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# A model for the phosphorylation of the $Ca^{2+}+Mg^{2+}$-activated ATPase by phosphate

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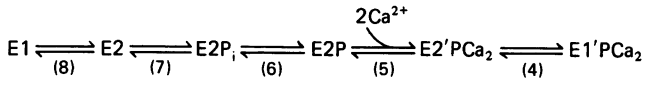
We have shown that changes in fluorescence intensity for the $Ca^{2+}+Mg^{2+}$-activated ATPase of sarcoplasmic reticulum labelled with fluorescein isothiocyanate following the addition of $Ca^{2+}$ can give the ratio of the two conformations (E1 and E2) of the ATPase. We show that the fluorescence response to $Ca^{2+}$ is unaffected by $Mg^{2+}$, phosphate or $K^+$, implying that these ions bind equally well to the E1 and E2 conformations. A model is presented for phosphorylation of the ATPase by phosphate as a function of pH, $Mg^{2+}$, $K^+$ and $Ca^{2+}$.

## INTRODUCTION

A key step in the mechanism of the $Ca^{2+}+Mg^{2+}$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) is hydrolytic cleavage of a phosphorylated intermediate. Much useful information about this step has been obtained by studying its reverse, the phosphorylation of the ATPase by phosphate (de Meis, 1981). For the ATPase in sealed vesicles of SR in the absence of external $Ca^{2+}$, the reaction is represented according to the reaction scheme proposed by de Meis & Vianna (1979), as shown in Scheme 1. It is clear that phosphorylation of the ATPase by phosphate will depend on the E1–E2 transition (step 8) and, in the presence of $Ca^{2+}$ within the SR vesicle, on the transition $E2'PCa_2$–$E1'PCa_2$ (step 4).




**Scheme 1. Phosphorylation of ATPase by phosphate, according to de Meis & Vianna (1979)**

Most analyses of phosphorylation by phosphate have ignored these steps (Punzengruber *et al.*, 1978; Martin & Tanford, 1981; Suko *et al.*, 1981; de Meis *et al.*, 1982; Inesi *et al.*, 1984; Guillain *et al.*, 1984; Champeil *et al.*, 1985). If the E1–E2 equilibrium were to favour overwhelmingly the E2 conformation (i.e. if the equilibrium constant E1/E2 were less than approx. 0.01) at all pH values, then such analyses would be valid. However, we have shown in the preceding paper (Froud & Lee, 1986) that the ratio E1/E2 is not small and varies from 0.14 to 1.9 with changing pH from 6 to 8. Further, the extent of phosphorylation of ATPase by phosphate is known to depend, not only on the concentration of phosphate, but also on the concentrations of $Mg^{2+}$, $K^+$ and $H^+$ (de Meis, 1981), and it is therefore important to study the effect of these ions on the E1–E2 equilibrium. In the preceding paper (Froud & Lee, 1986) we showed how the E1–E2 equilibrium could be studied by observation of the fluorescence of the ATPase covalently modified with FITC. The same approach is used here to study the effects of $Mg^{2+}$, $K^+$ and phosphate on the E1–E2 equilibrium. A model is then presented to describe the steps of phosphorylation.

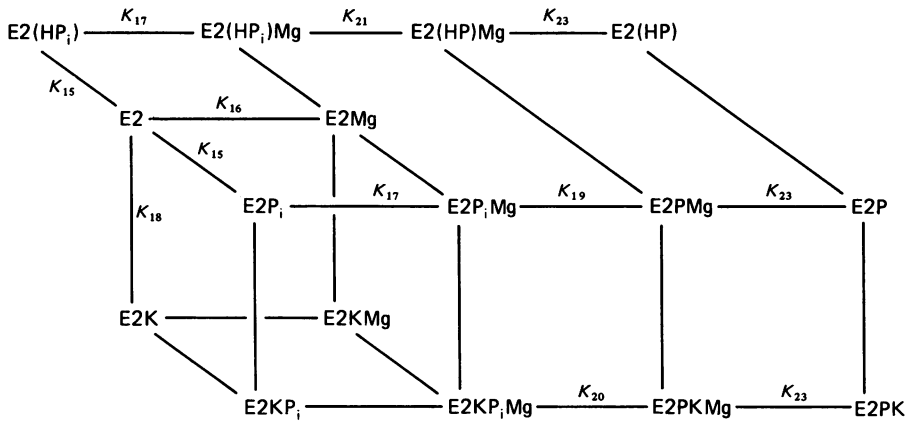
**Table 1. Responses of FITC-labelled ATPase to $Ca^{2+}$ and vanadate**

Changes in fluorescence intensity for FITC-labelled ATPase were recorded on addition of $Ca^{2+}$ and vanadate to labelled ATPase in 100 μM-EGTA/50 mM-Tris/50 mM-maleate buffer, at pH 6, 7 or 8, containing the additions listed.

| | pH 6 | | pH 7 | | pH 8 | |
|---|---|---|---|---|---|---|
| | Fluorescence response (%) | | Fluorescence response (%) | | Fluorescence response (%) | |
| Additions to buffer | To $Ca^{2+}$ | To vanadate | To $Ca^{2+}$ | To vanadate | To $Ca^{2+}$ | To vanadate |
| 100 mM-KCl | — | — | −5.1 | * | −3.1 | * |
| 1 mM-$Mg^{2+}$ | — | — | −5.1 | 15.4 | — | — |
| 100 mM-KCl, 5 mM-$Mg^{2+}$ | −8.1 | 5.4 | −4.7 | 13.2 | −2.4 | 14.3 |
| 100 mM-KCl, 20 mM-$Mg^{2+}$ | −8.2 | 8.2 | −5.2 | 10.3 | −3.2 | 8.8 |
| 100 mM-KCl, 20 mM-$Mg^{2+}$, 20 mM-phosphate | — | — | −6.1 | 10.3 | — | — |

* No response to vanadate in the absence of $Mg^{2+}$.

Abbreviations used: ATPase, $Ca^{2+}+Mg^{2+}$-activated ATPase; FITC, fluorescein 5'-isothiocyanate; SR, sarcoplasmic reticulum.




**Scheme 2. Phosphorylation of the E2 conformation of ATPase by phosphate**

$P_i$ represents $HPO_4^{2-}$ and ($HP_i$) $H_2PO_4^-$. $K_{22}$ is the equilibrium constant for $P_i + H^+ \rightleftharpoons HP_i$.

