Calcium ATPase of Sarcoplasmic Reticulum Has Four Binding Sites for Calcium†

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ABSTRACT: The calcium-transporting ATPase of sarcoplasmic reticulum is known to bind two Ca^{2+} ions from the cytoplasm to the free enzyme and two Ca^{2+} ions from the lumen to the phosphoenzyme. The concentration of phosphoenzyme formed at equilibrium from P_i and Mg^{2+} increases with increasing concentration of calcium in the lumen, which binds to the phosphoenzyme to form Ca_2·E~P·Mg. However, at subsaturating concentrations of Mg^{2+} increasing the concentration of lumenal Ca^{2+} does not drive phosphoenzyme formation to completion. The maximal levels of phosphoenzyme that are formed at saturating concentrations of lumenal Ca^{2+} increase with increasing concentrations of Mg^{2+}. This result requires that Ca^{2+} can bind to low-affinity lumenal sites on both the free enzyme and the phosphoenzyme, as well as to the high-affinity cytoplasmic calcium-binding sites. If there were no lumenal binding sites for Ca^{2+} on the free enzyme, high concentrations of lumenal Ca^{2+} would convert all of the enzyme to the same maximal concentration of Ca_2·E~P·Mg at subsaturating concentrations of Mg^{2+} and P_i. We conclude that there are two low-affinity lumenal sites as well as two high-affinity cytoplasmic sites for Ca^{2+} on the free enzyme. Phosphorylation by ATP results in translocation of Ca^{2+} from the high-affinity to the low-affinity sites.

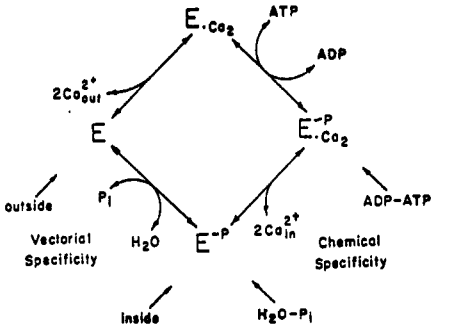
The coupling between the hydrolysis of ATP and the internalization of two Ca^{2+} ions that is brought about by the Ca^{2+}-ATPase[1] of sarcoplasmic reticulum vesicles can be defined by a simple set of rules that describe changes in chemical and vectorial specificity, as shown in Scheme I. The chemical specificity for catalysis of phosphorylation by ATP or P_i is controlled by the presence or absence of bound Ca^{2+} ions, while the vectorial specificity for Ca^{2+} binding or dissociation on the two sides of the membrane is controlled by the state of phosphorylation of the enzyme (Pickart & Jencks, 1984). This scheme was first described by Makinose (1973). The mechanism by which the two Ca^{2+} ions are transported across the membrane is of particular interest.

It is well-known that two Ca^{2+} ions bind to high-affinity sites of the unphosphorylated enzyme on the cytoplasmic side of the sarcoplasmic reticulum membrane and to low-affinity sites on the lumenal side of the phosphoenzyme (Makinose & Hasselbach, 1965; Carvalho & Leo, 1967; Dupont, 1978; Kalbitzer et al., 1978); i.e., phosphorylation acts as a vectorial switch that changes the side of the membrane at which calcium binds and dissociates during the transport process. Phosphorylation also decreases the affinity of the enzyme for calcium: the dissociation constants for the two Ca^{2+} ions from the unphosphorylated enzyme are in the micromolar range, while they are in the millimolar range for the phosphoenzyme (Makinose & Hasselbach, 1965; Carvalho & Leo, 1967; Dupont, 1978; Kalbitzer et al., 1978; Pickart & Jencks, 1984).

There are at least two different ways in which these changes in calcium binding and dissociation could occur:

(1) There can be a phosphorylation-dependent change in gating of the binding sites for two Ca^{2+} ions, such that calcium at these sites can bind and dissociate on the cytoplasmic side of the membrane with the unphosphorylated enzyme, E, and on the lumenal side of the membrane with the phosphoenzyme (Figure 1A).

