

Sequential Dissociation of Ca^{2+} from the Calcium Adenosinetriphosphatase of Sarcoplasmic Reticulum and the Calcium Requirement for Its Phosphorylation by ATP^{\dagger}

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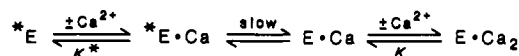
Received January 6, 1988; Revised Manuscript Received March 22, 1988

ABSTRACT: The kinetics for dissociation of the stable enzyme-calcium complex of the sarcoplasmic reticulum calcium ATPase, $\text{E}\cdot\text{Ca}_2$, were followed by assay with simultaneous addition of $[\text{}^{32}\text{P}]\text{ATP}$ and EGTA, which gives 70% phosphorylation of $\text{E}\cdot\text{Ca}_2$ with $k = 300 \text{ s}^{-1}$ (25 °C, pH 7.0, 5 mM MgSO_4 , 0.1 M KCl). The binding of ATP to $\text{E}\cdot\text{Ca}_2$ is described by $k_{\text{ATP}} = 1.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-\text{ATP}} = 120 \text{ s}^{-1}$, and $K_{\text{diss}} = 12 \text{ }\mu\text{M}$; ATP binding is partially rate limiting for phosphorylation at $<100 \text{ }\mu\text{M}$ ATP. The sequential dissociation of Ca^{2+} from $\text{E}\cdot\text{Ca}_2$ is described by $k_{-2} = 55\text{--}60 \text{ s}^{-1}$ for the first, "outer" Ca^{2+} , $k_{-1} = 25\text{--}30 \text{ s}^{-1}$ for the second, "inner" Ca^{2+} , and $K_{0.5} = 3.4 \text{ }\mu\text{M}$, $n = 1.9$ (from $K_{\text{diss}} = 7.4 \times 10^{-7} \text{ M}$ for $\text{Ca}\cdot\text{EGTA}$). Dissociation of the inner Ca^{2+} is inhibited by external Ca^{2+} , with $K_{0.5} = k_{-1}/k_2 = 0.7 \text{ }\mu\text{M}$. This confirms the conclusion that dissociation of the two Ca^{2+} ions is sequential. The ability of $\text{E}\cdot\text{Ca}_2$ to catalyze phosphorylation by ATP disappears in the presence of EGTA with $k = 50\text{--}55 \text{ s}^{-1}$, the same as k_{-2} for dissociation of the outer Ca^{2+} ion. This result, and the absence of the induction period that would occur if both $\text{E}\cdot\text{Ca}_2$ and $\text{E}\cdot\text{Ca}_1$ were catalytically competent, shows that both Ca^{2+} ions are required for phosphorylation. This conclusion is confirmed by the stoichiometry of $1.4/0.7 = 2.0$ for the ratio of Ca^{2+} internalized to phosphoenzyme formed after simultaneous addition of ATP and EGTA. Phosphorylation of $\text{E}\cdot\text{Ca}_2$ in the presence of ^{45}Ca gives 0.15, not 0.3, ^{45}Ca internalized, which corresponds to exchange of only 1 Ca^{2+} and is in agreement with this conclusion. The requirements for binding of two Ca^{2+} for catalytic specificity toward ATP and loss of two Ca^{2+} from $\text{E}\sim\text{P}\cdot\text{Ca}_2$ for specificity toward water account for the stoichiometry of Ca^{2+} transport and provide a possible reason for the two steps in the phosphorylation of $\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$.

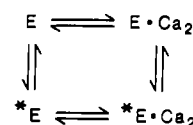
The binding and dissociation of calcium ions at the high-affinity exterior-facing sites of the calcium ATPase of sarcoplasmic reticulum vesicles cause a dramatic change in the chemical reactivity of the enzyme. In the absence of bound calcium, the enzyme reacts with inorganic phosphate (P_i),¹ but not with ATP, to form a covalent phosphoenzyme, $\text{E}\sim\text{P}$, that hydrolyzes readily. When calcium is bound, the enzyme reacts with ATP, but not with P_i , to form a covalent phosphoenzyme, $\text{E}\sim\text{P}\cdot\text{Ca}_2$, that can transfer its phosphate back to ADP but cannot hydrolyze. Covalent phosphoenzyme can only hydrolyze after the bound calcium has dissociated into the lumen of the vesicle to give $\text{E}\sim\text{P}$. Thus, bound calcium acts as a chemical "switch" that determines whether the enzyme can react reversibly with ATP and ADP, or with P_i and H_2O (de Meis & Vianna, 1979; Pickart & Jencks, 1984; Martonosi & Beeler, 1985).

Many efforts have been made to characterize the calcium-induced events that bring about these changes in chemical reactivity. Calcium-dependent conformational changes have been detected by changes in the intrinsic fluorescence of tryptophan residues in the protein (Dupont, 1976; Dupont & Leigh, 1978; Guillain et al., 1980, 1981; Champeil et al., 1983; Fernandez-Belda et al., 1984), changes in the ESR spectra of spin-labeled SRV (Ikemoto et al., 1978; Coan & Inesi, 1977; Coan et al., 1979; Inesi et al., 1980), changes in the susceptibility of the enzyme to sulfhydryl-modifying reagents (Ikemoto et al., 1978; Murphy, 1978; Kawakita et al., 1985), and changes in proteolytic digestion patterns in the presence and absence of calcium (Anderson & Jorgensen, 1985; Kawakita

Scheme I



Scheme II



et al., 1985). Although these physical methods do not necessarily monitor the actual changes in enzyme reactivity, Guillain et al. (1981) have shown that the same rate constant of $\sim 1.5 \text{ s}^{-1}$ is obtained for a fluorescence change after Ca^{2+} addition and the appearance of reactivity toward ATP at pH 6, and Dupont (1982, 1984) has shown identical rate constants for Ca^{2+} binding and a change in fluorescence. These workers also made the important observation that the rate of Ca^{2+} dissociation, as measured by a change of fluorescence, is inhibited by low concentrations of Ca^{2+} (Guillain et al., 1980, 1981; Dupont, 1982). Sumida et al. (1978) found that reactivity toward ATP disappears in a first-order reaction with a rate constant of 15 s^{-1} when enzyme-calcium was mixed with EGTA at pH 6.8, 20 °C, and Inesi (1987) has shown directly that the overall dissociation of two Ca^{2+} ions at pH 7 can be described approximately by a rate constant of 15 s^{-1} .

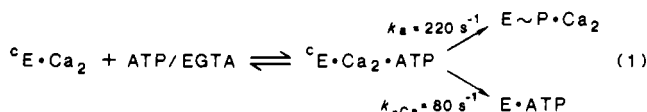
Mechanisms that have been proposed for calcium binding and dissociation include those shown in Schemes I and II

[†] Publication No. 1670. This research was supported in part by grants from the National Institutes of Health (GM20888) and the National Science Foundation (PCM-8117816).

¹ Abbreviations: SRV, sarcoplasmic reticulum vesicle(s); EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; P_i , inorganic phosphate.

(Dupont & Leigh, 1978; Sumida et al., 1978). Both mechanisms invoke two conformations of the enzyme, as in the classical E_1 - E_2 (or E - E^*) model (de Meis & Vianna, 1979), which can account for the inhibition of calcium dissociation at low $[Ca^{2+}]$ by postulating different high- and low-affinity states of the enzyme that can undergo slow interconversion.

We describe here a straightforward kinetic method to assay the changes in the chemical reactivity of the enzyme upon addition or removal of bound calcium. We have used this assay to characterize the dissociation of calcium from $^oE\cdot Ca_2$, which is defined as the stable form of the enzyme-calcium complex, and the change in chemical specificity for phosphorylation by ATP that accompanies calcium dissociation. The assay involves measurement of the amount of phosphoenzyme that is formed upon simultaneous addition of $[^{32}P]ATP$ and EGTA to the enzyme. Under these conditions, $^oE\cdot Ca_2$ undergoes phosphorylation at 220 s^{-1} and dissociation of Ca^{2+} at 80 s^{-1} , as shown in eq 1, to give a total rate constant of 300



s^{-1} for the disappearance of $^oE\cdot Ca_2$. This reaction yields 70% phosphorylation of the enzyme. Further phosphorylation is stopped by the binding of free Ca^{2+} to EGTA, the Ca^{2+} in $E\sim P\cdot Ca_2$ is rapidly occluded and subsequently internalized, and the $E\sim P\cdot Ca_2$ product is resistant toward hydrolysis if the vesicles are loaded with Ca^{2+} . The rate constant of $k_a = 220\text{ s}^{-1}$ represents a conformational change that converts the enzyme to a catalytically active form, $^oE\cdot Ca_2\cdot ATP$, which undergoes very fast phosphorylation (Petithory & Jencks, 1986).

The results reported here demonstrate directly that (a) dissociation of calcium ions from the exterior-facing transport sites follows an ordered process and (b) dissociation of a single calcium ion from the ATP-reactive form, $^oE\cdot Ca_2$, is sufficient to render the enzyme unreactive toward ATP. This finding is consistent with the fact that the stoichiometry of calcium ions transported per ATP molecule hydrolyzed is strictly maintained at 2:1 under the conditions used for these experiments. We speculate that dissociation of the single calcium ion is either accompanied by a conformational change that renders the enzyme unreactive toward ATP or else prevents a subsequent conformational change that is essential for the phosphorylation reaction from occurring. After this work was completed, G. Inesi kindly informed us of his findings that the overall dissociation of Ca^{2+} ions from $^oE\cdot Ca_2$ in the presence of EGTA proceeds with a rate constant of $\sim 15\text{ s}^{-1}$ and that the inner Ca^{2+} ion of $^oE\cdot Ca_2$ is the first ion to be internalized into intact SRV, which provides further evidence for a sequential mechanism of Ca binding and internalization (Inesi, 1987).

MATERIALS AND METHODS

Materials

Reagents were generally of the highest available purity and were used without further purification. Disodium ATP ("Sonderqualitat") and ADP were from Boehringer Mannheim. $[\gamma\text{-}^{32}P]ATP$ (>99% pure), $^{45}CaCl_2$, and Aquasol-2 scintillation fluid were from New England Nuclear. Ionophore A23187 was from Calbiochem.

Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the MacLennan (1970) procedure, as described previously (Khananshvilis &

Jencks, 1988). The preparations hydrolyzed ATP at $3.5\text{--}5.0\text{ }\mu\text{mol (mg of total protein)}^{-1}\text{ min}^{-1}$ when the vesicles were made permeable with the calcium ionophore A23187 ($2\text{ }\mu\text{L}$ of $1\text{--}2\text{ mM}$ in ethanol for 2 mL). SRV as isolated were $\sim 98\%$ sealed, as shown by an increase of ~ 50 -fold in the steady-state ATP hydrolysis rate upon addition of ionophore in the standard assay. The amount of phosphoenzyme observed with intact vesicles at saturating $[Ca^{2+}]$ and $[ATP]$ was $2.0\text{--}3.5\text{ nmol/mg}$ of total protein.

Calcium ATPase activity was measured spectrophotometrically by coupling ADP production to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard assay conditions were 40 mM MOPS, 100 mM KCl, 5.0 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.40 mM EGTA ($23\text{ }\mu\text{M}$ free Ca^{2+}), and 1.5 mM ATP, pH 7.0, 25°C .

Concentrations of free Ca^{2+} were calculated from a dissociation constant of $7.4 \times 10^{-7}\text{ M}$ for $Ca\cdot EGTA$ (Godt, 1974). This value was determined under conditions (pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, and 5 mM ATP) similar to those used in our experiments. Protein concentrations were estimated by the procedure of Lowry (Lowry et al., 1951) using bovine serum albumin as a standard.

