There Is Only One Phosphoenzyme Intermediate with Bound Calcium on the Reaction Pathway of the Sarcoplasmic Reticulum Calcium ATPase†

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ABSTRACT: Identical first-order rate constants for phosphorylation of the calcium ATPase of sarcoplasmic reticulum by bound inorganic phosphate (P_i) of 25 ± 2 s^{-1} with empty vesicles and 25 ± 1 s^{-1} with vesicles that were passively loaded with 40 mM Ca^{2+} were obtained by treating the reaction as an approach to equilibrium (4 mM [^{32}P]P_i, 20 mM $MgCl_2$, 10 mM EGTA, and 100 mM KCl at pH 7.0 and 25 °C). The formation of ADP-sensitive phosphoenzyme from P_i with Ca^{2+}-loaded vesicles also proceeds with a first-order rate constant of 25 s^{-1} and no detectable induction period. These identical rate constants show that lumenal Ca^{2+} does not inhibit the rate of phosphorylation of the enzyme by bound P_i and that there is no significant kinetic barrier for the conformational change that converts an ADP-insensitive to an ADP-sensitive phosphoenzyme intermediate with bound Ca^{2+}. We conclude that there is no evidence for the existence of two stable phosphoenzyme intermediates with bound Ca^{2+}, such as E_1~P·Mg·Ca_2 and Ca_2·E_2−P·Mg, that are included in the E_1−E_2 and related two-state models for calcium transport by this enzyme. In general, coupling of a physical reaction, such as muscle contraction or vectorial transport, to a chemical reaction, such as ATP hydrolysis, requires more than two states in the reaction cycle. It is not yet clear how the driving force that is provided by the movement of two Ca^{2+} ions from low-affinity to high-affinity sites is utilized to bring about ATP synthesis at a rate that is too fast to measure, when ADP is added to phosphoenzyme in Ca^{2+}-loaded vesicles.

The Ca^{2+}-ATPase[1] of sarcoplasmic reticulum transports two Ca^{2+} ions from the cytoplasm of muscle to the lumen of the sarcoplasmic reticulum at the expense of the hydrolysis of one molecule of ATP, in order to bring about relaxation of contracted muscle (de Meis, 1981; Martonosi & Beeler, 1983). The Ca^{2+}-ATPase is a member of the family of P-type cation pumps that includes the plasma membrane Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase. These enzymes pump cations across the membrane and form a covalent phosphoenzyme intermediate during the transport process (Sachs et al., 1982; Pedersen & Carafoli, 1987; Jencks, 1989a; Skou, 1990). Phosphorylation of the Ca^{2+}-ATPase by ATP is an important step that drives the movement of Ca^{2+} ions from high-affinity cytoplasmic sites to low-affinity lumenal sites and thus allows translocation of Ca^{2+} ions across the SR membrane (Jencks et al., 1993; Myung & Jencks, 1994b).

The E_1−E_2 and related two-state models have played an important role in the development of our understanding of vectorial transport of ions by the Ca^{2+}-ATPase (de Meis & Vianna, 1979; de Meis, 1981, 1988; MacLennan, 1990). However, several properties of the enzyme are difficult or impossible to interpret in terms of two-state models as they are usually defined (Pickart & Jencks, 1984; Stahl & Jencks, 1987; Petithory & Jencks, 1988b; Myung & Jencks, 1991). According to several of these models, translocation of Ca^{2+} ions occurs by a rate-limiting conformational change from an ADP-sensitive phosphoenzyme, E_1~P·Mg·Ca_2, which is formed from ATP and cytoplasmic Ca^{2+}, to an ADP-insensitive phosphoenzyme, Ca_2·E_2−P·Mg, from which Ca^{2+} ions dissociate to the lumen of the sarcoplasmic reticulum (Watanabe et al., 1981; Green et al., 1986; Brandl et al., 1986; Fernandez-Belda & Inesi, 1986). A slow conformational change in the reverse direction is thought to be responsible for passive Ca^{2+} efflux (Inao & Kanazawa, 1986). Previously, however, we have been unable to obtain evidence for the existence of separate E_1~P·Mg·Ca_2 and Ca_2·E_2−P·Mg species with a significant lifetime (Pickart & Jencks, 1982; Stahl & Jencks, 1987).