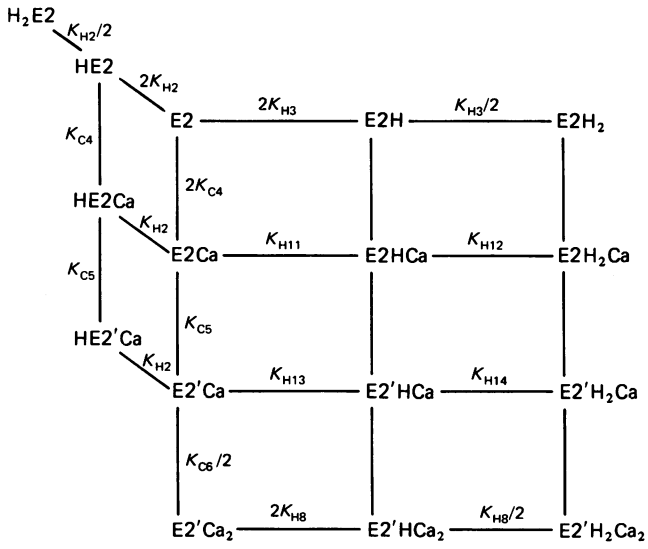
## MATERIALS AND METHODS

The SR and purified ATPase were prepared from female rabbit (New Zealand White) hindleg muscle as described in the preceding paper. The ATPase was labelled with FITC at a label/protein molar ratio of 0.4:1, incubated for 30 min at 30 °C in 100 $\mu$M-EGTA/50 mM-Tris/50 mM-maleate buffer at the given pH and then cooled at 20 °C for 5 min, and fluorescence intensities were measured, as described in the preceding paper (Froud & Lee, 1986).

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

## RESULTS

In the preceding paper (Froud & Lee, 1986) it was shown that addition of $Ca^{2+}$ to FITC-labelled ATPase or SR leads to a decrease in fluorescence intensity and addition of vanadate to an increase, as shown previously by Pick & Karlish (1982). We also showed that it was possible to estimate the ratio of the concentrations of the E1 and E2 conformations of the ATPase from the response to $Ca^{2+}$. If $Mg^{2+}$, $K^+$ or phosphate were to bind differently to the E1 and E2 conformations, then they would change the position of the E1/E2 equilibrium and hence the response to $Ca^{2+}$. The data in Table 1 show that in fact none of these ions has any significant effect on the fractional response to $Ca^{2+}$ and thus that the E1/E2 equilibrium is unaffected.

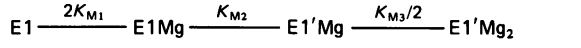



**Scheme 3. Binding of $Ca^{2+}$ to the E2 conformation of ATPase**

Binding of $Ca^{2+}$ is competitive with protonation of residue 1, with proton association constant $K_{H2}$. Binding of $Ca^{2+}$ is non-competitive with protonation of residue 2. For clarity, binding of protons to residue 1 is only indicated for the unprotonated state of residue 2.

## DISCUSSION

Any complete scheme for phosphorylation of the ATPase by phosphate must include the E2–E1 equilibrium. It is also known that pH, $Mg^{2+}$ and $K^+$ all affect phosphorylation and so must be included. Our scheme for the phosphorylation of the E2 form of the ATPase is shown in Scheme 2. We assume that the binding of phosphate, $Mg^{2+}$ and $K^+$ to E1 and E2 are identical, to account for the lack of effect of these ions on the E1–E2 equilibrium (Table 1). Binding of phosphate to E1 was previously suggested by Tanford & Martin (1982). However, whereas the binding of $Mg^{2+}$ and phosphate to E1 to give $E1P_iMg$ leads no further, the formation of $E2P_iMg$ leads to phosphorylation of the ATPase. We assume that binding of $K^+$ to E2 is unaffected by binding of phosphate and $Mg^{2+}$, although the binding constant for $K^+$ to the phosphorylated form of E2 is different from that to the unphosphorylated form, so that binding of $K^+$ affects the equilibrium level of phosphorylation. As in previous schemes, we suggest that the substrates are free phosphate and $Mg^{2+}$ and not an $Mg \cdot P_i$ complex (Punzengruber *et al.*, 1978; Martin & Tanford, 1981; de Meis *et al.*, 1982). Champeil *et al.* (1985) have suggested that the true substrate could be the $Mg \cdot P_i$ complex. However, since the binding constant of $Mg^{2+}$ to $HPO_4^{2-}$ is considerably greater than that to $H_2PO_4^-$, the




**Scheme 4. Binding of $Mg^{2+}$ to the $Ca^{2+}$-binding sites of the E1 conformation of ATPase**

concentration of the $Mg \cdot P_i$ complex would increase rapidly with increasing pH and thus the level of phosphorylation would be expected to increase with increasing pH: the opposite is observed.

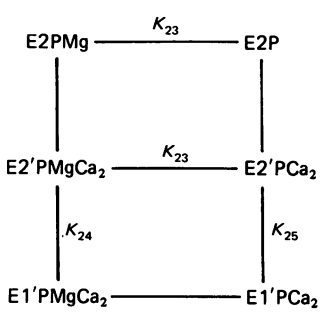
In developing a kinetic model for the ATPase we have tried to keep to a minimum the number of postulated interactions in the model, and, in particular, have tried to keep effects at the $Ca^{2+}$-binding sites as distinct as possible from effects at the phosphorylation site. We therefore assume that binding of $H^+$ or $Ca^{2+}$ at the $Ca^{2+}$-binding sites has no effect on the binding of phosphate, $K^+$ or $Mg^{2+}$ to the ATPase. The assumption is reasonable, since these ions bind equally well to the two conformations E1 and E2, which have very different $Ca^{2+}$-binding properties. The assumption could be relaxed but would result in considerable further complexity, not justified from the results described below. We further assume that, although the binding of $Ca^{2+}$ to $E2P_iMg$ and E2PMg affects the rates of phosphorylation and dephosphorylation, it has no effect on the equilibrium constant for the phosphorylation step; this assumption is justified below. This then means that $Ca^{2+}$ binds equally well to E2, to E2PMg and to all intermediate forms, again resulting in a considerable simplification of the resulting equations.

We describe binding of $Ca^{2+}$ to the E2 form of the ATPase in terms of a scheme (Scheme 3) exactly analogous to that used to describe the binding of $Ca^{2+}$ to E1 (Froud & Lee, 1986). We assume that the two $Ca^{2+}$-binding sites are identical and independent, so that the binding constants $K_{H11}$–$K_{H14}$ can be expressed in a form exactly analogous to that used to describe binding to the E1 form (Froud & Lee, 1986):

$$K_{H11} = K_{H3} + K_{H15}$$
$$K_{H12} = K_{H3}K_{H15}/(K_{H3} + K_{H15})$$
$$K_{H13} = K_{H8} + K_{H16}$$
$$K_{H14} = K_{H8}K_{H16}/(K_{H8} + K_{H16})$$

where $K_{H15}$ and $K_{H16}$ are binding constants for the protonation of residue 2 at the site occupied by $Ca^{2+}$ in E2Ca and at the site unoccupied by $Ca^{2+}$ in E2'Ca respectively (see Froud & Lee, 1986). Results obtained at very high concentrations of $Mg^{2+}$ have been interpreted in terms of binding of $Mg^{2+}$ to the $Ca^{2+}$-binding sites in the E1 conformation (Loomis *et al.*, 1982). Assuming that binding of $Mg^{2+}$ at these sites is independent of pH (see below), it can be described as in Scheme 4.