Rapid Mix-Quench Experiments. The formation or decay of covalent phosphoenzyme was followed by using a rapid mix-quench apparatus that can be used with either three or four syringes of 1-mL volume, as described previously (Stahl & Jencks, 1984). The temperature-equilibrated contents of syringes A and B were driven into a mixing block containing a Durrum jet mixer, and, after flowing through a length of narrow-bore Teflon tubing for an amount of time t_1 , the reaction was quenched in a second mixing block by hydrochloric acid (at a final concentration of 0.5 M) from syringe C. For four-syringe experiments, another reactant was placed in syringe C, and quenching by acid from syringe D took place in an additional mixing block after time t_2 . The reaction times were calibrated from measurements of 2,4-dinitrophenyl acetate hydrolysis by hydroxide ion (Barman & Gutfreund, 1964).

For the measurement of time courses $>300\text{ ms}$, a pulsed-mode feature of the rapid mix-quench apparatus was used. The contents of syringes A and B were mixed and allowed to age in a length of temperature-equilibrated Teflon tubing for a set delay time, after which the reactants were pushed out by buffer and mixed with the appropriate quench solution in a second mixing block.

Passively Loaded Vesicles. SRV were passively loaded with high $[Ca^{2+}]$ in some experiments in order to inhibit phosphoenzyme hydrolysis. SRV were incubated for $12\text{--}16\text{ h}$ at 4°C in a solution containing $\sim 30\text{ mg/mL}$ SRV protein, 40 mM MOPS, pH 7.0, 100 mM KCl, 5.0 mM $MgSO_4$, 0.2 M sucrose, and 20 mM $CaCl_2$. For each reaction, $10\text{ }\mu\text{L}$ of this stock SRV solution was mixed with 0.99 mL of a solution containing appropriate concentrations of calcium or EGTA. This solution was then loaded into syringe A of the rapid mixing apparatus, and the reaction was started within $15\text{--}30\text{ s}$.

Determination of $[^{32}P]EP$. The amount of $[^{32}P]EP$ in the quenched reaction mixtures was determined essentially as described by Verjovski-Almeida et al. (1978). The solution used to quench the phosphorylation reactions contained 1.5 M HCl and 40 mM KH_2PO_4 for three-syringe experiments or 2 M HCl plus 60 mM KH_2PO_4 for four-syringe experiments. Bovine serum albumin was added to the quenched reaction mixtures to give a final concentration of total protein of $0.25\text{--}0.30\text{ mg/mL}$. Cold 6 M trichloroacetic acid was then

added to give a final concentration of $\sim 12\%$. The tubes were kept on ice for ≤ 1 h and were centrifuged at ~ 2000 rpm for 15 min. The supernatant was decanted, and the pellets were resuspended, filtered over glass fiber filters (Whatman GF/C), and washed with 3×5 mL of an ice-cold solution of 5% trichloroacetic acid and 10 mM KH_2PO_4 . The filters were then counted in vials containing ~ 7 mL of Aquasol-2.

$[\text{E}]_{\text{tot}}$ was taken as the amount of covalent phosphoenzyme formed after a 5-s reaction of intact vesicles with saturating amounts of $[\text{P}^{32}\text{P}]\text{ATP}$ and Ca^{2+} , quenched manually with acid.

Determination of ^{45}Ca Accumulation in Vesicles. Uptake of ^{45}Ca into tightly sealed SRV using the rapid mix-quench apparatus was initiated by the addition of a saturating amount of ATP plus excess EGTA to ^{45}Ca -bound enzyme. The solution in the collection tube was filtered within <5 s over nitrocellulose filters (Millipore HAW/P 0.45 μm) previously soaked in chilled 10 mM CaCl_2 , 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 . The filter and collection tube were washed with $3 \times \sim 5$ mL of the same chilled solution. The filters were dried briefly by an aspirator vacuum and counted.

Occasionally, when buffered ^{45}Ca solutions were employed that contained a large total amount of ^{45}Ca , 1 mL of a solution containing 50 mM CaCl_2 , 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 was placed in the collection tube prior to initiating the reaction, in order to minimize ^{45}Ca uptake in the collection tube prior to filtration. The final concentration of unlabeled Ca^{2+} after the reactants emptied into the collection tube was ~ 10 mM for a four-syringe reaction. The high unlabeled $[\text{Ca}^{2+}]$ served both to decrease the specific activity of the ^{45}Ca in the collection tube and to inhibit the ATPase reaction after the first turnover by forming $\text{Ca}\cdot\text{ATP}$, which reacts with the ATPase substantially slower than $\text{Mg}\cdot\text{ATP}$ (Shigekawa et al., 1983).

Computer Programs. Rate constants were estimated by using a program written for IBM-compatible microcomputers that allows fitting of experimental data by a nonlinear least-squares procedure, as described previously (Stahl & Jencks, 1987).

RESULTS

Assay of $^{\circ}\text{E}\cdot\text{Ca}_2$. The formation of the ATP-reactive species $^{\circ}\text{E}\cdot\text{Ca}_2$ was assayed by addition of $[\text{P}^{32}\text{P}]\text{ATP}$ and EGTA to the enzyme followed in ~ 30 ms by an acid quench and assay of the amount of $\text{E}\sim\text{P}\cdot\text{Ca}_2$ formed, as described in the introduction (eq 1). This reaction was characterized further by measurement of its dependence on ATP concentration and the accompanying incorporation of $^{45}\text{Ca}^{2+}$ into intact vesicles.

When vesicles preincubated with ^{45}Ca ($^{\circ}\text{E}\cdot\text{Ca}_2^*$) are mixed with an equal volume of 600 μM ATP plus 10 mM EGTA, a burst of ^{45}Ca uptake equal to 1.4 mol/mol of enzyme is observed (Figure 1, closed triangles). Reaction with ATP in the absence of EGTA gives a rapid incorporation of 2.0 mol of ^{45}Ca , followed by a slow further uptake from subsequent turnover of the enzyme (closed circles, Figure 1); phosphorylation with $[\text{P}^{32}\text{P}]\text{ATP}$ under the same conditions gives 1.0 mol of phosphoenzyme (open circles, Figure 1).

These are the results expected for the reaction of eq 1, with 70% phosphorylation and a stoichiometry of 2.0 Ca^{2+} ions occluded in each mole of phosphoenzyme, $\text{E}\sim\text{P}\cdot\text{Ca}_2$ (eq 1). In particular, they show no detectable phosphorylation of enzyme from which a single calcium ion has been removed in the presence of EGTA (see below); i.e., $<5\%$ of the internalized ^{45}Ca is from reaction of ATP with the species $\text{E}\cdot\text{Ca}_1\cdot\text{ATP}$. The internalization of Ca^{2+} involves at least two steps: first occlusion of the two Ca^{2+} ions, so that they can

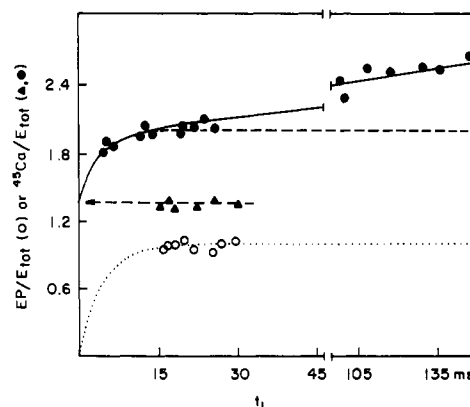


FIGURE 1: Reaction of $^{\circ}\text{E}\cdot\text{Ca}_2$ with ATP \bullet EGTA, using ^{45}Ca (\bullet , \blacktriangle) or $[\text{P}^{32}\text{P}]\text{ATP}$ (\circ). Intact vesicles were preincubated with ^{45}Ca (\bullet , \blacktriangle) or unlabeled Ca^{2+} (\circ) and reacted for varying times t_1 with either 0.3 mM $[\text{P}^{32}\text{P}]\text{ATP}$ (\circ), 0.3 mM ATP (\bullet), or 0.3 mM ATP plus 5 mM EGTA (\blacktriangle). Syringes A and B, and syringe C except for (\circ), contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 , 25 $^{\circ}\text{C}$. In addition, syringe A contained 0.20 mg/mL SRV and 50 μM $^{45}\text{CaCl}_2$ (\bullet , \blacktriangle) or 50 μM unlabeled CaCl_2 (\circ). Syringe B contained either 0.6 mM ATP and 50 μM $^{45}\text{CaCl}_2$ (\bullet), 0.6 mM ATP and 10 mM EGTA (\blacktriangle), or 0.6 mM $[\text{P}^{32}\text{P}]\text{ATP}$ and 50 μM unlabeled CaCl_2 (\circ). Syringe C contained either 15 mM EGTA (\bullet), 5 mM EGTA (\blacktriangle), or 1.5 M HCl and 40 mM KH_2PO_4 (\circ). The upper dashed line is drawn for a first-order rate constant of 220 s^{-1} for $[\text{Ca}^{2+}]/[\text{E}]_{\text{tot}} = 2.0$ (\bullet), and the center dashed line is drawn for $[\text{Ca}^{2+}]/[\text{E}]_{\text{tot}} = 1.4$ (\blacktriangle). The solid line is drawn for ^{45}Ca uptake due to turnover occurring at 3 s^{-1} in addition to ^{45}Ca uptake resulting from phosphorylation of $^{\circ}\text{E}\cdot\text{Ca}_2^*\cdot\text{ATP}$ at 220 s^{-1} . The dotted line is drawn for a first-order rate constant of 220 s^{-1} for $[\text{P}^{32}\text{P}]\text{EP}/[\text{E}]_{\text{tot}} = 1.0$ (\circ).

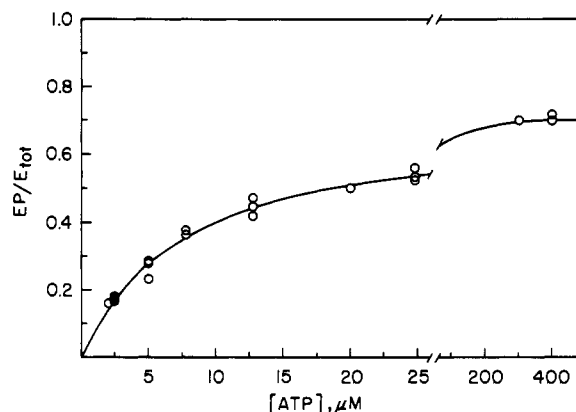
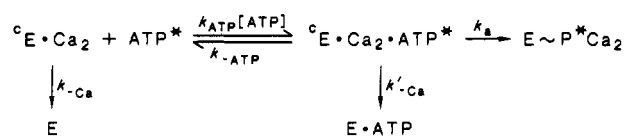


FIGURE 2: Dependence on $[\text{ATP}]$ of $[\text{EP}]/[\text{E}]_{\text{tot}}$ in the reaction of $^{\circ}\text{E}\cdot\text{Ca}_2$ with ATP plus EGTA. Final conditions were 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 , 25 $^{\circ}\text{C}$. Syringe A contained ~ 0.3 mg/mL passively loaded SRV, 0.40 mM CaCl_2 , and 0.39 mM EGTA (22 μM free Ca^{2+}). Syringe B contained 10 mM EGTA plus $[\text{P}^{32}\text{P}]\text{ATP}$ at twice the final concentration. Syringe C contained 1.5 M HCl and 40 mM KH_2PO_4 . The reactions were quenched after 25–35 ms. The line is drawn for half-maximal phosphorylation at 7.6 μM ATP, with $[\text{EP}]_{\text{max}} = 70\%$ of $[\text{E}]_{\text{tot}}$.

no longer dissociate to the outside of the vesicle, and then internalization at a slower rate. It is well established that phosphorylation is accompanied by rapid occlusion of the two Ca^{2+} ions (Kurzmack et al., 1977; Sumida et al., 1978; Dupont, 1980; Ikemoto et al., 1981; Takisawa & Makinose, 1981, 1983; Fernandez-Belda et al., 1984).