In this paper, we report another property of the enzyme that is not satisfactorily described in terms of the E_1−E_2 and related two-state models. The formation of ADP-sensitive phosphoenzyme from P_i with loaded vesicles proceeds with a rate constant of 25 s^{-1} and no detectable induction period. This rate constant is identical to the rate constant for the formation of phosphoenzyme from P_i with loaded vesicles; *i.e.*, the phosphoenzyme becomes ADP-sensitive as soon as it is formed from P_i and loaded vesicles. This result shows that there is no significant kinetic barrier for a conformational change that converts an ADP-insensitive Ca_2·E_2−P·Mg species to an ADP-sensitive E_1~P·Mg·Ca_2 species. We conclude that all of the phosphoenzyme with bound Ca^{2+}, Ca_2·E~P·Mg, is ADP-sensitive phosphoenzyme that loses its ADP-sensitivity only when Ca^{2+} dissociates from it into the lumen of the vesicles to form E−P·Mg, which is ADP-insensitive. The coupling between ATP hydrolysis and the transport of Ca^{2+} is brought about by alternating changes of chemical and vectorial specificities in the reaction cycle.

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

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EXPERIMENTAL PROCEDURES

Materials. MOPS, KCl, Tris, EGTA, and EDTA were purchased from Fluka, K•ADP and $CaCl_2$•$2H_2O$ were from Sigma, $MgCl_2$•$6H_2O$ was from Aldrich, and calcium ionophore A23187 was from Calbiochem. Carrier-free [^{32}P]P_i was obtained from New England Nuclear. All solutions were prepared with Milli-Q-grade water (Millipore Co.) and stored in polypropylene bottles.

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit back and hind leg white muscles by a slight modification of the procedure of MacLennan (1970) as described previously (Pickart & Jencks, 1982), and were stored at −80 °C. The SRV preparations were tightly sealed, as shown by a 20−25-fold increase in the steady-state activity of the Ca^{2+}-ATPase upon addition of the calcium ionophore A23187 to the standard assay solution. The amount of phosphoenzyme formed with saturating concentrations of either P_i, Mg^{2+}, and lumenal Ca^{2+} or ATP, Mg^{2+}, and cytoplasmic Ca^{2+} was 1−2.5 nmol•(mg of total protein)$^{-1}$.

Methods. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as protein standard. Carrier-free [^{32}P]P_i was treated as described previously (Myung & Jencks, 1994b). The tightly sealed SRV preparations were dialyzed at 4 °C overnight against 400 mL of solutions containing 0.4 M sucrose, 100 mM KCl, 40 mM MOPS/Tris, pH 7.0, and either no added $CaCl_2$, to give empty SRV, or 40 mM $CaCl_2$, to give passively loaded SRV.

The formation and disappearance of ^{32}P-labeled phosphoenzyme from [^{32}P]P_i were measured with a rapid-mixing-quench apparatus that can be used with three or four syringes, as described previously (Stahl & Jencks, 1984; Petithory & Jencks, 1988a). For each reaction, 10 μL of the stock solution of SRV was mixed with 0.99 mL of buffer solution and loaded into syringe A of the rapid-mixing-quench apparatus. Reactions were started within ~10 s. The solutions from syringes A and B were mixed and allowed to react in an aging tube; the reactions were quenched by the addition of quench solution from syringe C. Alternatively, the reactions were chased with a solution containing ADP and EDTA from syringe C for 4 ms and then quenched by the addition of quench solution from syringe D. Bovine serum albumin and KH_2PO_4 were added to the acid-quenched solutions to give final concentrations of ~0.3 mg/mL bovine serum albumin and ~25 mM KH_2PO_4; this was followed by the addition of trichloroacetic acid to give a final concentration of ~12% trichloroacetic acid (w/v).

The concentration of ^{32}P-labeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. (1978). The acid-quenched solutions were kept on ice not longer than 2 h and were then centrifuged at 1500*g* for 15 min at 4 °C. The supernatant solutions were decanted, and the pellets were resuspended in 5 mL of ice-cold 5% trichloroacetic acid and 10 mM KH_2PO_4. The proteins were collected by vacuum filtration with Whatman GF/C glass microfiber filters and were rinsed with 15 mL of resuspension solution. The filters had been soaked in resuspension solution containing ~50 mM KH_2PO_4. ^{32}P-Labeled phosphoenzyme was measured by liquid scintillation counting of the samples in glass vials containing ~7 mL of Aquasol-2.