Finally, it is necessary to consider the $E2'PCa_2$–$E1'PCa_2$ equilibrium (Scheme 5). In the $Ca^{2+}$-bound form, $E2'PCa_2$ is in equilibrium with $E1'PCa_2$ and, if the




**Scheme 5. $E2'PCa_2$–$E1'PCa_2$ equilibrium of ATPase**

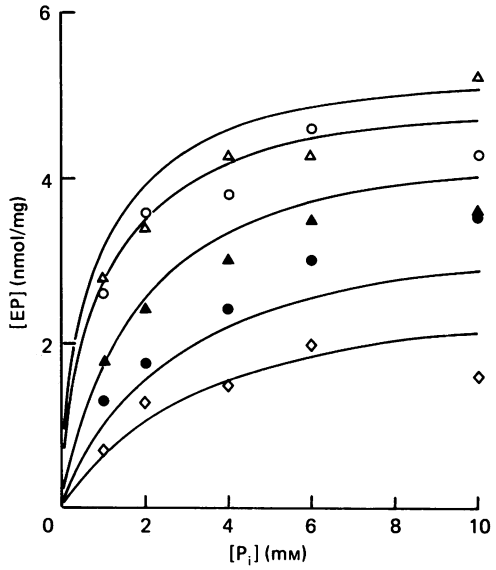



**Fig. 1 Effect of pH on phosphorylation of ATPase**

The experimental data from Inesi *et al.* (1984) show the equilibrium level of phosphoenzyme (nmol/mg of protein) at a free $Mg^{2+}$ concentration of 30 mM, as a function of free phosphate concentration at pH 6.0 (○), pH 6.5 (△), pH 7.0 (▲), pH 7.5 (●) and pH 8.0 (◇). The curves are simulations with the parameters in Table 1 and $[EP]_{max.}$ = 6.0 nmol/mg of protein.

**Table 2. Kinetic parameters for phosphorylation of ATPase at 25 °C**

Parameters were obtained by simulation as described in the text.

| Reaction | Equilibrium constant: Symbol | Equilibrium constant: Value | Forward rate constant value ($s^{-1}$) |
|---|---|---|---|
| $E2+P_i \rightleftharpoons E2P_i$ | $K_{15}$ | 120.0 | |
| $E2+(HP_i) \rightleftharpoons E2(HP_i)$ | $K_{15}$ | 120.0 | |
| $E2+Mg \rightleftharpoons E2Mg$ | $K_{16}$ | 110.0 | |
| $E2P_i+Mg \rightleftharpoons E2P_iMg$ | $K_{17}$ | 130.0 | |
| $E2(HP_i)+Mg \rightleftharpoons E2(HP_i)Mg$ | $K_{17}$ | 130.0 | |
| $E2+K \rightleftharpoons E2K$ | $K_{18}$ | 227.6 | |
| $E2P_iMg \rightleftharpoons E2PMg$ | $K_{19}$ | 5.1 | 90.6* |
| $E2KP_iMg \rightleftharpoons E2PKMg$ | $K_{20}$ | 0.55 | 65.1* |
| $E2(HP_i)Mg \rightleftharpoons E2(HP)Mg$ | $K_{21}$ | 18.2 | 36.4* |
| $P_i+H^+ \rightleftharpoons HP_i$ | $K_{22}$ | $5\times10^6$† | |
| $E2P+Mg \rightleftharpoons E2PMg$ | $K_{23}$ | $6\times10^6$ | |
| $E1'PMgCa_2 \rightleftharpoons E2'PMgCa_2$ | $K_{24}$ | 0.03 | |
| $E1'PCa_2 \rightleftharpoons E2'PCa_2$ | $K_{25}$ | $2.5\times10^{-4}$ | |
| $E1+Mg \rightleftharpoons E1Mg$‡ | $K_{M1}$ | 0.5 | |
| $E1Mg \rightleftharpoons E1'Mg$‡ | $K_{M2}$ | 0.1 | |
| $E1'Mg+Mg \rightleftharpoons E1'Mg_2$‡ | $K_{M3}$ | $1.2\times10^3$ | |
| $E1+P_i \rightleftharpoons E1P_i$ | $K_{15}$ | 120.0 | |
| $E1+(HP_i) \rightleftharpoons E1(HP_i)$ | $K_{15}$ | 120.0 | |
| $E1+Mg \rightleftharpoons E1Mg$ | $K_{16}$ | 110.0 | |
| $E1P_i+Mg \rightleftharpoons E1P_iMg$ | $K_{17}$ | 130.0 | |
| $E1(HP_i)+Mg \rightleftharpoons E1(HP_i)Mg$ | $K_{17}$ | 130.0 | |
| $E1+K \rightleftharpoons E1K$ | $K_{18}$ | 227.6 | |

* Rate constants for all $Ca^{2+}$-bound forms 0.05 of the corresponding rates for $Ca^{2+}$-free forms.

† From Smith & Martell (1976).

‡ Binding of $Mg^{2+}$ at the $Ca^{2+}$-binding site.

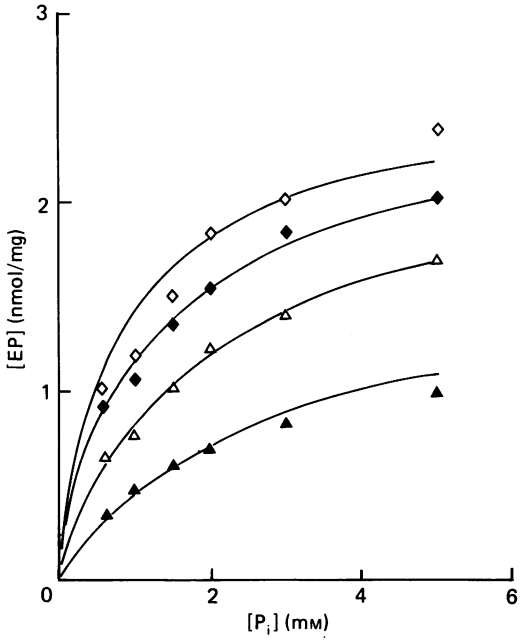



**Fig. 2. Phosphorylation of ATPase as a function of phosphate concentration at pH 6**

The experimental data from de Meis *et al.* (1982) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free phosphate concentration at pH 6.0 and free $Mg^{2+}$ concentrations of 10 mM (◇), 5.0 mM (◆), 2.5 mM (△) and 1.0 mM (▲). The curves are simulations with the parameters in Table 1 and $[EP]_{max.}$ = 3.0 nmol/mg of protein.

equilibrium constant for this step greatly favours $E1'PCa_2$, then the level of phosphorylation will increase on addition of $Ca^{2+}$.