The reaction of $^{\circ}\text{E}\cdot\text{Ca}_2$ with varying concentrations of $[\text{P}^{32}\text{P}]\text{ATP}$ plus 5 mM EGTA for $t_1 \sim 30$ ms, followed by an acid quench, results in a hyperbolic dependence of $[\text{EP}]/[\text{E}]_{\text{tot}}$ on $[\text{ATP}]$ that levels off at 0.70 $[\text{EP}]/[\text{E}]_{\text{tot}}$ (Figure 2). These experiments were carried out with vesicles that were passively loaded with 20 mM Ca^{2+} in order to prevent hydrolysis of phosphoenzyme. A plot of $[\text{E}]_{\text{tot}}/[\text{E}\sim\text{P}]$ against

Scheme III



$1/[\text{ATP}]$ gave a value of $K_{0.5} = 7.6 \mu\text{M}$ that provides a satisfactory fit to the data, as shown by the calculated solid line in Figure 2. $K_{0.5}$ is the concentration of ATP at which k_f , the net rate constant (Cleland, 1975) for the phosphorylation reaction in the presence of EGTA at varying $[\text{ATP}]$, equals $k_{-\text{Ca}}$, the first-order rate constant for irreversible dissociation of calcium from ${}^o\text{E} \cdot \text{Ca}_2$ (Scheme III).

$$k_f = \frac{k_{\text{ATP}}[\text{ATP}](k_a + k'_{-\text{Ca}})}{k_{-\text{ATP}} + k_a + k'_{-\text{Ca}}} = k_{-\text{Ca}} \quad (2)$$

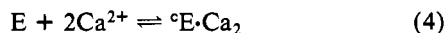
Rearranging gives

$$k_{\text{ATP}} = \frac{k_{-\text{Ca}}(k_a + k'_{-\text{Ca}} + k_{-\text{ATP}})}{(k_a + k'_{-\text{Ca}})K_{0.5}} \quad (3)$$

A value of $k_{\text{ATP}} \approx (1.0 \pm 0.2) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for the binding of ATP to ${}^o\text{E} \cdot \text{Ca}_2$ was estimated from eq 3 and the rate constants for the steps of Scheme I. This estimate is not exact because there is some accumulation of the intermediate ${}^o\text{E} \cdot \text{Ca}_2 \cdot \text{ATP}$ at high ATP concentrations, so that the system is not strictly at steady state; however, the uncertainty is probably within the experimental error of the measurements. A very similar rate constant of $0.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for ATP binding has been found to be consistent with the time course of enzyme phosphorylation by ATP at pH 6.8 (Sumida et al., 1980), but a larger rate constant of $6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ was obtained from a simulation for fast kinetics (Fernandez-Belda et al., 1984). Rate constants for ATP dissociation from ${}^o\text{E} \cdot \text{Ca}_2 \cdot \text{ATP}$ of $k_{-\text{ATP}} = 100\text{--}140 \text{ s}^{-1}$ and for Ca^{2+} dissociation of $k'_{-\text{Ca}} = 80 \text{ s}^{-1}$ have been reported previously (Petithory & Jencks, 1986), and a value of $k_{-\text{Ca}} = 55\text{--}60 \text{ s}^{-1}$ was measured in this work (see below). A dissociation constant for ATP from ${}^o\text{E} \cdot \text{Ca}_2 \cdot \text{ATP}$ of $K_s = 12 \pm 3 \mu\text{M}$ was calculated from the ratio of the rate constants for dissociation and binding, $k_{-\text{ATP}}/k_{\text{ATP}}$. This agrees with a previously reported value of $K_s = 15 \mu\text{M}$ that was determined at equilibrium (Pickart & Jencks, 1984).

The time course for phosphorylation of ${}^o\text{E} \cdot \text{Ca}_2$ at different ATP concentrations is shown in Figure 3. The reactions are consistent with first-order kinetics in the presence of 400 and $5 \mu\text{M}$ ATP. At these concentrations, the reactions represent predominantly rate-limiting phosphorylation, which is controlled by a conformational change (Petithory & Jencks, 1986), and ATP binding, respectively. At intermediate ATP concentrations of 20 and $60 \mu\text{M}$, both steps are kinetically significant, and the reactions are biphasic. All of the experiments described below were carried out at concentrations of ATP that were sufficiently high that the binding step was not kinetically significant.

Equilibrium Binding of Ca^{2+} to E. Figure 4 shows the use of this assay to measure the overall equilibrium constant for the binding of calcium to the free enzyme to give ${}^o\text{E} \cdot \text{Ca}_2$ (eq 4). The overall reaction involves several steps and may include



a conformational change, but E and ${}^o\text{E} \cdot \text{Ca}_2$ undergo reactions with first-order kinetics and appear to be homogeneous species so that the overall reaction can be described by eq 4. The binding shows a sigmoidal dependence on calcium concentration and a Hill slope of $n = 1.9$ (Figure 4, inset), which is

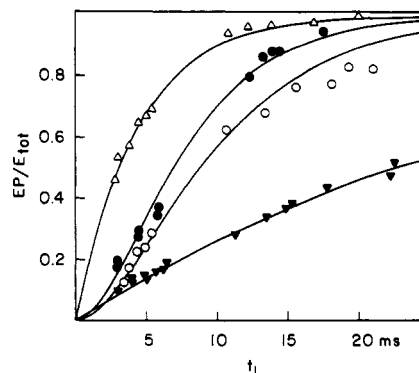


FIGURE 3: Reaction of ${}^o\text{E} \cdot \text{Ca}_2$ with varying $[\text{ATP}]$. The reactions were performed essentially as described for the open symbols in Figure 1. Final conditions were 40 mM MOPS, pH 7.0, 100 mM KCl, 5 mM MgSO_4 , 1.96 mM CaCl_2 , 2.00 mM EGTA (23 μM free Ca^{2+}), and 400 (Δ), 60 (\bullet), 20 (\circ), and 5 μM (∇) $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 25 $^\circ\text{C}$. Syringe A contained $\sim 0.3 \text{ mg/mL}$ SRV that were passively loaded with 20 mM Ca^{2+} . Syringe B contained $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at twice the final concentration. Syringe C contained 1.5 M HCl and 40 mM KH_2PO_4 . All other components were present in syringes A and B at their final concentrations. The rate constants for the first-order reactions are 220 s^{-1} (Δ) and 30 s^{-1} (∇).

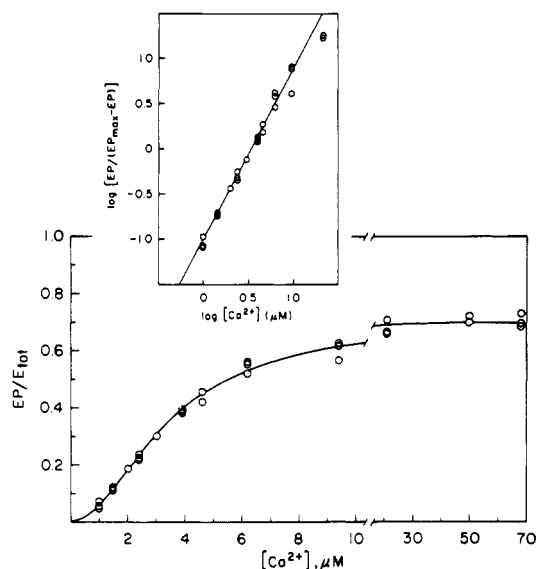


FIGURE 4: Dependence on $[\text{Ca}^{2+}]$ of the activation of ATP reactivity. Passively loaded vesicles were preincubated in Ca-EGTA buffers to give the indicated free $[\text{Ca}^{2+}]$ and reacted with 0.3 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus 5 mM EGTA for $t_1 = 25\text{--}35 \text{ ms}$ followed by acid quench. Syringes A and B contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 , at 25 $^\circ\text{C}$. In addition, syringe A contained 0.25 mg/mL passively loaded SRV, 1.72 mM EGTA, and either 1.0 (1.0 μM), 1.15 (1.5 μM), 1.32 (2.4 μM), 1.45 (3.9 μM), 1.54 (6.2 μM), 1.60 (9.4 μM), 1.68 (20.7 μM), or 1.77 mM (68 μM) CaCl_2 , to give the final free $[\text{Ca}^{2+}]$ indicated in parentheses. Alternatively, syringe A contained 0.25–0.3 mg/mL passively loaded SRV and 4.86 mM EGTA plus either 3.54 (2.0 μM) or 4.19 mM (4.6 μM) CaCl_2 , or 6.32 mM EGTA plus 5.14 mM CaCl_2 (3.2 μM), or 0.89 mM EGTA plus 0.93 mM CaCl_2 (53 μM free Ca^{2+}). Syringe B in all experiments contained 0.6 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ plus 10 mM EGTA. Syringe C contained 1.5 M HCl and 40 mM KH_2PO_4 . The final free calcium concentration after mixing the contents of syringes A and B was between 0.07 and 0.12 μM . The line is drawn for $K_{0.5} = K_{\text{ov}}^{1/2} = 3.4 \mu\text{M}$ and a Hill coefficient of 1.90, obtained from a Hill plot of the data (inset).

consistent with a high degree of cooperativity. The value of $K_{0.5} = K_{\text{ov}}^{1/2} = 3.4 \mu\text{M}$ corresponds to $K_{\text{ov}} = 1.2 \times 10^{-11} \text{ M}^2$, based on free calcium concentrations in EGTA buffers that were calculated from $K_{\text{diss}} = 7.4 \times 10^{-7} \text{ M}$ for the Ca-EGTA complex under the conditions of these experiments (Godt, 1974). This result is close to the value of $K_{0.5} = 3.5 \mu\text{M}$ that

Table I: Reported Values of the Apparent Dissociation Constant, $K_{0.5}$, for Exterior Ca^{2+} from the ATPase of SRV in the Absence of ATP

$K_{0.5}$ (μM)	n_H	pH	T ($^{\circ}\text{C}$)	method
0.5 ^a		7.0	20	fast filtration ^d
0.41	1.8–1.9	6.8	24	ESR spectrum ^e
0.43	1.9	6.8	24	column chromatography ^e
0.63 ^b		7.0	20	fluorescence ^f
1.3	1.6	7.2	–10	fast filtration ^g
1.3	1.6	7.2	20	fluorescence ^h
1.9		6.8	25	fluorescence ^h
2.4 ^c	1.7	7.0	25	equilibrium with [E–P] in reaction with P_i ⁱ
3.5 ^c	1.6	7.0	25	steady-state AcP hydrolysis ^j
3.4 ^c	1.9	7.0	25	this work

^a $K_{\text{diss}}(\text{Ca}\cdot\text{EGTA}) = 2.2 \times 10^{-7}$ M. ^b $K_{\text{diss}} = 4 \times 10^{-7}$ M. ^c $K_{\text{diss}} = 7.4 \times 10^{-7}$ M. ^d Dupont (1980). ^e Inesi et al. (1980). ^f Guillain et al. (1980). ^g Dupont (1982). ^h Fernandez-Belda et al. (1984). ⁱ Pickart & Jencks (1984). ^j Bodley & Jencks (1987).

was obtained from the dependence of the rate of acetyl phosphate hydrolysis on calcium concentration under V_m/K_m conditions (Bodley & Jencks, 1987). The binding of calcium and the formation of $^{\circ}\text{E}\cdot\text{Ca}_2$ are at equilibrium for this relatively slow reaction under these conditions; the much faster reaction with ATP occurs through several different pathways (Stahl & Jencks, 1987) so that the dependence of the rate of ATP hydrolysis on calcium concentration reflects kinetic as well as equilibrium constants for these pathways. Some other reported values of $K_{0.5}$ for calcium, determined under similar conditions by a number of methods, are summarized in Table I. The wide range of reported values reflects, in part, the choice of different values of K_{diss} for the calcium–EGTA complex. The value of $K_{\text{diss}} = 4 \times 10^{-7}$ M used by Dupont and Champeil (Champeil et al., 1983; Guillain et al., 1980; Dupont, 1982) gives $K_{0.5} = 1.8 \mu\text{M}$ and $n = 1.9$ for our measurements.

