) compares the magnitude of the total fluorescence response observed on addition of Mg^{2+} and P_i to DMC-labelled SR at pH 6.0 with the level of phosphorylation determined using [^{32}P]P_i; the fluorescence changes showed the same dependence on P_i concentration as the level of phosphoenzyme formation. Similarly, at pH 7.0 fluorescence changes for DMC-labelled SR showed the same dependence on P_i concentration as levels of phosphoenzyme formation when the fluorescence changes were corrected for the intrinsic pH-dependence, as described above (Figure 4A).

Higher levels of phosphorylation were observed for Ca^{2+}-loaded SR vesicles than for unloaded vesicles. Vesicles (10 mg of protein/ml) were passively loaded with Ca^{2+} by incubation with the required concentration of Ca^{2+} for 2 h. They were then diluted 50–100-fold into buffer containing 6 mM EGTA, giving, at the highest loading concentrations, an extravesicular Ca^{2+} concentration of 0.1 μM. Measurements of phosphorylation by

[^{32}P]P_i or fluorescence responses of DMC-labelled SR were made within 30 s of dilution, to minimize any effects of leakage of Ca^{2+} from the vesicles; measurements taken 2 min after dilution gave identical results, suggesting that any leakage of Ca^{2+} was negligible under these conditions. As shown in Figure 4(B), identical results for the effects of vesicular Ca^{2+} were obtained using [^{32}P]P_i and from fluorescence responses of DMC-labelled SR. Fluorescence changes as a function of Ca^{2+} loading at pH 7.0 are shown in Figure 5.

DISCUSSION

The level of phosphorylation of the Ca^{2+}-ATPase by P_i is known to be markedly dependent on pH, but very different pH-dependencies have been published in the literature [22,23]. Experimental problems in measuring low levels of ^{32}P-labelled protein against a much greater background of [^{32}P]P_i mean that alternative techniques for validating the measurements are required. Here we show that changes in the fluorescence of DMC-labelled ATPase on phosphorylation can be used in this way.

It has been suggested that pH affects phosphorylation of the ATPase because only $H_2PO_4^-$ can bind to the enzyme [23]. Figure 3(B) (curve a) shows the effect of pH that would be expected if this were the case, for phosphorylation of the ATPase with free concentrations of P_i and Mg^{2+} of 10 mM, calculated using the values for the constants of Scheme 2 given in Table 2. Comparison with the experimental pH profile shows that the effects of pH are much more marked than those calculated according to this model. Alternatively, if both $H_2PO_4^-$ and HPO_4^{2-}can bind to the ATPase with equal affinity, but only $H_2PO_4^-$ can phosphorylate it (Scheme 3), then a larger effect of pH would be seen, because of the competition between $H_2PO_4^-$ and HPO_4^{2-} for binding (Figure 3B, curve b). The influence of pH will become even greater if the effects of pH and Mg^{2+} concentration on the E1–E2 equilibrium of the ATPase are included, since the E1–E2 equilibrium is shifted towards E1 with increasing pH, and only E2 is phosphorylated by P_i (Scheme 1). The effects of pH and Mg^{2+} on the E1–E2 equilibrium constant can be described using the constants given in Lee et al. [31]. Assuming that binding of P_i and Mg^{2+} to the phosphorylation site (binding constants K_1–K_4) is identical for E1 and E2, then curve c of Figure 3(B) is obtained. The effect of pH on phosphorylation calculated in this way matches the experimental data (Figure 3A).

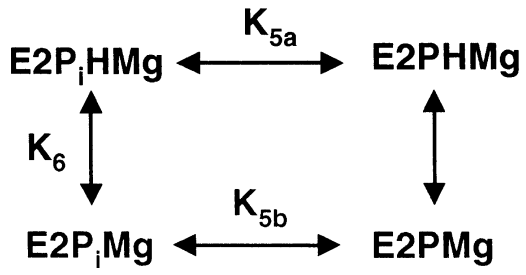
A further difference between the two possible explanations for the effects of pH is observed in an analysis of the level of phosphorylation of the ATPase as a function of P_i concentration at different pH values (Figure 6). If only $H_2PO_4^-$ can bind to the ATPase, then the maximal levels of phosphorylation observed at high P_i concentrations should be independent of pH, the effective K_d value for P_i increasing with increasing pH (Figure 6A).