A set of equations can be derived for the equilibrium level of phosphorylation in terms of the model as outlined (see the Appendix).

Consider first the effect of pH on phosphorylation. Inesi *et al.* (1984) produced a model for the effect of pH on the equilibrium level of phosphorylation involving two $H^+$-binding sites per phosphorylation site, with the p$K$ values changing on phosphorylation. However, we find that the experimental data of Inesi *et al.* (1984) fit equally well to a single ionization when the effect of pH on the E1–E2 equilibrium is also taken into account (Fig. 1). This ionization could correspond either to a group on the ATPase or to the phosphate group itself. Since the p$K$ value giving the best fit to the data (6.7) is equal to that of phosphate, we have assumed that it is indeed ionization of phosphate that is affecting phosphorylation and not ionization of a group on the ATPase. Measurements with FITC-labelled ATPase failed to detect any ionizations near the ATP-binding site in this pH region (Froud & Lee, 1986). We considered the possibility that $HPO_4^{2-}$ and $H_2PO_4^-$ had different binding constants for the ATPase, but found that any differences were too small to be significant. Values for the binding constants of phosphate and $Mg^{2+}$ (Table 2) were obtained by comparing simulations obtained by using the equations in the Appendix to the experimental data on phosphorylation as a function of phosphate and $Mg^{2+}$ concentrations given by de Meis *et al.* (1982) at pH 6.2 (Fig. 2) and by Punzengruber *et al.* (1978) at pH 7.0 (Fig. 3). These data also served to define the equilibrium constants for phosphorylation. In the simulations, an important parameter is $[EP]_{max.}$, the maximum observable level of phosphorylation. This is generally unknown, and studies have suggested that in many SR preparations active ATPase may constitute only 50% of the total protein (Gafni & Boyer, 1984; Coll & Murphy, 1984). As discussed previously (Martin & Tanford, 1981; de Meis *et al.*, 1982; Inesi *et al.*, 1984), this makes less certain the determination of the equilibrium constant for phosphorylation. However, we find that the possible range of values is limited by the requirement that the effect of pH on the effective value of this constant be affected by the effect of pH on the E1–E2 equilibrium ($K_B$ term in the Appendix). The value of the equilibrium constant for phosphorylation is also determined by the rate measurements described below. It was not found possible to fit the experimental data on the effects of $P_i$ and $Mg^{2+}$ on phosphorylation as a function of pH assuming different binding constants of phosphate and $Mg^{2+}$ to the E1 and E2 conformations.

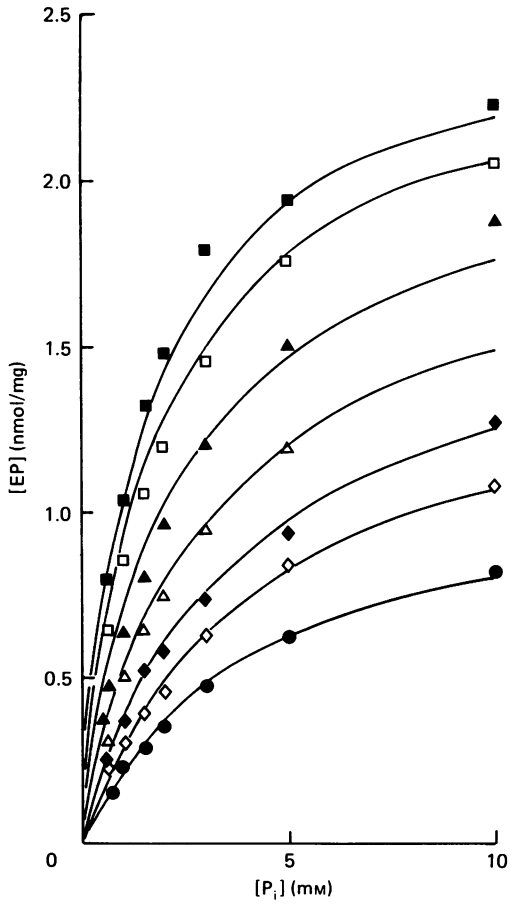



**Fig. 3. Phosphorylation of ATPase as a function of phosphate concentration at pH 7**

The experimental data from Punzengruber *et al.* (1978) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free phosphate concentration at pH 7.0 at free $Mg^{2+}$ concentrations of 20 mM (■), 10 mM (□), 5.0 mM (▲), 3.0 mM (△), 2.0 mM (◆), 1.5 mM (◇) and 1.0 mM (●). The curves are simulations with the parameters in Table 1 and $[EP]_{max.}$ = 3.3 nmol/mg of protein.

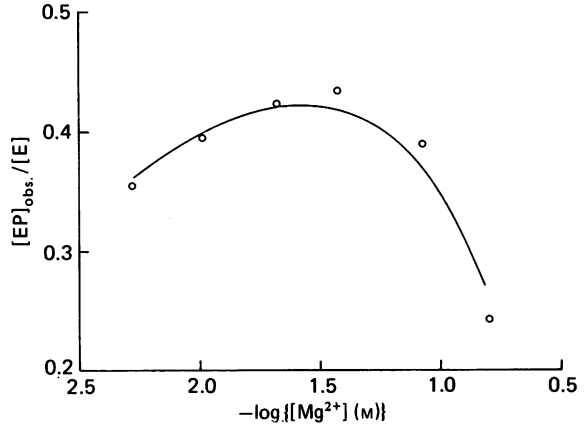



**Fig. 4. Effect of high concentrations of $Mg^{2+}$ on the phosphorylation of ATPase**

The experimental data from Martin & Tanford (1984) show the fractional equilibrium level of phosphorylation ($[EP]_{obs.}/[E]$) at pH 6.2 and free phosphate concentration of 5 mM as a function of log{free $[Mg^{2+}]$ (M)}, where [E] is the molar concentration of ATPase. The curve is a simulation with the parameters in Table 1 and a maximum possible fractional level of phosphorylation ($[EP]_{max.}/[E]$) of 0.54.