Exchange of Enzyme-Bound Ca^{2+} for ^{45}Ca in Solution. (A) Exchange of the First Calcium Ion. Exchange of enzyme-bound Ca^{2+} with ^{45}Ca in the external medium was monitored by mixing enzyme (empty vesicles) preincubated with $30 \mu\text{M}$ unbuffered Ca^{2+} with an equal volume of $30 \mu\text{M}$ ^{45}Ca , followed after varying times (t_1) with a second mixing to give $300 \mu\text{M}$ ATP and 5 mM EGTA (Figure 5, open circles). This exchange was found to be biphasic. The amount of ^{45}Ca taken up into the vesicles due to $\text{E}\sim\text{P}\cdot\text{Ca}_2$ formation as a result of the exchange of $^{\circ}\text{E}\cdot\text{Ca}_2$ with ^{45}Ca during t_1 (circles) follows a first-order time course with a rate constant of $55\text{--}60 \text{ s}^{-1}$. The amount of ^{45}Ca taken up levels off at an amount equal to half of the total amount of ^{45}Ca that was taken up when ^{45}Ca was present initially, in both syringes A and B at the final specific activity (Figure 5, triangles). When t_1 for the exchange reaction (circles) was extended to $\sim 30 \text{ s}$ and the reaction was stopped by the manual addition of ATP plus EGTA, essentially all of the enzyme-bound ^{40}Ca was exchanged for ^{45}Ca (upper circles, Figure 5).

The data shown by the circles in Figure 5 are therefore consistent with the exchange of a single enzyme-bound Ca^{2+} for ^{45}Ca in the solution with a rate constant of $55\text{--}60 \text{ s}^{-1}$ resulting in $^{\circ}\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$, 70% of which becomes $\text{E}\sim\text{P}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ upon addition of ATP plus EGTA. Identical results were obtained with $60 \mu\text{M}$ unbuffered calcium throughout (closed symbols).

(B) Exchange of the Second Calcium Ion. Exchange of the second enzyme-bound Ca^{2+} ion with ^{45}Ca in solution occurs very slowly compared to exchange of the first bound Ca^{2+} ion. A rate constant of 0.6 s^{-1} was measured for the appearance of $^{\circ}\text{E}\cdot\text{Ca}_2^*$ from $^{\circ}\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ in the presence of $60 \mu\text{M}$ external

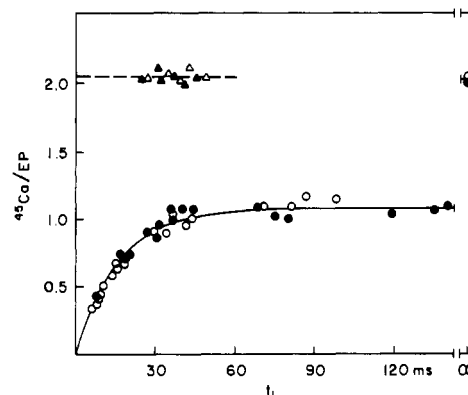


FIGURE 5: Exchange of enzyme-bound calcium for Ca^{2+} in solution. Intact vesicles preincubated with unlabeled calcium were mixed with a ^{45}Ca solution of the same free $[\text{Ca}^{2+}]$ for varying times t_1 , followed by addition of ATP plus EGTA. Syringe A contained 0.15 mg/mL SRV plus either 30 (\circ) or $60 \mu\text{M}$ (\bullet) unbuffered CaCl_2 or 30 (Δ) or $60 \mu\text{M}$ (\blacktriangle) $^{45}\text{CaCl}_2$ at the final specific activity. Syringe B contained either 30 (\circ , Δ) or $60 \mu\text{M}$ (\bullet , \blacktriangle) $^{45}\text{CaCl}_2$ at the final specific activity (Δ , \blacktriangle) or at twice the final specific activity (\circ , \bullet). Syringe C contained 0.9 mM ATP plus 15 mM EGTA. Syringe D contained 5 mM EGTA. All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 . For $t_1 = \infty$ (\circ , \bullet), the contents of syringes C and D were added manually 30 s after the contents of syringes A and B were mixed. The solid line (\circ , \bullet) is drawn for a first-order rate constant of 58 s^{-1} to $^{45}\text{Ca}/[\text{EP}] = 1.05$ ($[\text{EP}]/[\text{E}]_{\text{tot}} = 0.70$). The dashed line (Δ , \blacktriangle) at $^{45}\text{Ca}/[\text{EP}] = 2.04$ indicates the amount of ^{45}Ca uptake resulting from phosphorylation of $^{\circ}\text{E}\cdot\text{Ca}_2^*$ in the control reaction in which ^{45}Ca was present in both syringes A and B.

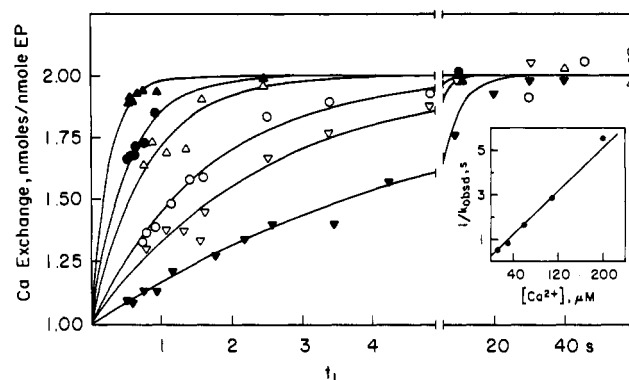
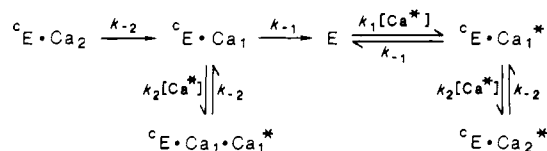


FIGURE 6: Exchange of the second calcium in $^{\circ}\text{E}\cdot\text{Ca}_2$ for calcium in solution. The rapid mix-quench apparatus was used in the pulsed mode. Two different experimental protocols were followed: Intact vesicles preincubated in varying unlabeled free $[\text{Ca}^{2+}]$ were mixed with ^{45}Ca solutions of the same free $[\text{Ca}^{2+}]$ for varying times followed by 0.3 mM ATP plus 5 mM EGTA (Δ , \circ , ∇); alternatively, intact vesicles preincubated with $15 \mu\text{M}$ $^{45}\text{CaCl}_2$ were mixed with unlabeled $\text{Ca}\cdot\text{EGTA}$ buffers of varying free $[\text{Ca}^{2+}]$ for t_1 , followed again by ATP plus EGTA (∇ , \blacktriangle , \bullet). Syringe A contained 0.17 mg/mL intact SRV and either 20 (Δ), 50 (\circ), or $100 \mu\text{M}$ (∇) added calcium to give approximately 30 , 60 , and $110 \mu\text{M}$ ($\pm 5 \mu\text{M}$) total free Ca^{2+} , or $15 \mu\text{M}$ $^{45}\text{CaCl}_2$ (\blacktriangle , \bullet , ∇). Syringe B contained either 20 (Δ), 50 (\circ), or $100 \mu\text{M}$ (∇) added $^{45}\text{CaCl}_2$ to give approximately 30 , 60 , and $110 \mu\text{M}$ ($\pm 5 \mu\text{M}$) total free ^{45}Ca , or 2.0 mM CaCl_2 plus either 2.25 (\blacktriangle), 2.10 (\bullet), or 1.60 (∇) mM EGTA to give final free $[\text{Ca}^{2+}]$ of 5.7 (\blacktriangle), 12.0 (\bullet), or $200 \mu\text{M}$ (∇). Syringe C contained 0.9 mM ATP plus 15 mM EGTA. All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 . The lines are drawn for first-order rate constants of 4 (\blacktriangle), 1.9 (\bullet), 1.2 (Δ), 0.6 (\circ), 0.4 (∇), and 0.18 s^{-1} (∇). Inset: Dependence on exterior $[\text{Ca}^{2+}]$ of the reciprocal of the observed rate constant for exchange of the inner ion of $^{\circ}\text{E}\cdot\text{Ca}_2$ with Ca^{2+} in the solution (data of Figure 6 at $>10 \mu\text{M}$ $[\text{Ca}^{2+}]$). The line is drawn for a slope of $2.5 \times 10^4 \text{ s M}^{-1}$.

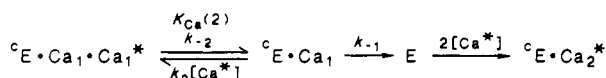
^{45}Ca , using the pulsed-mode feature of the rapid mix-quench device (Figure 6, open circles).

Two different protocols were used to follow the replacement of the slow-exchanging bound Ca^{2+} ion with Ca^{2+} from the

Scheme IV



Scheme V



external solution. In one method (Figure 6, open symbols), enzyme preincubated with varying concentrations of unlabeled Ca^{2+} (${}^c\text{E} \cdot \text{Ca}_2$) was mixed with an equal volume of the same concentration of ${}^{45}\text{Ca}$, to give ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ within $t_1 = 100$ ms (as shown in Figure 5) and ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1^*$ after much longer t_1 . ATP plus EGTA was added after t_1 , which phosphorylates 70% of all (labeled and unlabeled) ${}^c\text{E} \cdot \text{Ca}_2$. Alternatively, enzyme was preincubated with $\sim 20 \mu\text{M}$ ${}^{45}\text{Ca}$ to give ${}^c\text{E} \cdot \text{Ca}_2^*$, which was mixed with a solution containing unlabeled Ca^{2+} (Figure 6, closed symbols). Dissociation of bound ${}^{45}\text{Ca}$ from the enzyme was rendered irreversible by the large excess of unlabeled calcium in the medium, which was buffered with EGTA to give the desired free $[\text{Ca}^{2+}]$. ATP plus EGTA was added after t_1 , as in the first method. The second method follows the replacement of enzyme-bound ${}^{45}\text{Ca}$ with unlabeled Ca^{2+} (to give ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1$ and ${}^c\text{E} \cdot \text{Ca}_2$), which appears as a decrease in the amount of bound ${}^{45}\text{Ca}$ with increasing t_1 . To avoid confusion, these experiments are both described in terms of the replacement of enzyme-bound unlabeled calcium with ${}^{45}\text{Ca}$ in solution (to give ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ and ${}^c\text{E} \cdot \text{Ca}_2^*$), with the understanding that the opposite exchange was also measured.

Figure 6 shows that the observed rate constant for the appearance of the second ${}^{45}\text{Ca}$ on ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ decreases with increasing exterior $[\text{Ca}^{2+}]$. The observed first-order rate constant for the formation of ${}^c\text{E} \cdot \text{Ca}_2^*$ from ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ decreases from $\geq 10 \text{ s}^{-1}$ at $1.2 \mu\text{M}$ exterior $[\text{Ca}^{2+}]$ to 0.18 s^{-1} at $200 \mu\text{M}$ exterior $[\text{Ca}^{2+}]$.