Table 2 Binding and equilibrium constants describing the phosphorylation of the Ca^{2+}-ATPase by P_i

Step	Symbol	Value
$E2 + P_i \rightarrow E2P_i$	K_1*	160 M^{-1}
$E2 + Mg^{2+} \rightarrow E2Mg$	K_2	64 M^{-1}
$E2Mg + P_i \rightarrow E2P_iMg$	K_3*	755 M^{-1}
$E2P_i + Mg^{2+} \rightarrow E2P_iMg$	K_4	306 M^{-1}
$E2P_iHMg \rightarrow E2PHMg$	K_{5a}†	2.3
$E2P_iMg \rightarrow E2PMg$	K_{5b}†	0

* Same value for $H_2PO_4^-$ and HPO_4^{2-}.

† In the presence of saturating concentrations of luminal Ca^{2+}, the value of the equilibrium constant is increased by a factor of 2.



Scheme 3 Effect of pH on phosphorylation of the Ca^{2+}-ATPase

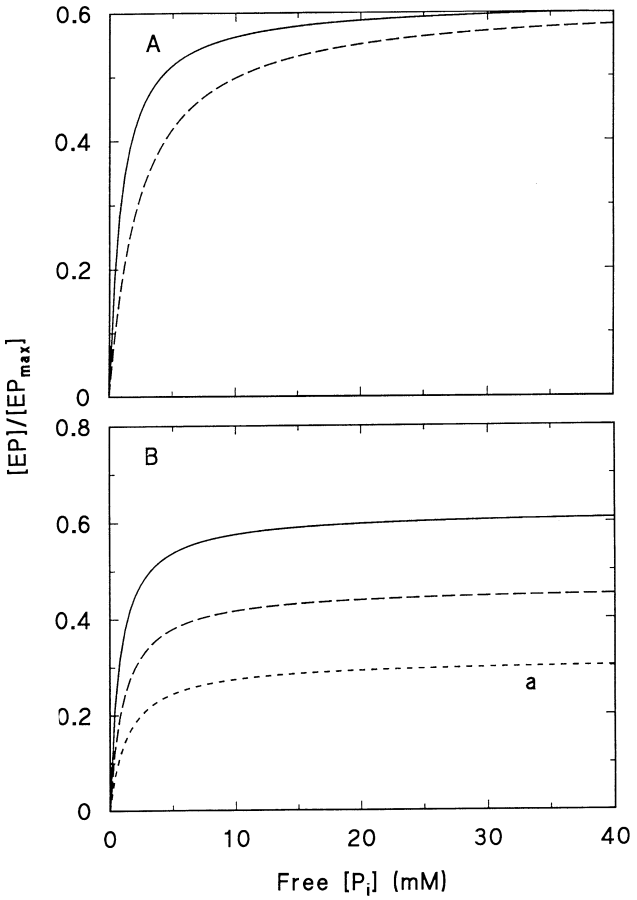


Figure 6 Calculated P_i-dependencies of the levels of phosphoenzyme formation

Levels of phosphorylation were calculated for the Ca^{2+}-ATPase in 20 mM free Mg^{2+} at pH 6.0 (solid line) or 7.0 (broken line) as a function of the concentration of free P_i: (**A**) assuming that only $H_2PO_4^-$ can bind to the ATPase, with $K_5 = 1.9$; and (**B**) according to Scheme 3, with values of K_{5a} and K_{5b} of 2.3 and 0 respectively. In (B), the dotted line (curve a) for pH 7.0 was calculated according to Scheme 3, but also taking into account the effect of pH and Mg^{2+} on the E1—E2 equilibrium. (At pH 6.0, inclusion of the E1—E2 equilibrium had no significant effect on the simulation, with the calculated phosphorylation curve being identical to the solid line shown.)