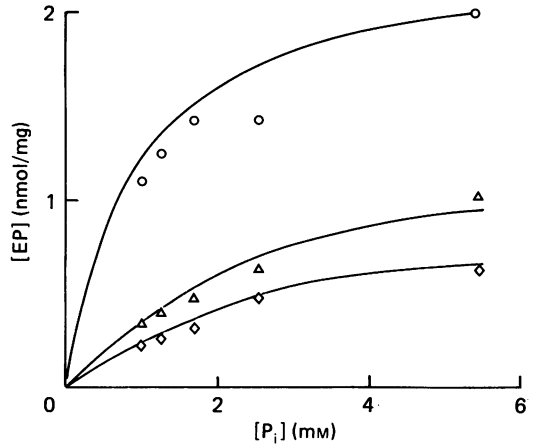



**Fig. 5. Effect of $K^+$ on the phosphorylation of ATPase**

The experimental data from Chaloub & de Meis (1980) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free phosphate concentration at pH 6.2 and free $Mg^{2+}$ concentration of 10 mM at $K^+$ concentrations of 0 mM (○), 100 mM (△) and 200 mM (◇). The curves are simulations with the parameters in Table 1 and $[EP]_{max.}$ = 2.7 nmol/mg of protein.

Although levels of phosphorylation of the enzyme generally increase with increasing concentrations of $Mg^{2+}$ (see Figs. 2 and 3), at high $Mg^{2+}$ concentrations these levels fall. This has been attributed to the binding of $Mg^{2+}$ to the $Ca^{2+}$-binding sites of the E1 conformation of the ATPase (Loomis *et al.*, 1982; Martin & Tanford, 1984). Fig. 4 shows a fit to the experimental data of Martin & Tanford (1984) in terms of such a model, with the parameters listed in Table 2. It was necessary to assume that binding of $Mg^{2+}$ to these sites was not affected by pH, since otherwise binding constants that give inhibition

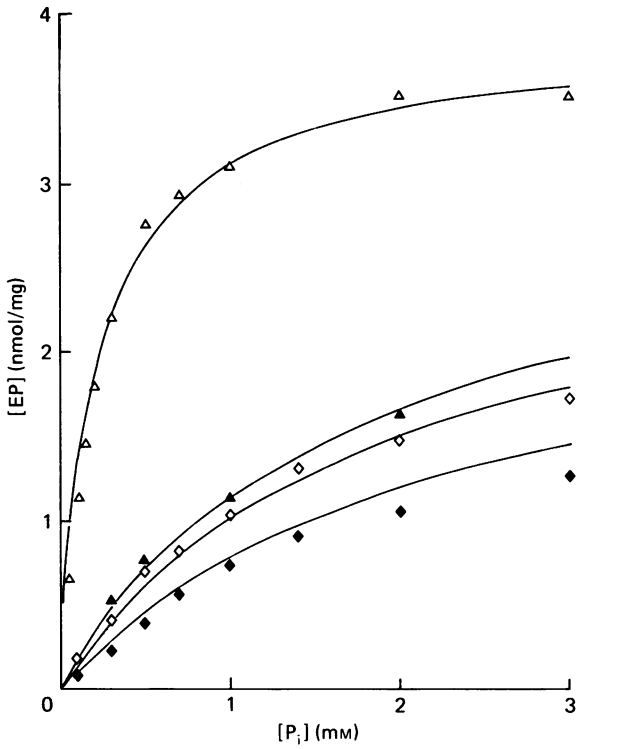



**Fig. 6. Phosphorylation of ATPase as a function of phosphate concentration in the presence of internal $Ca^{2+}$ in SR vesicles**

The experimental data from Prager *et al.* (1979) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free phosphate concentration at pH 7 for $Ca^{2+}$-loaded vesicles at a free $Mg^{2+}$ concentration of 10 mM (△) and for non-loaded vesicles at free $Mg^{2+}$ concentrations of 20 mM (▲), 10 mM (◇) and 5 mM (◆). The curves are simulations with the parameters in Table 1 and $[EP]_{max.}$ = 4.0 nmol/mg of protein and, for the $Ca^{2+}$-loaded vesicles, $[Ca^{2+}]_{int.}$ = 1.7 mM.

at pH 6.2 gave too large inhibitions at pH 8.0. Even so, it was impossible to simulate the large decrease in phosphorylation levels reported to occur at the highest concentrations of $Mg^{2+}$ (Loomis *et al.*, 1982; Martin & Tanford, 1984) without obtaining inhibitions inconsistent with the data of Punzengruber *et al.* (1978) and Inesi *et al.* (1984). However, since Loomis *et al.* (1982) found that up to 40% of the binding of [$^{32}$P]phosphate was non-specific at high $Mg^{2+}$ concentrations, we believe the simulations to be acceptable. The binding constant for $K^+$ was derived by comparison with the data of Chaloub & de Meis (1980) (Fig. 5). We considered the possibility that $K^+$ could bind to both the protonated and the unprotonated forms $E2P_i$ and $E2(HP_i)$, but the simulations suggest such weak binding of $K^+$ to $E2(HP_i)$ that it can be ignored.

Effects of $Ca^{2+}$ were determined by comparison with the data of Punzengruber *et al.* (1978), Chaloub & de Meis (1980) and Suko *et al.* (1981). $Ca^{2+}$ binding was described by a scheme (Scheme 3) exactly analogous to that used to describe $Ca^{2+}$ binding to E1 (Froud & Lee, 1986). The $Ca^{2+}$-binding site is postulated to contain two ionizable groups, with protonation of site 1 being competitive with $Ca^{2+}$ binding, and protonation of site 2 being non-competitive with $Ca^{2+}$ binding. The binding constant for protons at site 1, $K_{H2}$, was determined previously by the

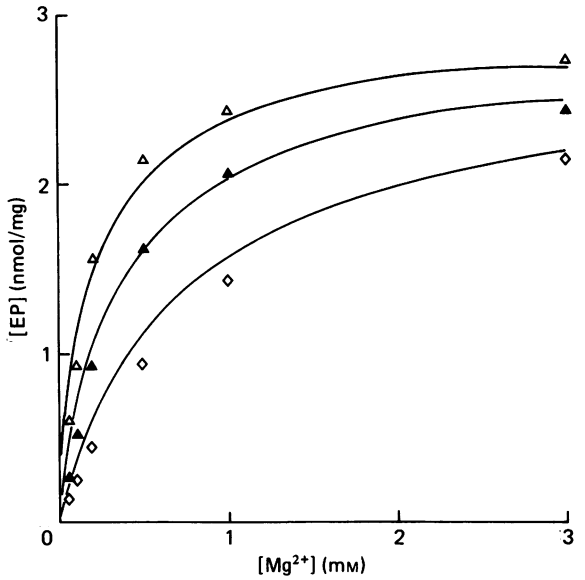



**Fig. 7. Phosphorylation of ATPase as a function of $Mg^{2+}$ concentration in the presence of internal $Ca^{2+}$ in SR vesicles**

The experimental data from Suko *et al.* (1981) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free $Mg^{2+}$ concentration at pH 7 for $Ca^{2+}$-loaded vesicles at free phosphate concentrations of 2.0 mM (△), 1.0 mM (▲) and 0.5 mM (◇). The curves are simulations with the parameters in Table 1 and $[Ca^{2+}]_{int.} = 7.0$ mM and $[EP]_{max.} = 3.0$ nmol/mg of protein.