Since the rate constant for exchange of the second calcium ion decreases with increasing $[\text{Ca}^{2+}]$ in the solution, exchange of the second ion must proceed through an intermediate that partitions between rebinding of ${}^{45}\text{Ca}$ and dissociation of unlabeled bound Ca^{2+} . The simplest model that can account for these observations is described in Scheme IV. Exchange of the first enzyme-bound Ca^{2+} with ${}^{45}\text{Ca}$ occurs with $k_{\text{obs}} = k_{-2} = 60 \text{ s}^{-1}$ to give ${}^c\text{E} \cdot \text{Ca}_1$, which immediately binds ${}^{45}\text{Ca}$ to form ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$. Subsequent replacement of the second bound calcium ion to give ${}^c\text{E} \cdot \text{Ca}_2^*$ depends on a competition between Ca^{2+} dissociation from ${}^c\text{E} \cdot \text{Ca}_1$ (k_{-1}) and ${}^{45}\text{Ca}$ binding to reform ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ ($k_2[\text{Ca}^*]$). At the concentrations of exterior ${}^{45}\text{Ca}$ used in these experiments, which are generally much larger than $K_{0.5}$, the pseudo-first-order rate constants for ${}^{45}\text{Ca}$ binding to the enzyme ($k_1[\text{Ca}^*]$ and $k_2[\text{Ca}^*]$) are fast compared with the time of the measurements and do not contribute to the exchange kinetics, so that ${}^c\text{E}$ is converted rapidly to ${}^c\text{E} \cdot \text{Ca}_2^*$. Therefore, as the external $[\text{Ca}^{2+}]$ increases, k_{obs} for the exchange of the inner Ca^{2+} ion with ${}^{45}\text{Ca}$ decreases due to the partitioning of ${}^c\text{E} \cdot \text{Ca}_1$ more toward ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ than toward ${}^c\text{E}$.

The observed rate constant for the replacement of the inner Ca^{2+} of ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$ by ${}^{45}\text{Ca}$ (Scheme V) equals the product of k_{-2} , the rate constant for dissociation of the outer bound ion to give ${}^c\text{E} \cdot \text{Ca}_1$, times the fraction of ${}^c\text{E} \cdot \text{Ca}_1$ that dissociates unlabeled Ca^{2+} irreversibly into the ${}^{45}\text{Ca}$ -containing medium rather than binding ${}^{45}\text{Ca}$ to give back ${}^c\text{E} \cdot \text{Ca}_1 \cdot \text{Ca}_1^*$.

$$k_{\text{obs}} = \frac{k_{-2}k_{-1}}{k_{-1} + k_2[\text{Ca}^*]} \quad (5)$$

Equation 5 describes the observed rate constant for exchange of the inner Ca^{2+} ion according to Scheme V when ${}^c\text{E} \cdot \text{Ca}_1$ is a steady-state intermediate in the pathway; i.e., when $k_{-1} + k_2[\text{Ca}^*] \gg k_{-2}$ and the amount of ${}^c\text{E} \cdot \text{Ca}_1$ does not change significantly over the course of the measurement. At low exterior $[\text{Ca}^*]$, this approximation does not hold because ${}^c\text{E} \cdot \text{Ca}_1$ accumulates significantly in the absence of exterior Ca^{2+} (which will be shown presently).

A plot of the reciprocal of the observed rate constants for exchange of the inner Ca^{2+} ion against external $[\text{Ca}^{2+}]$ (Figure 6, inset) gives a straight line for $[\text{Ca}^{2+}] \geq 12 \mu\text{M}$ with a slope of $(2.5 \pm 0.3) \times 10^4 \text{ s M}^{-1}$. From eq 5, the slope of this line equals $k_2/k_{-1}k_{-2}$. The value of $k_{-2} = 60 \text{ s}^{-1}$ (Figure 5) gives $k_{-1}/k_2 = \{[(2.5 \pm 0.3) \times 10^4 \text{ s M}^{-1}](60 \text{ s}^{-1})\}^{-1} = 0.7 \pm 0.1 \mu\text{M}$. This is the concentration of calcium at which $k_2[\text{Ca}^{2+}] = k_{-1}$ and the observed rate constant is half-maximal. The data at lower $[\text{Ca}^{2+}]$ are not included in this plot, and the intercept is not accurate because of its small size and the failure of the steady-state approximation at low $[\text{Ca}^{2+}]$.

Loss of ATP Reactivity upon Addition of EGTA to ${}^c\text{E} \cdot \text{Ca}_2$. Addition of EGTA to loaded vesicles preincubated with saturating amounts of external calcium (${}^c\text{E} \cdot \text{Ca}_2$ or ${}^c\text{E} \cdot \text{Ca}_2^*$) for varying times t_1 , followed by ATP plus EGTA, results in a time-dependent decrease in ATP reactivity, as monitored by $\text{E} \sim \text{P} \cdot \text{Ca}_2$ formation (Figure 7A) or ${}^{45}\text{Ca}$ uptake into vesicles as a consequence of $\text{E} \sim \text{P} \cdot \text{Ca}_2^*$ formation (Figure 7B). The data do not fit well to a single exponential. In Figure 7A, the time course for the loss of enzyme that is phosphorylated by ATP fits a first-order rate constant of $\sim 50 \text{ s}^{-1}$ at very short reaction times ($t_1 < 10$ ms, lower line) and $\sim 42 \text{ s}^{-1}$ at reaction times of 15–40 ms (upper line); at reaction times > 50 ms the data may fit an even smaller rate constant. The data for ${}^{45}\text{Ca}$ uptake in Figure 7B show similar behavior; the solid lines are drawn for rate constants of 55 s^{-1} (lower line) and 45 s^{-1} (upper line).

This biphasic behavior cannot be explained by accumulation of a second ATP-reactive intermediate in the reaction of ${}^c\text{E} \cdot \text{Ca}_2$ with EGTA. Such an intermediate would result in lags in the time courses shown in Figure 7A,B. Instead, what is observed is consistent with a reaction that follows a first-order rate constant of $50\text{--}55 \text{ s}^{-1}$ but becomes slightly inhibited over the course of the measurement.

Trapping of ${}^c\text{E} \cdot \text{Ca}_1$. The ${}^c\text{E} \cdot \text{Ca}_1^*$ intermediate, which forms when EGTA is added to ${}^c\text{E} \cdot \text{Ca}_2^*$, may be trapped by the addition of excess unlabeled calcium to give ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1$ prior to phosphorylation by ATP (Figure 8). Intact vesicles preincubated with $15 \mu\text{M}$ exterior ${}^{45}\text{Ca}$ were mixed with an equal volume of 5.0 mM EGTA (to give $[\text{Ca}^{2+}] = 10 \text{ nM}$) for varying times t_1 , followed by 6.0 mM unlabeled Ca^{2+} for $t_2 = 110\text{--}180$ ms (final free $[\text{Ca}^{2+}]$ in $t_2 = 300 \mu\text{M}$), followed by 1.2 mM ATP. The unlabeled Ca^{2+} added in t_2 immediately traps any ${}^c\text{E} \cdot \text{Ca}_1^*$ present after t_1 as ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1$. In addition, any ${}^c\text{E} \cdot \text{Ca}_2^*$ remaining after t_1 will exchange the outer bound ${}^{45}\text{Ca}$ for an unlabeled Ca^{2+} to also form ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1$; this exchange occurs at 55 s^{-1} (Figure 5) and is complete within 130 ms. If no ${}^c\text{E} \cdot \text{Ca}_1^*$ is present after t_1 , the only ${}^{45}\text{Ca}$ uptake observed in this experiment would be from ${}^c\text{E} \cdot \text{Ca}_1^* \cdot \text{Ca}_1$ formed from this exchange of the outer ion of ${}^c\text{E} \cdot \text{Ca}_2^*$ with unlabeled calcium during t_2 . This would result in a decrease in radioactive calcium (Figure 8, dashed line), with a time course identical with that observed when ${}^c\text{E} \cdot \text{Ca}_2^*$ is mixed with EGTA without unlabeled calcium added prior to ATP (Figure 7B) (except that the stoichiometry of $[\text{Ca}^{2+}]/[\text{E}]_{\text{tot}}$ would be

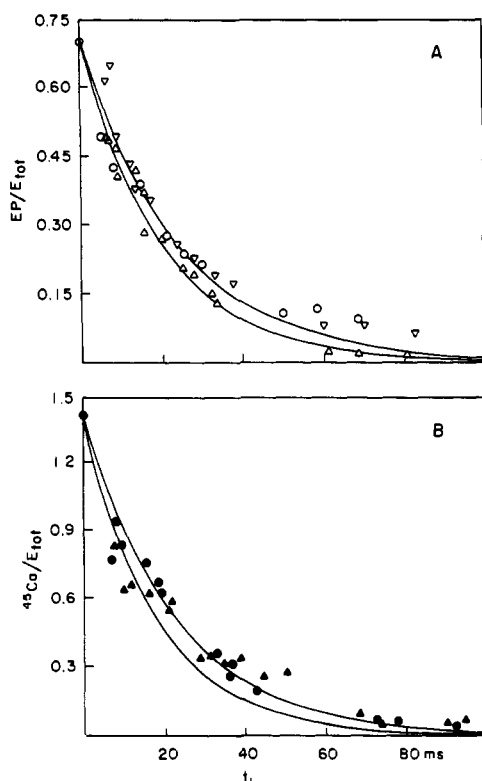


FIGURE 7: (A) Loss of reactivity of $^E\text{-Ca}_2$ in the presence of EGTA, assayed with $[^{32}\text{P}]\text{ATP}$. Enzyme preincubated in saturating amounts of exterior Ca^{2+} was mixed with EGTA for varying times t_1 and then reacted with ATP plus EGTA. Syringe A contained loaded vesicles preincubated with either 1.72 mM EGTA and 1.68 mM CaCl_2 (21 μM free Ca^{2+}) (○), 0.86 mM EGTA and 0.85 mM CaCl_2 (21 μM free Ca^{2+}) (Δ), or 0.40 mM EGTA and 0.41 mM CaCl_2 (22 μM free Ca^{2+}) (▽). Syringe B contained 10 mM EGTA, except (Δ) which contained 12 mM EGTA. Syringe C contained 0.9 mM $[^{32}\text{P}]\text{ATP}$ plus 5 mM EGTA. Syringe D contained 2 M HCl and 60 mM KH_2PO_4 . All syringes except D also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 . Final free Ca^{2+} concentrations in t_1 were 0.12 (○), 0.05 (▽), and 0.03 μM (Δ). For $t_1 = 0$ points, syringe B contained buffer of the same concentrations as syringe A, and syringe C contained 15 mM EGTA plus 0.9 mM $[^{32}\text{P}]\text{ATP}$. The lines are drawn for first-order rate constants of 42 and 50 s⁻¹. (B) Loss of reactivity of $^E\text{-Ca}_2^*$ in the presence of EGTA, assayed with unlabeled ATP. Syringe A contained loaded vesicles preincubated with 0.40 mM EGTA and 0.41 mM $^{45}\text{CaCl}_2$ (22 μM free Ca^{2+}) (●) or empty vesicles preincubated with 0.05 mM $^{45}\text{CaCl}_2$ (▲). Syringe B contained 10 mM EGTA. Syringe C contained 0.9 mM ATP plus 5 mM EGTA. All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM MgSO_4 . Final free Ca^{2+} concentrations in t_1 were 0.03 μM (●) and 0.004 μM (▲). For $t_1 = 0$ points, syringe B contained buffer of the same concentrations as syringe A, and syringe C contained 15 mM EGTA plus 0.9 mM ATP. The lines are drawn for first-order rate constants of 45 and 55 s⁻¹.

1:1 for the experiment of Figure 8 due to the exchange of the outer ^{45}Ca with unlabeled Ca^{2+} during t_2). Instead, Figure 8 shows a distinct lag in the time course for the decrease of $^E\text{-Ca}_1^*\text{-Ca}_1$. This biphasic time course is consistent with the formation and decay in the presence of EGTA of a second ^{45}Ca -containing intermediate, $^E\text{-Ca}_1^*$, which is trapped by unlabeled calcium added after t_1 to give $^E\text{-Ca}_1^*\text{-Ca}_1$. The $^E\text{-Ca}_1^*$ intermediate is not detected when the unlabeled calcium chase is not added, as shown by the absence of a lag in the kinetics of the loss of ATP-reactive enzyme in the presence of EGTA (Figure 7A,B). The reaction rate decreases in the final part of the reaction, as in the experiments shown in Figure 7.