Scheme I



(2) Calcium on the unphosphorylated enzyme can dissociate from one pair of binding sites to the cytoplasmic side of the membrane, while calcium bound to the phosphorylated enzyme can dissociate to the lumenal side of the membrane from a different set of binding sites (Figure 1B). According to this mechanism, phosphorylation brings about a translocation of the two calcium ions from one set of binding sites to another, whereas according to the first mechanism there is only a change in the gating of a single pair of binding sites upon phosphorylation.

Several investigators (Makinose & Hasselbach, 1965; Dupont, 1978; Chaloub et al., 1979) and notably Suko et al. (1981) have proposed that low-affinity calcium-binding sites exist on the lumenal side of the membrane in the free enzyme, as well as the phosphoenzyme. This conclusion is based upon an extensive series of measurements of the concentration of phosphoenyzme that is formed at equilibrium in the presence of different concentrations of Mg^{2+} and P_i with vesicles that were passively loaded with 30 or 40 mM calcium (Suko et al., 1981). It was shown that characteristic patterns for the dependence of phosphoenzyme concentration on the concentration of Mg^{2+} and P_i are expected if there are lumenal calcium binding sites in the free enzyme and these binding sites are saturated with calcium. The results of these

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[1] Abbreviations: Ca^{2+}-ATPase, calcium-transporting ATPase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EP, phosphoenzyme; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P_i, inorganic phosphate; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; Tris, tris(hydroxymethyl)aminomethane.

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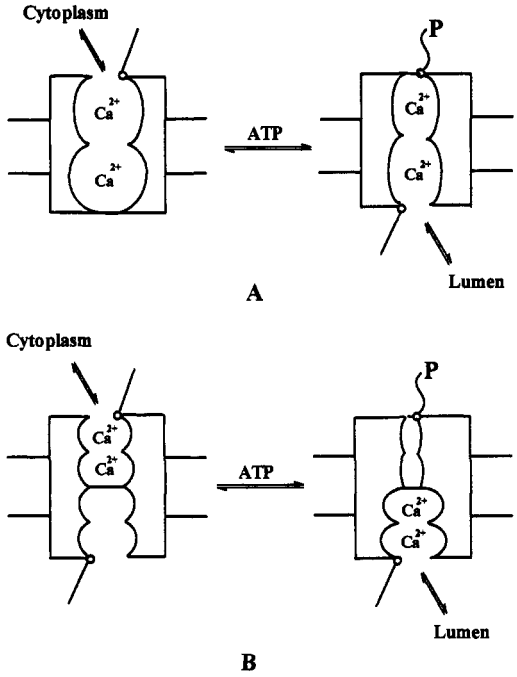
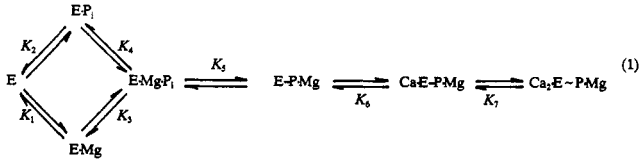


FIGURE 1: Schematic models for the binding and dissociation of two Ca^{2+} ions on the cytoplasmic side of the membrane with the free enzyme and the lumenal side of the membrane with the phosphoenzyme. (A) A two-site model, with a change in gating upon phosphorylation. (B) A four-site model, in which the two Ca^{2+} ions are translocated from high- to low-affinity sites upon phosphorylation.

experiments, especially the finding that the dependence of the concentration of phosphoenzyme on the concentration of Mg^{2+} at equilibrium does not differ significantly at 30 and 40 mM lumenal Ca^{2+}, are consistent with the existence of lumenal Ca^{2+}-binding sites on the free enzyme that are saturated with calcium. However, it is difficult to be certain that a sufficiently large range of calcium concentrations was examined in these experiments to establish the existence of interior binding sites beyond question. We undertook a reinvestigation of this problem because of the importance of the question of whether lumenal binding sites for Ca^{2+} exist on the free enzyme, in addition to the cytoplasmic sites, and because we wished to determine what concentration of lumenal calcium is required to saturate these sites, if they do exist.