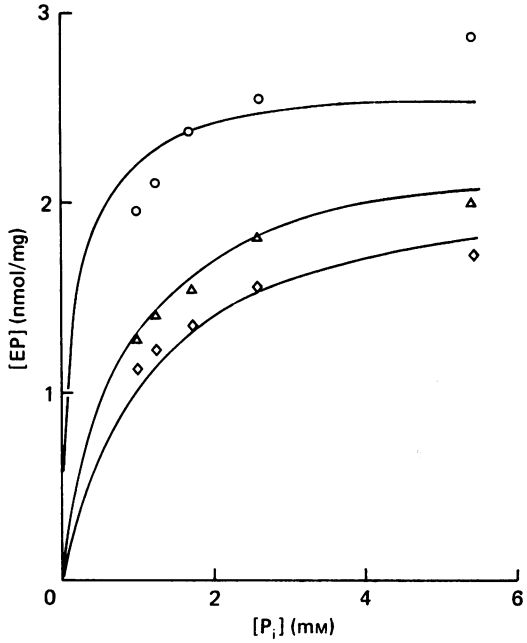



**Fig. 8. Effect of $K^+$ on the phosphorylation of ATPase in the presence of internal $Ca^{2+}$ in SR vesicles**

The experimental data from Chaloub & de Meis (1980) show the equilibrium level of phosphoenzyme (nmol/mg of protein) as a function of free phosphate concentration at pH 6.2 and at a free $Mg^{2+}$ concentration of 10 mM for $Ca^{2+}$-loaded vesicles at $K^+$ concentrations of 0 mM (○), 100 mM (△) and 200 mM (◇). The curves are simulations with the parameters in Table 1 and $[Ca^{2+}]_{int.} = 7.0$ mM and $[EP]_{max.} = 2.7$ nmol/mg of protein.

effect of pH on the E1–E2 equilibrium (Froud & Lee, 1986). Protonations at site 2 have all been put equal to those for site 2 in the E1 conformation, with only the binding constants for $Ca^{2+}$ being different. The effects of $Ca^{2+}$ on the level of phosphorylation then depend upon the binding constant for $Ca^{2+}$ and on the equilibrium constant $E2'PCa_2$–$E1'PCa_2$. The binding constants for $Ca^{2+}$ can only be determined from these experiments if the internal concentrations of $Ca^{2+}$ within the SR vesicles are known accurately. Unfortunately, this is not so, because the internal $Ca^{2+}$ concentrations are not related in a simple manner to the external concentrations used to load the SR vesicles (Prager *et al.*, 1979), because the internal volumes of the vesicles are not known, and because $Ca^{2+}$ tends to leak from the vesicles (Suko *et al.*, 1981). We have thus chosen to treat the internal concentrations of $Ca^{2+}$ as a variable. The binding constants for $Ca^{2+}$ were then determined from the inhibitory effects of high concentrations of $Ca^{2+}$ on the activity of the ATPase (Gould *et al.*, 1986; Table 2), leaving the values of $K_{23}$, $K_{24}$ and $K_{25}$ (Scheme 5) to be obtained by simulation. In this scheme it is assumed that there is no significant dissociation of $Ca^{2+}$ from $E1'PCa_2$ or $E1'PCa_2Mg$, in agreement with much experimental data (Gould *et al.*, 1986). The value of the binding constant for $Mg^{2+}$ to E2P ($K_{23}$) affects the level of phosphorylation observed at low concentrations of free $Mg^{2+}$. The value of the equilibrium constant $K_{25}$ describing the $E2'PCa_2$– $E1'PCa_2$ equilibrium, however, has no effect on the level of phosphorylation if it is equal to or less than the value of the equilibrium constant $K_{24}$ describing

**Table 3. Comparison of observed and calculated rates of dephosphorylation of phosphorylated ATPase**

| | Rate of dephosphorylation ($s^{-1}$) | | | |
|---|---|---|---|---|
| | $K = 0$ | | $K = 100$ mM | |
| pH | Observed* | Calculated | Observed† | Calculated |
| 6.0 | 3.0–3.8 | 2.8 | 14–16 | 13.8 |
| 6.8 | — | — | 43 | 44.2 |
| 7.0 | 7.9 | 7.7 | — | — |
| 8.0 | 15.8 | 15.4 | 63–73 | 79.7 |

* Decay rates calculated from data of Inesi *et al.* (1984).
† Decay rates calculated from data of Inesi & Hill (1983).

the $E2'PCa_2Mg$–$E1'PCa_2Mg$ equilibrium. As described in Gould *et al.* (1986), the value of $K_{24}$ must also be consistent with data on the inhibition of ATPase activity by high concentrations of $Ca^{2+}$. Also, as described in Gould *et al.* (1986), pH is assumed to have no effect on the value of $K_{24}$. Figs. 6 and 7 show that, despite the very much higher levels of phosphorylation in the presence of internal $Ca^{2+}$, the experimental data can be matched by assuming no effect of $Ca^{2+}$ on the binding of phosphate

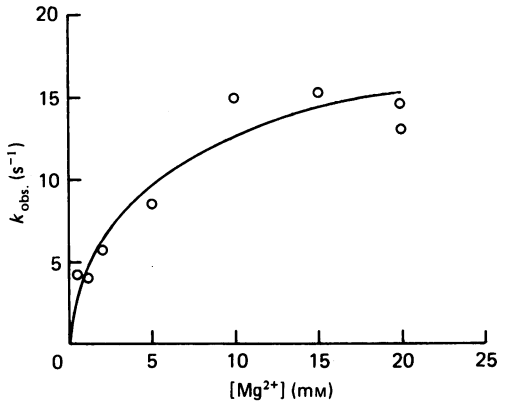



**Fig. 9. Effect of $Mg^{2+}$ on the rate of phosphorylation of ATPase**

The experimental data from Inesi *et al.* (1984) show the dependence of the apparent rate constant of phosphorylation ($k_{obs.}$) on the free $Mg^{2+}$ concentration at pH 6.0 and at a free phosphate concentration of 4 mM. The curve is a simulation with the parameters in Table 1.