The data of Figure 8 can be fit to two consecutive first-order processes with rate constants k_{-2} and k_{-1} , for dissociation of

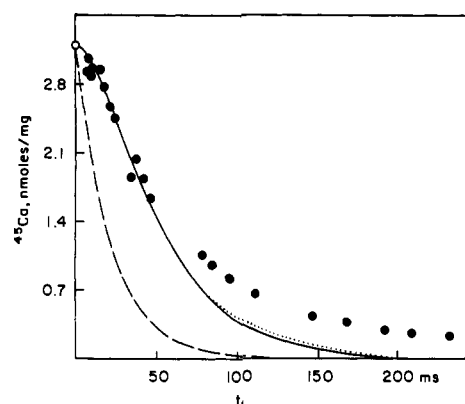


FIGURE 8: Trapping of $^E\text{-Ca}_1$ formed in EGTA. Enzyme preincubated with exterior ^{45}Ca was mixed with EGTA for varying times t_1 , followed by excess unlabeled CaCl_2 for $t_2 = 110\text{--}180$ ms, followed by ATP. Syringe A contained 0.10 mg/mL intact vesicles plus 15 μM $^{45}\text{CaCl}_2$. Syringe B contained 5.0 mM EGTA. Syringe C contained 6 mM unlabeled CaCl_2 plus 9 mM Tris base. Syringe D contained 1.2 mM ATP plus 0.1 mM unlabeled CaCl_2 . All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 . Free $[\text{Ca}^{2+}] = 10$ nM during t_1 . Free $[\text{Ca}^{2+}]$ in $t_2 = 300$ μM . For the reaction point at $t_1 = 0$ (○), $^E\text{-Ca}_2^*$ was mixed with unlabeled Ca^{2+} for 110–180 ms without prior reaction with EGTA. Syringe A was as above, syringe B contained 5 mM EGTA plus 6 mM unlabeled CaCl_2 (to give free $[\text{Ca}^{2+}] = 0.5$ mM during t_1), syringe C contained 1.2 mM ATP and 0.1 mM CaCl_2 , and syringe D contained buffer. All other conditions were as (●). These conditions were chosen to give the same final concentrations of reactants in the collection tube as in (○). The single data point (○) represents the average of five data points for the exchange of a single ^{45}Ca . For blanks, syringe A contained enzyme preincubated with equal volumes of 15 μM ^{45}Ca and 5 mM EGTA–6 mM CaCl_2 . Syringe B also contained equal volumes of 15 μM ^{45}Ca and 5 mM EGTA–6 mM CaCl_2 . Syringes C and D were as (○). These conditions again were chosen to give the same final conditions in the collection tube as (○, ●) in order to quantitate the amount of ^{45}Ca uptake in the collection tube due to turnover prior to filtering the vesicles. The collection tubes for all reactions contained 1 mL of 10 mM CaCl_2 plus 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 in order to minimize ^{45}Ca uptake due to turnover. The solid line is drawn for two consecutive first-order reactions with rate constants of 45 and 30 s⁻¹ and the dotted line for 60 and 25 s⁻¹, with $[^{45}\text{Ca}] = 3.18$ nmol/mg at $t_1 = 0$. The dashed line is drawn for a first-order reaction with a rate constant of 45 s⁻¹, corresponding to the loss of ATP reactivity observed when EGTA is added to $^E\text{-Ca}_2^*$ for varying t_1 and assayed directly by addition of ATP plus EGTA without the intervening unlabeled Ca^{2+} (Figure 7B). $[E]_{tot} = 3.2$ nmol/mg.

the two calcium ions from $^E\text{-Ca}_2^*$. Since the value of k_{-2} , for dissociation of the outer calcium ion (Scheme IV), is known, k_{-1} can be obtained from the optimal fit of the data to two consecutive first-order reactions with one of the rate constants set to 60 s⁻¹. Fitting the time course using a range of $k_{-2} = 60\text{--}45$ s⁻¹ gives a range of $k_{-1} = 25\text{--}30$ s⁻¹ (Figure 8, solid and dotted lines, respectively). The rate constant for dissociation of Ca^{2+} from $^E\text{-Ca}_1$, $k_{-1} = 25\text{--}30$ s⁻¹, is half as large as the rate constant for dissociation of the outer Ca^{2+} from $^E\text{-Ca}_2$, with $k_{-2} = 55\text{--}60$ s⁻¹.

Exchange of ^{45}Ca into $^E\text{-Ca}_2\text{-ATP}$. As described previously, $^E\text{-Ca}_2$ partitions in the presence of ATP and EGTA between phosphorylation with a rate constant of 220 s⁻¹ and irreversible dissociation of calcium with a rate constant of 80 s⁻¹ (eq 1). The product of the 80 s⁻¹ step cannot be phosphorylated. The following experiment was undertaken to determine how many calcium ions are bound to the product of the 80 s⁻¹ step when phosphorylation occurs in the presence of ^{45}Ca instead of EGTA (Scheme VI).

Intact vesicles, suspended in a Ca-EGTA buffer to give 26 μM free calcium, were mixed with the same Ca-EGTA buffer containing ^{45}Ca and 0.3 mM ATP for various times t_1 followed

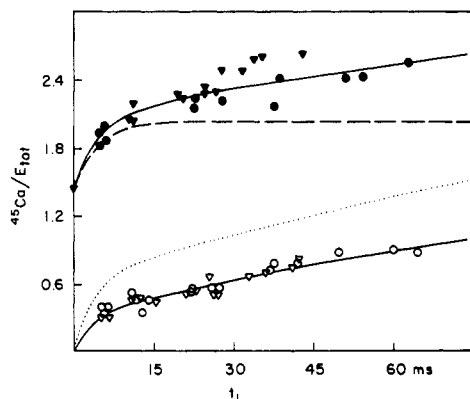
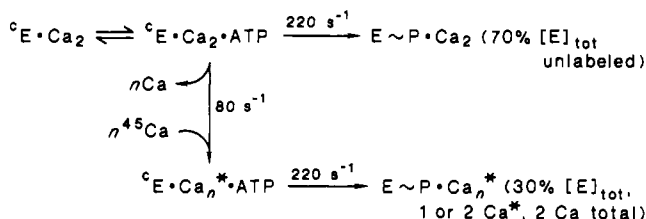


FIGURE 9: Exchange of ^{45}Ca into $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$. Intact vesicles preincubated in either unlabeled Ca^{2+} or ^{45}Ca were mixed with ATP plus ^{45}Ca for varying times t_1 followed by a quench of excess EGTA. Syringe A contained 0.17 mg/mL SRV and 0.20 mM EGTA plus either 0.22 mM CaCl_2 (∇), 0.25 mM CaCl_2 (\circ), 0.22 mM $^{45}\text{CaCl}_2$ (\blacktriangledown), or 0.25 mM $^{45}\text{CaCl}_2$ (\bullet) at the final specific activity. Syringe B contained 0.7 mM ATP and 0.20 mM EGTA plus 0.22 (∇ , \blacktriangledown) or 0.25 mM (\circ , \bullet) $^{45}\text{CaCl}_2$ at either the final specific activity (closed symbols) or twice the final specific activity (open symbols). Syringe C contained 15 mM EGTA. Free $[\text{Ca}^{2+}]$ during $t_1 = 26$ (∇ , \blacktriangledown) or 53 μM (\circ , \bullet). All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM MgSO_4 . For $t_1 = 0$ (\blacktriangledown), reactions were as (\blacktriangledown) above except syringe B contained 10 mM EGTA plus 0.7 mM ATP and syringe C contained 5 mM instead of 15 mM EGTA. The solid upper line is calculated for the sum of the products of a first-order reaction from 70% to 100% of $[\text{E}]_{\text{tot}}$ with $k_{\text{obs}} = 220 \text{ s}^{-1}$ (dashed line), plus a first-order rate constant representing turnover from 100% to 200% of $[\text{E}]_{\text{tot}}$ with $k_{\text{obs}} = 6 \text{ s}^{-1}$. The lower lines are calculated for the uptake of 1 (solid line) or 2 (dotted line) ^{45}Ca ions by 30% of $[\text{E}]_{\text{tot}}$ with $k_{\text{obs}} = 300 \text{ s}^{-1}$ followed by turnover of $[\text{E}]_{\text{tot}}$ with $k_{\text{obs}} = 6 \text{ s}^{-1}$.

Scheme VI



by an EGTA quench. For the control (Figure 9, closed symbols), the solutions in syringes A and B contained ^{45}Ca of equal specific activity. The control represents the amount of ^{45}Ca taken up as a result of the phosphorylation of all enzyme as $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$, plus any subsequent hydrolysis and rephosphorylation during t_1 . Since the vesicles are not preloaded with Ca^{2+} in this experiment, turnover during t_1 prior to quenching with EGTA is significant and gives rise to a slow additional uptake of Ca^{2+} after the first turnover. The control data fit the upper solid line, which describes ^{45}Ca accumulation resulting from phosphorylation of $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ at 220 s^{-1} followed by hydrolysis and rephosphorylation at $\sim 7 \text{ s}^{-1}$.

For the exchange reaction (open symbols), only syringe B contained ^{45}Ca , at twice the final specific activity. When the contents of syringes A and B were mixed, the ^{45}Ca in t_1 had the same specific activity as in the control reaction, and any nonradioactive calcium that dissociated from $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ during t_1 was replaced by ^{45}Ca of the same specific activity as in the control reaction. Since 30% of $[\text{E}]_{\text{tot}}$ is available to undergo exchange, if the amount of ^{45}Ca uptake observed in the exchange reaction equaled 30% of the amount of ^{45}Ca uptake observed in the control reaction, then two ^{45}Ca ions would be exchanged with $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$. If the ^{45}Ca uptake observed in the exchange reaction equals 15% of the control, then it could be concluded that $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ exchanges a single

calcium ion with a rate constant of 80 s^{-1} .

The observed exchange (Figure 9, open symbols) gives an excellent fit to the lower solid line, which was calculated as described in the figure legend for exchange of a single calcium ion from the $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ intermediate that partitions toward calcium dissociation. This shows that a single Ca^{2+} ion dissociates from $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ with a rate constant of 80 s^{-1} . This experiment does not give a rate constant for dissociation of the second Ca^{2+} ion from $^{\circ}\text{E}\cdot\text{Ca}_1\cdot\text{ATP}$ because the absence of dissociation of the second Ca^{2+} ion could mean either that in the presence of EGTA only one Ca^{2+} ion dissociates at 80 s^{-1} or that two Ca^{2+} ions dissociate, but in the presence of exterior Ca^{2+} dissociation of the second Ca^{2+} ion is inhibited in the same way as dissociation of the second Ca^{2+} from $^{\circ}\text{E}\cdot\text{Ca}_1$.

The observed uptake of 15% ^{45}Ca also excludes a mechanism in which very fast dissociation of two Ca^{2+} ions from $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ is followed by a conformational change with a rate constant of 80 s^{-1} that prevents phosphorylation (eq 6), as well



as a similar mechanism in which loss of a single Ca^{2+} ion induces the conformational change. These mechanisms would give uptake of 100% or 50% ^{45}Ca , respectively.