We report here what we believe to be simple and direct evidence in support of the existence of low-affinity lumenal binding sites for calcium on the free enzyme. If there are no such lumenal binding sites, the binding of calcium to form Ca_2·E~P·Mg can be described by



(1)

According to this mechanism, phosphoenzyme formation can be driven to completion by high concentrations of lumenal calcium at subsaturating concentrations of Mg^{2+} and P_i (Figure 2A).

If there are lumenal Ca^{2+}-binding sites on the free enzyme, the reaction is described by eq 2. With this mechanism, an increase in the concentration of lumenal Ca^{2+} will not drive the reaction to completion at subsaturating concentrations of Mg^{2+} and P_i, because Ca^{2+} can bind to the lumenal sites of both the free enzyme and the phosphoenzyme (Figure 2B).

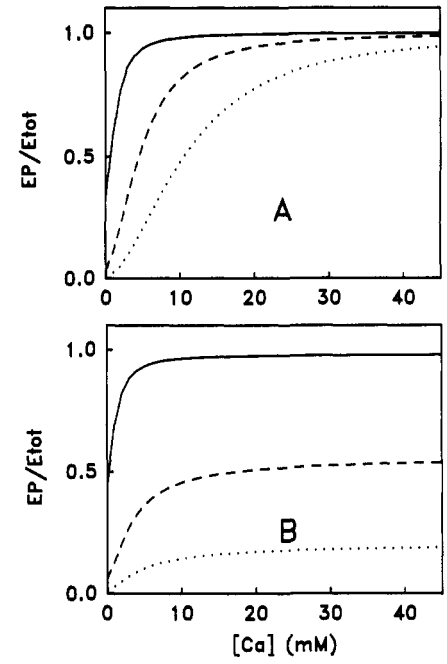
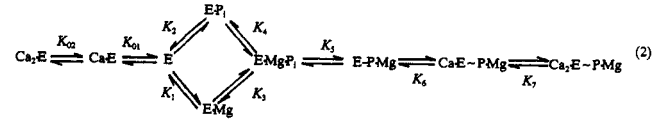


FIGURE 2: (A) If there are no lumenal binding sites for Ca^{2+} in the free enzyme, increasing the concentration of lumenal Ca^{2+} at different concentrations of Mg^{2+} or P_i will drive phosphoenzyme formation to completion. (B) If there are lumenal binding sites for Ca^{2+} in the free enzyme, increasing the concentration of lumenal Ca^{2+} will drive phosphoenzyme formation to completion when the concentrations of Mg^{3+} and P_i are saturating but will give lower maximal concentrations of phosphoenzyme in the presence of subsaturating concentrations of Mg^{2+} and P_i. The curves were calculated from eqs 3 and 4 for the mechanisms of eqs 1 and 2, respectively, with K_{01} = 5 mM, K_{02} = 6 mM, $K_1 = K_2$ = 10 mM, $K_3 = K_4$ = 1 mM, K_5 = 1.0, $K_6 = K_7$ = 1 mM, $[P_i]$ = 2 mM, and $[Mg^{2+}]$ = 0.05 (···), 0.25 (- - -), and 10 (—) mM.



(2)

We have derived eqs 3 and 4 for the reaction schemes shown in eqs 1 and 2, respectively. If two Ca^{2+} ions bind to the

$$\frac{E_{tot}}{\Sigma E\text{–}P} = 1 + \frac{K_1K_3 + K_4[P_i] + K_3[Mg] + [Mg][P_i]}{K_5[Mg][P_i]\left(1 + \frac{[Ca]}{K_6} + \frac{[Ca]^2}{K_6K_7}\right)} \quad (3)$$

$$\frac{E_{tot}}{\Sigma E\text{–}P} = 1 + \frac{K_1K_3\left(\frac{[Ca]^2}{K_{01}K_{02}} + \frac{[Ca]}{K_{01}} + 1\right) + K_4[P_i] + K_3[Mg] + [Mg][P_i]}{K_5[Mg][P_i]\left(1 + \frac{[Ca]}{K_6} + \frac{[Ca]^2}{K_6K_7}\right)} \quad (4)$$