However, if the effect of pH follows Scheme 3, then maximal levels of phosphorylation will decrease with increasing pH, due to a decrease in the effective value of the equilibrium constant K_5 (Figure 6B). The effective K_d value for P_i will also increase with increasing pH since, as shown by eqn. (4), the effective association constant depends on the value of K_5. Maximal levels of phosphorylation observed at high concentrations of P_i are clearly lower at pH 7.0 than at 6.0 (Figure 4), consistent with Scheme 3 but not with a scheme in which only $H_2PO_4^-$ can bind to the ATPase.

Very different values of K_5 of between about 1 and 16 have been reported in the literature for unloaded SR vesicles at pH 6.0 [4,5,7,9,20–22]. As described above, comparing the total fluorescence decrease seen on phosphorylation of the DMC-labelled SR by ATP in the presence of Ca^{2+} with that seen on phosphorylation with 20 mM Mg^{2+} and 20 mM P_i at pH 6.0 fixes K_5 as about 2.0. The observed pH-dependence of phosphorylation (Figure 3) can be fitted to Scheme 3, with a value of K_6 for the protonation of HPO_4^{2-} of 5.0×10^6 [27], and values of 2.3 and 0 for K_{5a} and K_{5b} respectively (Table 2); these constants result in effective values for K_5 of 1.9 at pH 6.0 and 0.8 at pH 7.0.

Analysis of the level of phosphorylation as a function of the concentrations of P_i and Mg^{2+} allows estimation of the constants K_1–K_4. These are given in Table 2, and simulations calculated using these parameters are shown in Figures 3 and 4. The values are comparable with those reported by Punzengruber et al. [7] and Martin and Tanford [5], who reported values of K_1 and K_2 between 70 and 140 M^{-1}, and values of K_3 and K_4 between 400 and 800 M^{-1}. Noticeable is the synergism between binding of Mg^{2+} and P_i, with the binding of one ion increasing the affinity of the other by approx. 5-fold (Table 2).

Binding of Ca^{2+} to luminal sites on the ATPase increases the level of phosphorylation of the ATPase by P_i. Since at high concentrations of P_i and Mg^{2+} not all of the ATPase is phosphorylated, luminal binding sites for Ca^{2+} must exist on both the unphosphorylated and the phosphorylated forms of the ATPase, with stronger binding to the phosphorylated form resulting in an effective increase in the equilibrium constant K_5 for phosphorylation [3,8,9]. As shown in Figures 3 and 4(B), data in the presence of 20 mM luminal Ca^{2+} can be simulated by assuming a doubling of the value of K_5. Figure 5 shows a plot of the fluorescence change observed for DMC-labelled SR as a function of luminal Ca^{2+} concentration at pH 7.0. The data fit well to a single luminal binding site for Ca^{2+} with a K_d value of 1.0 ± 0.1 mM. However, it is known that two Ca^{2+} ions bind to the ATPase and are transported [10], and thus the phosphorylated ATPase must possess two luminal Ca^{2+}-binding sites. The good fit of the data to a single-site-binding equation shows that there is no co-operativity in the binding of Ca^{2+} to the luminal sites on the ATPase, as reported previously by Jencks et al. [8]. The effective doubling of K_5 in the presence of saturating concentrations of luminal Ca^{2+} can be simulated by assuming two luminal Ca^{2+}-binding sites of identical affinities, with association constants of 1.3×10^3 M^{-1} and 1.7×10^3 M^{-1} for the non-phosphorylated and phosphorylated forms of the ATPase respectively (Figure 5).

Ca^{2+}-ATPase, in unsealed preparations, has been shown to be inhibited by Ca^{2+} concentrations in the millimolar range; this has been attributed both to binding of Ca^{2+} to ATP, with CaATP being a poor substrate for the ATPase, and to Ca^{2+} binding to the luminal sites on the phosphorylated ATPase, decreasing its rate of dephosphorylation [32–34]. The concentration of Ca^{2+} resulting in 50 % inhibition of ATPase activity has been found to be dependent on the concentration of ATP, changing from a pCa value of 3.57 for 50 % inhibition at 2 mM ATP to one of 3.33 at 2 μM ATP, at pH 7.2 [33]. The Ca^{2+} affinity of 1.7×10^3 M^{-1} for the phosphorylated ATPase at pH 7.0, estimated above, would correspond to 50 % occupation of the luminal sites at a pCa value of 3.23, in good agreement with the inhibition data.

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