DISCUSSION

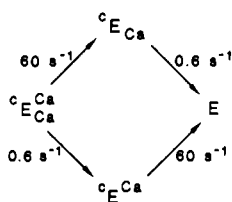
Assay of $^{\circ}\text{E}\cdot\text{Ca}_2$ and Binding of ATP. The rapid uptake of 1.4 Ca^{2+} ions per mole of enzyme when ATP and EGTA are added simultaneously to $^{\circ}\text{E}\cdot\text{Ca}_2$, compared with 2.0 Ca^{2+} ions with ATP alone (Figure 1), confirms the conclusion that $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ in the presence of EGTA undergoes competitive phosphorylation and dissociation of Ca^{2+} with rate constants of 220 and 80 s^{-1} , respectively (eq 1). In the absence of EGTA, phosphorylation occurs at 220 s^{-1} , which is 73% of $220 + 80 = 300 \text{ s}^{-1}$. This agrees well with the observed phosphorylation of 70% of the enzyme in the presence of ATP and EGTA (Petithory & Jencks, 1986).

An important conclusion from this experiment is that the stoichiometry of $1.4/0.7 = 2.0$ calcium ions taken up for each mole of phosphoenzyme shows that there is no detectable phosphorylation of enzyme containing a single bound Ca^{2+} ion upon addition of ATP and EGTA. It will be shown below that dissociation of the two Ca^{2+} ions is sequential. Therefore, this result means that the $^{\circ}\text{E}\cdot\text{Ca}_1$ species that is initially formed when Ca^{2+} dissociates in the presence of EGTA is not competent to undergo phosphorylation at a significant rate.

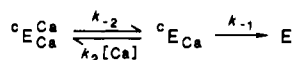
Although the binding of ATP is close to diffusion-controlled, with $k_{\text{ATP}} = 1.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, the binding step becomes rate-limiting at low ATP concentrations because of the very fast rate of subsequent phosphorylation, with $k_p = 220 \text{ s}^{-1}$. At intermediate concentrations, the binding and phosphorylation steps are both partially rate-limiting, so that biphasic kinetics are observed (Figure 3). However, at the higher ATP concentrations that were used for assaying the formation of $^{\circ}\text{E}\cdot\text{Ca}_2$, the binding step is fast; with $300 \mu\text{M}$ ATP, the pseudo-first-order rate constant for ATP binding is 3000 s^{-1} . This kinetic behavior, the first-order kinetics for phosphorylation in the absence as well as in the presence of EGTA, and the yield of 70% phosphoenzyme in the presence of EGTA (Petithory & Jencks, 1986) are consistent with the reaction of a single enzyme species; they provide no evidence for sequential reactions of different subunits.

The rate constants of $1.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for binding and 120 s^{-1} for dissociation of ATP with $^{\circ}\text{E}\cdot\text{Ca}_2$ may be compared with the rate constants of $5 \times 10^6 \text{ M}^{-1}$ for binding and 24 s^{-1} for dissociation of ATP with free E. There is not a large difference

Scheme VII



Scheme VIII



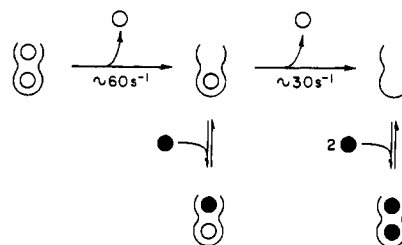
between the rate constants for the two enzyme species. The dissociation constants for ATP of 13 and 5 μM with ${}^o\text{E}\cdot\text{Ca}_2$ and E, respectively, suggest that strong binding of ATP does not provide a significant driving force for the conversion of E to ${}^o\text{E}\cdot\text{Ca}_2$ (or " $\text{E}_1\cdot\text{Ca}_2$ ") (Stahl & Jencks, 1987).

Ordered Dissociation of Calcium from ${}^o\text{E}\cdot\text{Ca}_2$. Dissociation of the two calcium ions from ${}^o\text{E}\cdot\text{Ca}_2$ is an ordered process. Figure 5 shows that the exchange of a single calcium ion from ${}^o\text{E}\cdot\text{Ca}_2$ with ${}^{45}\text{Ca}$ in solution to give ${}^o\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ occurs with a first-order rate constant of 55–60 s^{-1} . This rate constant is independent of exterior calcium at concentrations greater than 30 μM . It is very similar to the rate constant of 80 s^{-1} for calcium dissociation from ${}^o\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ (Petithory & Jencks, 1986); i.e., ATP has little effect on the rate constant for dissociation of calcium. Exchange of the second enzyme-bound unlabeled calcium ion with ${}^{45}\text{Ca}$ to give ${}^o\text{E}\cdot\text{Ca}_2^*$ occurs very slowly compared to the first exchange under these conditions, with an observed rate constant of 0.6 s^{-1} in a solution containing 60 μM ${}^{45}\text{Ca}$ (Figure 6).

A nearly identical result was obtained by Dupont at 20 $^\circ\text{C}$, pH 7.2, by mixing enzyme, preincubated with 50 μM unlabeled calcium, with 50 μM ${}^{45}\text{Ca}$ and measuring the amount of ${}^{45}\text{Ca}$ bound to the enzyme by using a rapid filtration technique (Dupont et al., 1985). Similar biphasic Ca^{2+} dissociation under various conditions has been observed by a number of workers including Ikemoto et al. (1981), Dupont (1984), and Inesi (1987). Inesi and Lewis (1987) have also reported in an abstract that lanthanum inhibits release of the second Ca^{2+} and gives a species that is active for phosphorylation by ATP. In principle, biphasic Ca^{2+} dissociation could be attributed to dissociation of Ca^{2+} from nonequivalent binding sites, with rate constants of 60 and 0.6 s^{-1} (Scheme VII), or to strictly ordered dissociation from two sequential sites such that the second ("inner") calcium ion cannot dissociate until the site occupied by the first ("outer") calcium ion is vacant (Scheme VIII). When the concentration of calcium in the medium is high, dissociation of the inner calcium is inhibited because of re-binding of calcium to the outer site in the ordered mechanism.

These two possibilities were distinguished by measuring the dependence on exterior $[{}^{45}\text{Ca}]$ of k_{obs} for exchange of the inner Ca^{2+} ion to give ${}^o\text{E}\cdot\text{Ca}_2^*$. If the two bound calcium ions dissociate independently with rate constants that differ by 100-fold (Scheme VII), then changes in exterior $[{}^{45}\text{Ca}]$ would have no effect on the exchange kinetics. However, if dissociation of the inner bound calcium ion can occur only if the outer calcium site is vacant (Scheme VIII), ${}^o\text{E}\cdot\text{Ca}_1$ would partition between binding of ${}^{45}\text{Ca}$ to re-form ${}^o\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ and dissociation of unlabeled Ca^{2+} to form E, which would then bind two ${}^{45}\text{Ca}$ ions. As the concentration of ${}^{45}\text{Ca}$ in the medium increases, $k_2[\text{Ca}^*]$ would increase and become much larger than k_{-1} so that ${}^o\text{E}\cdot\text{Ca}_1$ would partition to form ${}^o\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ rather than E. The observed rate constant for exchange of the inner calcium ion by the mechanism of Scheme

Scheme IX



VIII would therefore decrease with increasing $[{}^{45}\text{Ca}]$.

The reaction was monitored by measuring either the loss of label from ${}^o\text{E}\cdot\text{Ca}_1^*\cdot\text{Ca}_1$ in solution containing unlabeled calcium (open symbols, Figure 6) or the appearance of a second bound ${}^{45}\text{Ca}$ ion on ${}^o\text{E}\cdot\text{Ca}_1\cdot\text{Ca}_1^*$ in a solution containing ${}^{45}\text{Ca}$ (closed symbols). Figure 6 shows that the rate of exchange of the inner calcium ion decreases with increasing concentration of calcium in the solution and is therefore consistent with ordered dissociation of calcium from ${}^o\text{E}\cdot\text{Ca}_2$ and the minimal mechanism of Scheme VIII, as well as the similar mechanisms of Dupont (1982, 1984, 1985) and Inesi (1987).

Figure 6 shows that the observed rate constant for exchange of the inner Ca^{2+} approaches zero as the calcium concentration in solution increases. A plot of the reciprocal of the observed rate constant for exchange of the inner calcium ion against free calcium concentration in the medium (Figure 6, inset) fits a line described by $k_{-1}/k_2 = 0.7 \pm 0.1 \mu\text{M}$. Half-maximal inhibition of calcium dissociation from ${}^o\text{E}\cdot\text{Ca}_1$ occurs at a calcium concentration approximately 5-fold lower than $K_{0.5} = 3.4 \mu\text{M}$ for the calcium-dependent activation of ATP reactivity (Figure 4). This k_{-1}/k_2 ratio and the rate constant for Ca^{2+} dissociation of $k_{-1} = 30 \text{ s}^{-1}$ give $k_2 = 4.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for binding of the second Ca^{2+} . The rate constant of $k_{-2} = 60 \text{ s}^{-1}$ then gives $K_2 = k_{-2}/k_2 = 1.4 \mu\text{M}$ for the dissociation constant of this Ca^{2+} from ${}^o\text{E}\cdot\text{Ca}_2$. A dissociation constant in this range is expected because positive cooperativity for calcium binding requires that the dissociation constant for the second calcium ion be smaller than that for the first calcium.

Exchange resulting from ordered dissociation can be envisioned if the calcium ions bind sequentially in a channellike site (Scheme IX). The outer calcium ion physically blocks the dissociation of the inner calcium ion. Only when the outer calcium site is unoccupied, which is a rare occurrence at high exterior free calcium concentrations, can the inner calcium ion dissociate and be replaced sequentially by two calcium ions from the medium. The alternative possibility is that dissociation of the first calcium ion from one of two nonequivalent binding sites triggers a conformational change which enables the second calcium ion to dissociate. However, MacLennan and co-workers (MacLennan et al., 1985; Brandl et al., 1986) have proposed, on the basis of predictions of secondary structure from sequence and chemical modification data, that the calcium binding sites are located in a "stalk" region made up of parallel helical segments. This arrangement of helices could form a channellike site for the ions that extends partway into the membrane domain. A twisting of the helical segments upon phosphorylation of enzyme-calcium could then switch the side of access of the channellike transmembrane helical domain, as suggested by Tanford (1982). None of the results reported here requires a conformational change, but they do not exclude a conformational change that either accompanies or follows calcium dissociation.

Khananshvilii and Jencks (1988) found that the two calcium ions dissociate from $\text{E}\sim\text{P}\cdot\text{Ca}_2$ to the vesicle interior in an

ordered, two-step reaction with observed rate constants of 34 and 17 s⁻¹. It is interesting to note that there is only about a 2-fold difference in the observed rate constants for dissociation of calcium ions to the vesicle interior from E~P-Ca₂, and dissociation of calcium ions to the vesicle exterior from ^oE-Ca₂, although the apparent dissociation constant of Ca²⁺ from ^oE-Ca₂, $K_{0.5} \sim 10^{-6}$ M, is approximately 3 orders of magnitude smaller than that for dissociation of Ca²⁺ from E~P-Ca₂, $K_{0.5} \sim 10^{-3}$ M. This means that with the phosphoenzyme there is a large kinetic barrier (or "slow gating") for the binding and dissociation of calcium.

The 2-fold difference in the rate constants for dissociation of the first and second Ca²⁺ ions would be expected if the rate constant for dissociation from the first site is constant and the second Ca²⁺ equilibrates rapidly between the two sites with an equilibrium constant of 1.0, so that this site is occupied half of the time; the same point has been made for dissociation of Rb⁺ from the Na,K-ATPase by Forbush (1987).

A similar mechanism for ordered dissociation of bound ions has been shown to occur in the Na,K-ATPase. Forbush and Glynn have concluded that ordered release of occluded Rb⁺ and K⁺ ions occurs upon addition of inorganic phosphate to the E₂(Rb) or the E₂(K) form of the Na,K-ATPase. They observed that the "slow-dissociating" Rb⁺ ion cannot dissociate until the site occupied by the "fast-dissociating" Rb⁺ ion is vacant (Forbush, 1985, 1987; Glynn et al., 1985a,b). The similarity in the ordered mechanisms for dissociation of transported ions from these enzymes is an indication that ordered dissociation of ions may be a common mechanism in the ion-transporting ATPases.