lumenal sites of the free enzyme, with dissociation constants K_{01} and K_{02} (eq 2), the concentration of lumenal calcium, [Ca], appears in both the numerator and denominator of eq 4 and an increase in the lumenal calcium concentration will not force all of the enzyme into the species Ca_2·E~P·Mg. However, if there is no binding of lumenal calcium to the free enzyme and, therefore, no K_{01} and K_{02} terms (eq 1), the term for calcium binding inside the vesicle appears only in the denominator of eq 3 and a high concentration of lumenal

Table I: Dependence of Phosphoenzyme Concentration at Equilibrium on the Concentrations of Magnesium and Lumenal Calcium[a]

	phosphoenzyme (nmol/mg)			
$[Ca^{2+}]_{lumen}$ (mM)	$[Mg^{2+}]$ 0.25 mM	$[Mg^{2+}]$ 0.5 mM	$[Mg^{2+}]$ 1.0 mM	$[Mg^{2+}]$ 10 mM
0	0.11	0.20	0.31	0.67
0	0.11	0.20	0.29	0.64
2	0.33	0.56	0.75	1.47
2	0.33	0.58	0.76	1.52
5	0.60	0.90	1.24	1.80
5	0.61	0.90	1.26	1.88
10	0.81	1.19	1.67	2.34
10	0.82	1.17	1.69	2.33
15	0.72	1.20	1.65	2.00
15	0.83	1.25	1.58	2.05
23	0.99	1.38	1.75	2.19
23	0.99	1.40	1.75	2.24
31	1.22	1.68	1.98	2.51
31	1.29	1.62	2.05	2.45
40	1.13	1.58	1.74	2.29
40	1.11	1.57	1.72	2.34

[a] Vesicles passively loaded with calcium were added to a solution containing 2 mM ^{32}P-labeled inorganic phosphate and the indicated concentrations of $MgCl_2$, 30 mM KCl, 10 mM EGTA, and 40 mM MOPS/Tris buffer, pH 7.0, at 25 °C. The amount of phosphoenzyme formation was determined after 25 s by precipitation with acid, filtration, and scintillation counting, as described in the text.

calcium will convert all of the enzyme to Ca_2·E~P·Mg. Equation 4 reduces to eq 3 when the concentration of lumenal Ca^{2+} is $\ll K_{01}$.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles from white rabbit muscle were prepared as described previously (Khananshvili & Jencks, 1988) and were stored at −80 °C. Protein concentrations were estimated by using the method of Lowry et al. (1951), with bovine serum albumin as the protein standard, and ^{32}P-labeled inorganic phosphate was obtained from New England Nuclear, allowed to stand for 1 h in 1 M HCl, and neutralized with Tris base. Charcoal, 0.1 g, was added to 0.5 mL of the solution, which was filtered through a glass fiber membrane and a Millipore membrane after standing for 15 min at room temperature.

Vesicles were loaded with calcium by dialysis overnight at 5 °C against solutions containing 0.4 M sucrose, 40 mM MOPS/Tris buffer at pH 7.0, 30 mM KCl, and different concentrations of $CaCl_2$. For each data point 20 μL of a suspension of vesicles was added to 0.48 mL of reaction solution containing 40 mM MOPS/Tris, pH 7.0, 2 mM ^{32}P-labeled inorganic phosphate, 30 mM KCl, 10 mM EGTA, and 0.25, 0.5, 1.0, or 10 mM $MgCl_2$ at 25 °C. The reactions were quenched with 0.5 mL of 0.4 M HCl containing 40 mM KH_2PO_4 at 25 s, at which time the concentration of phosphoenzyme was shown to have reached a stable equilibrium level. Bovine serum albumin (0.5 mL of 0.4 mg/mL) and 0.5 mL of 60% trichloroacetic acid were added to facilitate precipitation of the protein. Blanks were prepared at each concentration of magnesium and inorganic phosphate with identical reaction mixtures in which the quench solution was added before the addition of vesicles. The samples were centrifuged, washed with 20 mL of 5% trichloroacetic acid containing 50 mM KH_2PO_4, and collected on Whatman GF/C glass fiber filters. The radioactivity of the samples in glass vials containing 7 mL of Aquasol 2 was determined by liquid scintillation counting. All determinations were made in duplicate. The concentrations of free Mg^{2+} and P_i for the experiments shown in Figure 3 were calculated with the computer program of Fabiato (1988).