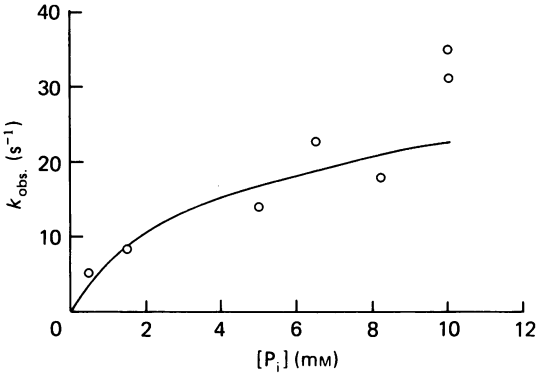



**Fig. 10. Effect of phosphate on the rate of phosphorylation of ATPase**

The experimental data from Inesi *et al.* (1984) show the dependence of the apparent rate constant of phosphorylation ($k_{obs.}$) on the free phosphate concentration at pH 6.0 and at a free $Mg^{2+}$ concentration of 17 mM. The curve is a simulation with the parameters in Table 1.

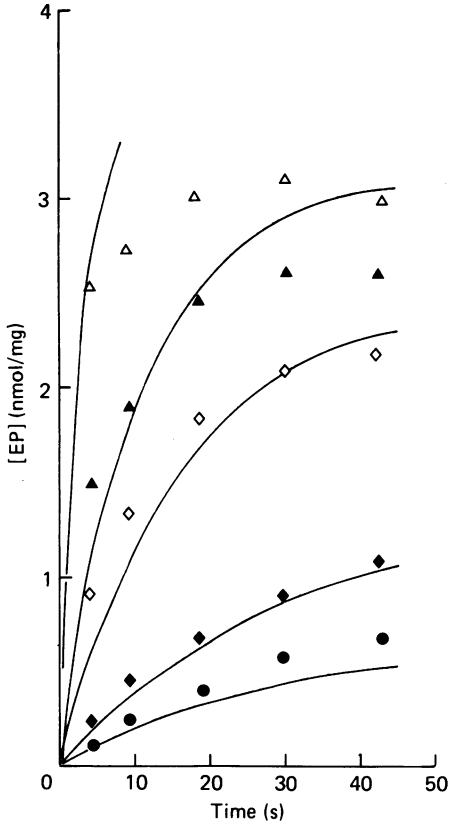



**Fig. 11. Effect of internal $Ca^{2+}$ in SR vesicles on the rate of phosphorylation of ATPase**

The experimental data from Suko *et al.* (1981) show the rate of phosphorylation at pH 7.0 and at a $P_i$ concentration of 1.0 mM and $Mg^{2+}$ concentrations of 5.0 mM (△), 1.0 mM (▲), 0.5 mM (◇), 0.15 mM (◆) and 0.07 mM (●). The curves are simulations with the parameters in Table 1 and $[Ca^{2+}]_{int.} = 5.4$ mM and $[EP]_{max.} = 4.5$ nmol/mg of protein.

or $Mg^{2+}$ or on the equilibrium constant for phosphorylation when the equilibrium constant $E2'PMgCa_2/E1'PMgCa_2$ considerably favours $E1'PMgCa_2$. Fig. 8 also shows that the effect of $K^+$ in the presence of internal $Ca^{2+}$ can only be matched by assuming no effect of $Ca^{2+}$ on the binding of $K^+$.

The concentrations of internal $Ca^{2+}$ assumed in these simulations are reasonable. Thus, for example, Suko *et al.* (1981) have shown that when SR vesicles are incubated with 40 mM-$Ca^{2+}$ the internal $Ca^{2+}$ load is 80 nmol/mg of protein. Prager *et al.* (1979) have shown that approx. 30 nmol of $Ca^{2+}$/mg of protein corresponds to saturable binding, leaving a free internal $Ca^{2+}$ concentration of approx. 50 nmol/mg of protein. The simulations (Figs. 7 and 8) were performed assuming an internal $Ca^{2+}$ concentration of 5.4 mM, corresponding to an internal volume of 9 μl/mg of protein. This can be compared to published estimates of the internal volume of approx. 1 (Chiu & Haynes, 1980), 4–5 (Duggan & Martonosi, 1970), 5.8 (McKinley & Meissner, 1978) and approx. 10 μl/mg protein (Weber *et al.*, 1966).

Rate constants for phosphorylation and dephosphorylation were estimated assuming that binding and release of $Mg^{2+}$ and phosphate are fast enough compared with the rates of phosphorylation and dephosphorylation to be treated by the quasi-equilibrium approach (Wong, 1975). Inesi *et al.* (1984) and Inesi & Hill (1983) have presented data for the dephosphorylation of E2P at three pH values in the presence and in the absence of $K^+$ (Table 3). With values for the binding constants and equilibrium constant for phosphorylation defined by the equilibrium experiments, it is possible to use an iterative procedure to obtain a best fit to the rate data, with the results listed in Table 3 and the rate parameters listed in Table 2. The rates of dephosphorylation of the forms E2PK, E2(HP) and E2P together with the respective equilibrium constants for phosphorylation define the phosphorylation rates. Figs. 9 and 10 illustrate the good agreement between the simulated values of the apparent rate constant ($k_{obs.}$) for phosphorylation as a function of

$Mg^{2+}$ and $P_i$ concentrations and the data reported by Inesi *et al.* (1984). The simulated values (not shown) also agree well with the phosphorylation rates reported by Pickart & Jencks (1984).

The data reported by Inesi & Hill (1983) on the rates of dephosphorylation in the presence of $K^+$ involved quenching with high concentrations of ATP. As described in the following paper (Gould *et al.*, 1986), there is much evidence that ATP accelerates the rate of E2P hydrolysis. However, the rates reported by Inesi & Hill (1983) are very similar to those reported by Inesi & Kurzmack (1983) and Chaloub & de Meis (1980), in which reactions were quenched with excess phosphate. It therefore appears that under these conditions ATP has no effect on dephosphorylation. A possible explanation is presented in the following paper (Gould *et al.*, 1986).

There is much evidence that $Ca^{2+}$ within SR vesicles lowers the rate of dephosphorylation (de Meis, 1981). Suko *et al.* (1981) have studied the effect of internal $Ca^{2+}$ on the rate of phosphorylation. Reasonable simulations of the experimental data can be obtained by assuming that the rates of phosphorylation and dephosphorylation of all $Ca^{2+}$-bound forms are 0.05 of those of the $Ca^{2+}$-free form (Fig. 11); agreement cannot be obtained by assuming a zero rate of reaction for the $Ca^{2+}$-bound forms.