Several of the results from a comprehensive series of experiments carried out by Ikemoto et al. (1981), which were interpreted in terms of a nonequivalent subunit model, are similar to those reported here and can be interpreted in the same way without invoking different subunits. The principal difference in the conditions of these experiments is that they were carried out at 2–4 °C and the EGTA quench generally contained a low concentration of ATP, usually ~5 μM. This gave ~50% internalization of bound Ca²⁺, as might be expected for a nonequivalent subunit model, but the amount of internalization was found to increase at higher ATP concentration in a manner similar to that reported here (Figure 2). Mixing E-Ca₂* with unlabeled Ca²⁺ gave rapid exchange of half the labeled calcium, while the other half was internalized in the presence of ATP. It was shown that calcium dissociation in the presence of EGTA can be monitored by the disappearance of reactivity toward ATP and follows a first-order course with a rate constant of 0.3 s⁻¹ at 2–4 °C.

A particularly important result from the work of Ikemoto et al. is that the Ca²⁺ that exchanges slowly in the presence of external Ca²⁺ is internalized rapidly in a first-order reaction upon addition of ATP and EGTA, while the Ca that exchanges rapidly is internalized more slowly, after an induction period. This shows that the two Ca²⁺ ions are not equivalent and that the "inner" Ca²⁺ is internalized rapidly, while the "outer", exchangeable Ca²⁺ is internalized later. The same result has recently been obtained by Inesi, who has reached the same conclusion (Inesi, 1987).

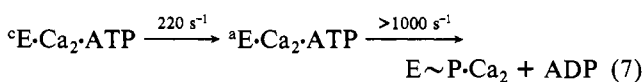
Accumulation of Enzyme with a Single Bound Calcium Ion, ^oE-Ca₁. The experiment shown in Figure 8 shows that enzyme containing a single bound calcium ion, ^oE-Ca₁, has a significant lifetime; it dissociates with a rate constant of $k_{-1} = 25\text{--}30$ s⁻¹. The reaction was initiated by the addition of EGTA to ^oE-Ca₂* and was quenched after t_1 by the addition of a large excess of unlabeled calcium. This stops further dissociation of the

inner ⁴⁵Ca and also allows exchange of any remaining outer ⁴⁵Ca with ⁴⁵Ca in solution before the addition of ATP, which brings about rapid phosphorylation and occlusion, and internalization of both calcium ions. If ⁴⁵Ca dissociated rapidly from ^oE-Ca₁*, the observed dissociation of both Ca²⁺ ions would be limited only by the rate of dissociation of the outer Ca²⁺ and would follow the first-order course that is shown by the dashed line in Figure 8.

The observed biphasic reaction course, with an initial induction period, shows that the outer Ca²⁺ dissociates first. The data may be fit with first-order rate constants for two consecutive reactions of 45 and 30 s⁻¹ or 60 and 25 s⁻¹, which are shown by the solid and dotted lines in Figure 8, respectively. The range of the rate constant for dissociation of the outer Ca²⁺ was taken to be $k_{-2} = 45\text{--}60$ s⁻¹, and a rate constant of 25–30 s⁻¹ was assigned to k_{-1} , for dissociation of the inner Ca²⁺ from ^oE-Ca₁. A small fraction of the total bound ⁴⁵Ca, ~5%, dissociates even more slowly; the reason for this is not known.

This experiment shows that enzyme containing a single bound Ca²⁺ ion accumulates significantly in the presence of EGTA. If the enzyme containing a single bound Ca²⁺ reacted with ATP, a lag would be seen in the time course for the loss of ATP reactivity upon addition of EGTA to ^oE-Ca₂. The absence of a lag in these experiments (Figure 7A,B) shows that ^oE-Ca₁, which accumulates substantially in the presence of EGTA, undergoes little or no phosphorylation by ATP.

Change in Chemical Specificity for Catalysis. A major goal of this work was to identify the step(s) that change(s) the substrate specificity of the enzyme from P_i reactive to ATP reactive upon addition of exterior calcium. Although a vast amount of work has gone into characterizing the physical changes that occur upon addition of calcium to the enzyme [reviewed in Dupont et al. (1985)], less effort has been made to characterize the changes in the catalytic reactivity of the enzyme under the same conditions. We have shown previously that the development of reactivity toward ATP is controlled by a conformational change that occurs at 220 s⁻¹ after the binding of ATP to ^oE-Ca₂ and is followed by very rapid phosphorylation by the active conformation of the enzyme, ^aE-Ca₂·ATP (eq 7); it was suggested that this conformational



change might involve a hinge-bending motion between domains of the kind postulated by MacLennan and co-workers (MacLennan et al., 1985; Brandl et al., 1986) that brings ATP into position to phosphorylate the β-aspartyl COO⁻ group at the active site (Petithory & Jencks, 1986).

The following evidence supports the conclusion that the ability of the ATPase to catalyze phosphorylation upon addition of ATP is lost if a single Ca²⁺ ion is removed from the enzyme, i.e., the species ^oE-Ca₁·ATP has little or no ability to undergo phosphorylation.

(1) The first-order rate constant of $k_{-2} = 50\text{--}55$ s⁻¹ for the loss of reactivity of ^oE-Ca₂ toward ATP in the presence of EGTA (Figure 7) agrees with the directly measured rate constant of 55–60 s⁻¹ for dissociation of the outer Ca²⁺ ion from ^oE-Ca₂ (Figure 5). In particular, the loss of ATP reactivity begins immediately upon addition of EGTA, with no induction period.

(2) Dissociation of the inner Ca²⁺ ion from ^oE-Ca₂ in the presence of EGTA follows a biphasic time course with an induction period because the outer Ca²⁺ ion must dissociate first (Figure 8). The rate constant for dissociation of the inner Ca²⁺ is 25–30 s⁻¹, about 2-fold slower than that for the outer

Ca^{2+} , which would give an easily detectable induction period if this dissociation were required for loss of ATP reactivity.

(3) The stoichiometry of ^{45}Ca uptake when ATP and EGTA are added simultaneously to $^{\circ}\text{E}\cdot\text{Ca}_2$ is 1.4 $\text{Ca}^*/0.7$ EP, which corresponds to 2.0 Ca^{2+} ions for each mole of phosphoenzyme formed (Figure 1). When ATP and EGTA are added together to $^{\circ}\text{E}\cdot\text{Ca}_2$, 70% of the enzyme is phosphorylated at 220 s^{-1} , and 30% of the enzyme is not phosphorylated because of Ca^{2+} dissociation from $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ at 80 s^{-1} (eq 1). The species $^{\circ}\text{E}\cdot\text{Ca}_1$ is formed initially in the presence of EGTA, and if this species were competent for phosphorylation, less ^{45}Ca would be internalized, and the ratio of $[\text{Ca}^*]_{\text{in}}/[\text{EP}]$ would be less than 2.0. We estimate that a 5% decrease in this ratio could have been detected.

(4) The stoichiometry of ^{45}Ca uptake when $^{\circ}\text{E}\cdot\text{Ca}_2$ is phosphorylated by ATP in the presence of $^{45}\text{Ca}^{2+}$ is 15%, not 30%, of the total calcium uptake (Figure 9). This shows that under the conditions of this experiment only a single Ca^{2+} ion dissociates from $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ in the 80 s^{-1} step that accompanies phosphorylation; this is the step that prevents phosphorylation in the presence of EGTA. This result does not exclude the possibility that two Ca^{2+} ions dissociate in the presence of EGTA, one at 80 s^{-1} and the second very rapidly; however, dissociation of the second Ca^{2+} from $^{\circ}\text{E}\cdot\text{Ca}_2$ is slower than the first Ca^{2+} , and ATP has little effect on the rate constant for dissociation of this Ca^{2+} , as noted above.

The concomitant dissociation of the first Ca^{2+} ion and the loss of ability for phosphorylation with ATP, with a rate constant of $\sim 55\text{ s}^{-1}$ and no induction period, could be associated with a fast conformational change. However, these results show that there is no additional slow conformational change that is required for this change of specificity for catalysis, such as might be expected from some " $\text{E}_1\text{--E}_2$ " models; the change in specificity arises directly from a change in the chemical composition of the enzyme-substrate complex.

It is physiologically desirable that the change in catalytic specificity which allows phosphorylation of the enzyme by ATP should occur only when two Ca^{2+} ions are bound. If phosphorylation could occur with a single bound Ca^{2+} ion, the overall reaction might occur with transport of only a single Ca^{2+} ion for each ATP that is hydrolyzed. There are several reports of a stoichiometry of one Ca^{2+} transported for each ATP that is cleaved under various conditions (Hasselbach & Makinose, 1963; Ikemoto, 1975; Meltzer & Berman, 1984; Rossi et al., 1979; Scott & Shamoo, 1984; Gafni & Boyer, 1985), but the results described here provide no indication that reaction with a single bound Ca^{2+} occurs at a significant rate under the conditions of our experiments.

It is equally important for a stoichiometry of two Ca^{2+} ions transported that the change in catalytic specificity of the phosphoenzyme to catalysis of phosphoryl transfer to water, instead of to ADP, should occur only after both Ca^{2+} ions have dissociated from $\text{E}\sim\text{P}\cdot\text{Ca}_2$ into the inside of the vesicle. If phosphoenzyme containing a single bound calcium could react with water to give hydrolysis, ATP hydrolysis might proceed with the transport of only a single Ca^{2+} ion. There is evidence that the change in specificity of the phosphoenzyme that allows hydrolysis does not occur until both Ca^{2+} ions have dissociated (Khananashvili & Jencks, 1988; unpublished experiments).

This requirement of two bound Ca^{2+} ions for the development of ATP reactivity and of two internalized Ca^{2+} ions for the loss of reactivity of the phosphoenzyme toward ADP in the reverse direction creates an apparent paradox with respect to the specificity of the intermediate species with one bound Ca^{2+} , because microscopic reversibility requires that a reaction

must have the same specificity in both directions. It is shown here that the species with one bound Ca^{2+} , $^{\circ}\text{E}\cdot\text{Ca}_1$, does not react with ATP to give phosphoenzyme and ADP, while the intermediate species in the internalization of Ca^{2+} from $\text{E}\sim\text{P}\cdot\text{Ca}_2$ is still quenched by ADP to give ATP with one Ca^{2+} retained in the SRV (Khananashvili & Jencks, 1988).

The paradox is avoided if phosphorylation and dephosphorylation proceed through two consecutive reactions that have different specificity requirements; there is evidence that this is the case. It should also be noted that it is the second Ca^{2+} ion to bind or dissociate that causes the change in specificity; this is the same ion for both steps because binding and dissociation are sequential and the second Ca^{2+} to bind from the outside is the second Ca^{2+} to dissociate inside the vesicle. The phosphorylation of $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ occurs with a rate-limiting conformational change at 220 s^{-1} , followed by very rapid phosphorylation of the catalytically active species $^{\circ}\text{E}\cdot\text{Ca}_2\cdot\text{ATP}$ in a second step (eq 7). The available data are consistent with the hypothesis that two bound Ca^{2+} ions are required for the conformational change, while the reversible phosphorylation step to give ATP can occur with either one or two Ca^{2+} ions. It appears that hydrolysis of the phosphoenzyme and phosphorylation of the enzyme by inorganic phosphate can occur only in the absence of bound Ca^{2+} (Makinose & Hasselbach, 1965; Kanazawa & Boyer, 1973; Guillain et al., 1981; Fernandez-Belda et al., 1984). The ADP-induced dephosphorylation of the intermediate species probably does not occur under physiological conditions because of the strong cooperativity for binding two Ca^{2+} ions to $\text{E}\sim\text{P}$ (Prager et al., 1979). This asymmetry in specificity may be the reason that evolution has produced a two-step mechanism, with a conformational change, for the phosphorylation reaction.

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