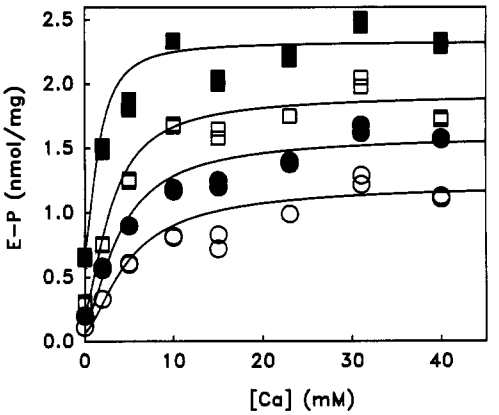


FIGURE 3: Dependence of phosphoenzyme concentration at equilibrium on the lumenal calcium concentration in sarcoplasmic reticulum vesicles passively loaded with the indicated concentrations of calcium. The concentration of phosphoenzyme was measured 25 s after the addition of 2 mM ^{32}P-labeled inorganic phosphate and 0.25 mM (○), 0.5 mM (●), 1 mM (□), or 10 mM (■) magnesium chloride at pH 7.0 and 25 °C; the conditions are described in Table I. The solid lines are drawn according to eq 4, with K_{01} = 5 mM, K_{02} = 6 mM, $K_1 = K_2$ = 10 mM, $K_3 = K_4$ = 1 mM, K_5 = 1.0, $K_6 = K_7$ = 1 mM, and E_{tot} = 2.4 nmol/mg. The concentrations of Mg^{2+} and P_i were calculated from an apparent association constant of 45.3 M^{-1} for Mg–inorganic phosphate with the computer program of Fabiato (1988): free $[Mg^{2+}]$ = 0.18 (○), 0.35 (●), 0.71 (□), and 7.52 mM (■); free $[P_i]$ = 1.99 (○), 1.97 (●), 1.94 (□), and 1.49 mM (■).

RESULTS

Table I and Figure 3 show the dependence of phosphoenzyme concentration on the concentration of lumenal calcium in sarcoplasmic reticulum vesicles that were equilibrated overnight with eight different concentrations of $CaCl_2$, in the range 0–40 mM, and then diluted into a solution containing 2 mM ^{32}P-labeled inorganic phosphate, 30 mM KCl, 10 mM EGTA, 40 mM MOPS/Tris buffer at pH 7.0, and 0.25, 0.5, 1.0, or 10 mM $MgCl_2$. Phosphoenzyme concentrations were determined at 0, 20, 40, 80, and 120 s after the addition of ^{32}P-labeled inorganic phosphate and $MgCl_2$ and were found to be stable between 20 and 40 s and to decrease by 35% between 40 and 120 s because of leakage of Ca^{2+} from the vesicles. The phosphoenzyme levels shown in Table I and Figure 3 were determined at 25 s, as described under Materials and Methods.

DISCUSSION

Figure 3 shows the dependence on lumenal calcium concentration of the concentration of phosphoenzyme at equilibrium, with SR vesicles that were passively loaded with eight different concentrations of calcium and then incubated for 25 s with ^{32}P-labeled inorganic phosphate and EGTA at pH 7 in the presence of different concentrations of $MgCl_2$. The solid lines are the best fit to the model of eq 2 and were calculated from eq 4 with values of K_{01} = 5 mM, K_{02} = 6 mM, $K_1 = K_2$ = 10 mM, $K_3 = K_4$ = 1 mM, K_5 = 1.0, and $K_6 = K_7$ = 1 mM for vesicles that were incubated with 10, 1, 0.5, and 0.25 mM $[Mg^{2+}]$, respectively. The corresponding slopes of Hill plots for [E–P] − $[E–P]_0$ (not shown; $[E–P]_0$ is the concentration of phosphoenzyme in the absence of lumenal Ca^{2+}) are n_H = 0.8, 1.1, 1.1, and 1.1. These Hill slopes do

not provide evidence for significant positive cooperativity of calcium binding to the phosphoenzyme.