The binding constants presented here for $Mg^{2+}$ and phosphate are very similar to those derived by others (Punzengruber *et al.*, 1978; Martin & Tanford, 1981; de Meis *et al.*, 1982; Inesi *et al.*, 1984). However, the data give no indication of synergy in binding of $Mg^{2+}$ and phosphate of the kind suggested by Martin & Tanford (1981) and de Meis *et al.* (1982). The values for the equilibrium constants for phosphorylation are, however, different from previous estimates. In our model the effect of $K^+$ and increasing pH in decreasing the level of phosphorylation of the enzyme follow from differences in binding constants for $K^+$ and $H^+$ to the phosphorylated and non-phosphorylated ATPase. Thus the binding constant for $K^+$ falls 10-fold on phosphorylation, whereas the binding constant for $H^+$ increases 3-fold. It is known that the group phosphorylated on the ATPase is an aspartate group (Degani & Boyer, 1973). Our data then suggest a $pK_a$ of 7.25 for the aspartyl phosphate group compared with a $pK_a$ of 6.7 for phosphate in free solution (Table 2). We do not need to postulate any effect of pH or $K^+$ on the strength of binding of phosphate (as suggested by Pickart & Jencks, 1984) to account for the experimental data.

The phosphorylation reactions have also been studied by measuring medium–$P_i$ oxygen exchange (McIntosh & Boyer, 1983; Guillain *et al.*, 1984). These studies have been interpreted in terms of rates of dissociation of $Mg^{2+}$ and phosphate from the ternary complex $E2P_iMg$ of about 100 and 400 $s^{-1}$ respectively. These rates are high compared with the rates of dephosphorylation, so that the quasi-equilibrium approach utilized above for dephosphorylation is justified. However, as discussed by Guillain *et al.* (1984), an 'off' rate for $Mg^{2+}$ dissociation of 100 $s^{-1}$ and an equilibrium binding constant of approx. 100 mean that for phosphorylation at millimolar concentrations of $Mg^{2+}$ the rate of $Mg^{2+}$ binding can be lower than the rate of phosphorylation. The good agreement between the experimental data of Inesi *et al.* (1984) on phosphorylation and the simulations obtained by using the quasi-equilibrium approach (Figs. 9 and 10) suggest that the rates reported by Guillain *et al.* (1984) may be underestimates.

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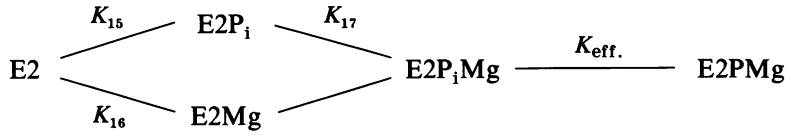
# APPENDIX

## DERIVATION OF EQUATIONS DESCRIBING THE EQUILIBRIUM LEVEL OF PHOSPHORYLATION OF ATPASE BY PHOSPHATE

The schemes for phosphorylation described in the main paper above are reducible to the following form:




Effects of pH and $Ca^{2+}$ on the E1–E2 equilibrium, the effects of pH and $K^+$ on phosphorylation and the effects of the $Ca_2$E1P–$Ca_2$E2P equilibrium are all contained in $K_{eff.}$. In terms of the scheme:

$$\frac{[EP]}{[EP]_{max.}} = \frac{K_{15}K_{17}K_{eff.}[Mg][P_i]}{1+K_{15}[P_i]+K_{16}[Mg]+K_{15}K_{17}[P_i][Mg]+K_{15}K_{17}K_{eff.}[P_i][Mg]}$$

where $[EP]_{max.}$ is the maximum obtainable concentration of phosphorylated ATPase (equal to the concentration of ATPase for 100%-pure active ATPase). $K_{eff.}$ can be written in the form:

$$K_{eff.} = K_A K_B K_C$$

The term $K_A$ describes the effect of pH and $K^+$ on phosphorylation (Scheme 2 in the main paper) and is:

$$K_A = (K_{19}+K_{21}K_{22}[H]+K_{20}K_{18}[K])/(1+K_{22}[H]+K_{18}[K])$$

The term $K_B$ describes the effect of pH, the binding of $Mg^{2+}$ to the $Ca^{2+}$-binding sites of E1 and the binding of $Ca^{2+}$ to the $Ca^{2+}$-binding sites of E2. For sealed SR vesicles containing $Ca^{2+}$ with no external $Ca^{2+}$, the following equations describe the relative amounts of the various bound forms of E1 and E2:

$$F_1 = (1+2K_{H1}[H]+K_{H1}^2[H]^2)(1+2K_{H3}[H]+K_{H3}^2[H]^2)(1+2K_{M1}[Mg]+2K_{M1}K_{M2}[Mg]+K_{M1}K_{M2}K_{M3}[Mg]^2)$$

$$F_2 = (1+2K_{H2}[H]+K_{H2}^2[H]^2)(1+2K_{H3}[H]+K_{H3}^2[H]^2)$$

$$F_3 = (1+K_{H11}[H]+K_{H11}K_{H12}[H]^2)(2K_{C4}[Ca]+2K_{C4}K_{H2}[H][Ca])$$

$$F_4 = (1+K_{H13}[H]+K_{H13}K_{H14}[H]^2)(2K_{C4}K_{C5}[Ca]+2K_{C4}K_{C5}K_{H2}[H][Ca])$$

$$F_5 = (1+2K_{H8}[H]+K_{H8}^2[H]^2)K_{C4}K_{C5}K_{C6}[Ca]^2$$

It then follows that:

$$K_B = (F_2+F_3+F_4+F_5)/(F_1K_1+F_2+F_3+F_4+F_5)$$

where $K_1$ is the equilibrium constant for the E2–E1 equilibrium (see Froud & Lee, 1986).

Finally, the effect of dissociation of $Mg^{2+}$ from the phosphorylated ATPase and the E2′$PCa_2$–E1′$PCa_2$ equilibrium (Scheme 5 in the main paper) give $K_C$:

$$K_C = 1+\frac{1}{K_{23}[Mg]}+\left(\frac{1}{K_{24}}+\frac{1}{K_{23}K_{25}[Mg]}\right)\left(\frac{F_5}{F_2+F_3+F_4+F_5}\right)$$

Here it is assumed that only the fully $Ca^{2+}$-bound form of E2′$PCa_2$ can transform into E1′$PCa_2$ (Scheme 5 in the main paper).

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