The concentration of phosphoenzyme increases with increasing concentrations of lumenal calcium and then levels off at different maximal values with different concentrations of Mg^{2+}. This shows that at the higher Mg^{2+} concentrations lumenal calcium binds to the phosphoenzyme, E–P·Mg, to form Ca_2·E~P·Mg and that at lower Mg^{2+} concentrations calcium binds to lumenal binding sites on the free enzyme, E, to form Ca_2·E. If there were no binding of calcium to the lumenal binding sites on the free enzyme, increasing the concentration of lumenal calcium would eventually force all of the enzyme into the species Ca_2·E~P·Mg, as described in the introduction. The presence of lumenal binding sites for calcium on the unphosphorylated enzyme provides an *alternative* to the formation of Ca_2·E~P·Mg; it allows calcium to bind to both E and E–P. Therefore, all of the enzyme is not converted to Ca_2·E~P·Mg at saturating concentrations of calcium when the concentrations of P_i and Mg^{2+} are not saturating. This result requires that calcium can bind to both high-affinity cytoplasmic sites and low-affinity lumenal sites on the free enzyme.

The binding of Ca^{2+} to lumenal sites on the free enzyme and the binding of Ca^{2+} to sites on the phosphoenzyme are *alternative* binding modes; two lumenal Ca^{2+} ions can bind to the phosphoenzyme *or* to the free enzyme. The data are not consistent with the binding of four Ca^{2+} ions from the lumen to either species.

Figure 1A shows a model in which there are only two binding sites for Ca^{2+}: cytoplasmic Ca^{2+} binds and dissociates at these sites with the free enzyme and lumenal Ca^{2+} binds and dissociates with the phosphoenzyme. This model does not permit occupancy of Ca^{2+} binding sites from the cytoplasm and the lumen of the vesicle simultaneously.

We have shown previously that high concentrations of lumenal Ca^{2+} have no significant effect on the rate constants and equilibrium constants for binding of Ca^{2+} to the high-affinity cytoplasmic sites (Petithory & Jencks, 1988; Myung & Jencks, 1991). This requires that the lumenal and the cytoplasmic calcium-binding sites are different and independent of each other and excludes the model of Figure 1A. The existence of independent cytoplasmic and lumenal sites for binding two Ca^{2+} ions requires that there are a total of four calcium-binding sites on the free enzyme.

A model that is consistent with the data is shown schematically in Figure 1B. The cytoplasmic and lumenal Ca^{2+} binding sites are independent; each pair of sites binds two Ca^{2+} ions, and there is no interaction between the sites (Petithory & Jencks, 1988; Myung & Jencks, 1991). Phosphorylation of E_{Ca_2} by ATP results in destabilization of the two Ca^{2+} ions in the high-affinity sites and forces their translocation to the low-affinity lumenal sites. The Ca^{2+} ions can then dissociate to the lumen with a rate constant of ~ 30 s^{-1}, under the conditions studied in this laboratory (Hanel & Jencks, 1991). Calcium ions can bind to the lumenal sites of the free enzyme and the phosphoenzyme. However, they can be translocated between the lumenal and the cytoplasmic sites only in the phosphoenzyme. When Ca_2·E~P·Mg reacts with ADP to give ATP and $^cE_{Ca_2}$, the Ca^{2+} ions can dissociate to the cytoplasm.

It should be noted that movement of the two Ca^{2+} ions from the low-affinity sites in Ca_2·E~P·Mg to the high-affinity sites in $^cE_{Ca_2}$ can account for the "energy-rich" nature of Ca_2·E~P·Mg that allows it to react with ADP and form ATP. Movement of two Ca^{2+} ions from low-affinity sites with millimolar dissociation constants to high-affinity sites with micromolar dissociation constants when Ca_2·E~P·Mg reacts with ADP to give E·Ca_2·Mg and ATP corresponds to a factor of $\sim 10^6$ in stronger binding or a favorable change in Gibbs energy of approximately -8 kcal mol^{-1}.

The results require that the lumenal low-affinity sites are no longer available for binding calcium from the lumen after $^cE_{Ca_2}$ is phosphorylated by ATP to give Ca_2·E~P·Mg, because they are occupied by the two Ca^{2+} ions. If two Ca^{2+} ions could bind to low-affinity lumenal sites in both E·Mg and Ca_2·E~P·Mg, then a total of four Ca^{2+} ions could bind to the phosphoenzyme at high concentrations of lumenal calcium. This would convert all of the enzyme to a phosphoenzyme species Ca_4·E~P·Mg, which is not observed. We conclude that the two Ca^{2+} ions are transported from the high-affinity sites to the low-affinity sites upon phosphorylation, as shown in Figure 1B.

Mészáros and Bak (1992) have recently proposed that two additional Ca^{2+} ions can bind to Ca_2·E~P·Mg from the cytoplasm to form a species containing four calcium ions bound to the phosphoenzyme, because there is no detectable lag phase for the uptake of Ca^{2+} after the addition of ATP to $^cE_{Ca_2}$. However, E·Ca_2·Mg is phosphorylated by ATP with a rate constant of 220 s^{-1} (Petithory & Jencks, 1986), calcium is internalized from Ca_2·E~P·Mg with a rate constant of ~ 30 s^{-1}, and the phosphoenzyme is hydrolyzed with a rate constant of ~ 115 s^{-1} (Hanel & Jencks, 1991). If calcium can bind to the enzyme after calcium dissociates into the lumen from Ca_2·E~P·Mg, the lag phase would have a half-time of ~ 5 ms. A lag phase of this duration might not have been detected under the conditions of their experiments.

We were surprised to find Hill slopes of 1.0 ± 0.1 for the binding of Ca^{2+} to the phosphoenzyme to form Ca_2·E~P·Mg, because it is well established that two Ca^{2+} ions bind to the enzyme and are transported (Hasselbach, 1978). The probable explanation for this result is that the two Ca^{2+} ions bind to the phosphoenzyme with no cooperativity or slight negative cooperativity. Hill slopes of 1.0 ± 0.3 have been reported previously for inhibition by Ca^{2+} of steady-state turnover of the enzyme in leaky vesicles (Khananshvili et al., 1990; Hanel & Jencks, 1991). This inhibition has been attributed to the binding of lumenal calcium to the phosphoenzyme that regenerates Ca_2·E~P·Mg, which undergoes hydrolysis very slowly; dissociation of Ca^{2+} from this species is largely rate-limiting for turnover (Hasselbach, 1978; Yamada et al., 1972). A Hill slope of 1.0–1.3 has also been observed for the binding of strontium to the high-affinity sites on the free enzyme (Fujimori & Jencks, 1992).

Prager et al. (1979) have reported a larger Hill slope of 1.72 for phosphoenzyme formation from Ca^{2+}-loaded vesicles. However, this slope was obtained from concentrations of lumenal calcium that were calculated by a complex procedure that takes account of the binding of calcium to calcium-binding proteins inside the vesicle. We have calculated the Hill slopes directly from the known concentrations of calcium in the solutions in which the vesicles were equilibrated before phosphorylation, because the activities of Ca^{2+} are the same on the two sides of the membrane at equilibrium. We have recalculated the results of Prager et al. in the same way, from the data reported in Figure 2 of Prager et al. (1979), and found that the dependence of phosphoenzyme concentration on the concentration of calcium in the medium in which the vesicles were incubated follows a Hill slope of 1.1 ± 0.1, which is in agreement with our results.

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