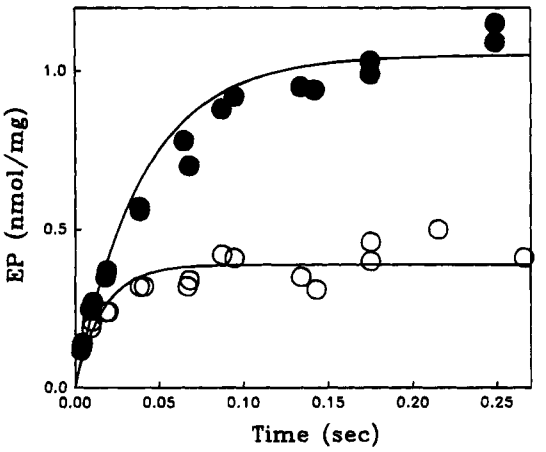


FIGURE 1: Kinetics for phosphorylation of the enzyme by P_i with empty SRV (○) and with SRV loaded with 40 mM Ca^{2+} (●). Phosphoenzyme was formed when the solutions from syringes A and B were mixed and allowed to react in an aging tube. The reaction was then quenched with the solution from syringe C. Syringes A and B contained 40 mM MOPS/Tris buffer, pH 7.0, 0.1 M KCl, and 10 mM EGTA at 25 °C. In addition, syringe A contained 1.02 mg/mL empty SRV or 0.96 mg/mL SRV loaded with 40 mM $CaCl_2$. Syringe B contained 8 mM [^{32}P]P_i and 40 mM $MgCl_2$. Syringe C contained 1.5 N HCl and 100 mM P_i. The solid lines were drawn for a rate constant of 60 s^{-1} and an end point of 0.39 nmol/mg (○) and a rate constant of 25 s^{-1} and an end point of 1.05 nmol/mg (●).

Scheme 1

$$E + P_i + Mg \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}P_i{\bullet}Mg \overset{K'_{int}}{\underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}}} E{-}P{\bullet}Mg$$

$$K$$

RESULTS

Kinetics for Phosphorylation of the Enzyme by P_i with Empty SRV and with Loaded SRV. Figure 1 shows the rate of phosphorylation of the enzyme upon the simultaneous addition of 4 mM [^{32}P]P_i and 20 mM $MgCl_2$ to empty SRV (○) and to tightly sealed SRV that were passively loaded with 40 mM Ca^{2+} (●). Phosphorylation of the enzyme in loaded SRV by P_i and Mg^{2+} proceeds with an observed first-order rate constant of 25 s^{-1}. Phosphorylation of the enzyme in empty SRV proceeds at essentially the same rate initially, but the reaction follows first-order kinetics with a much larger rate constant of 60 s^{-1} because the reaction is approaching equilibrium, instead of proceeding to completion.

Scheme 1 describes a simple model in which P_i and Mg^{2+} bind to the enzyme to form E•P_i•Mg, which then undergoes formation of a covalent phosphate bond to give E−P•Mg (Pickart & Jencks, 1984). The covalent step is largely rate-limiting for the formation of E−P•Mg, as shown by the value of k_p/k_{off} = 0.09 that was obtained from measurements of medium $P_i \rightleftarrows H_2O$ exchange (McIntosh & Boyer, 1983); *i.e.*, the covalent step is ~10 times slower than the dissociation of P_i. The absence of a detectable lag for phosphorylation shows that the binding of 4 mM P_i and 20 mM Mg^{2+} is fast. Thus, the observed rate constant of k_{obs} = 60 s^{-1} for phosphorylation of the enzyme by P_i with empty SRV may be assigned to approach to equilibrium for the covalent step, with the rate constants k_p and k_{-p}. Approximate values of k_p for the formation and k_{-p} for the hydrolysis of E−P•Mg were obtained by treating the reaction as an approach to equilibrium according to eqs 1−3 in which K_{obs} is the

$$K'_{obs} = \frac{[E{-}P{\cdot}Mg]}{[E] + [E{\cdot}P_i{\cdot}Mg]} = \frac{k_f}{k_r} \quad (1)$$

$$k_{obs} = k_f + k_r = k_f + k_{-p} \quad (2)$$

$$k_f = k_p \frac{[P_i][Mg]}{[P_i][Mg] + K} \quad (3)$$

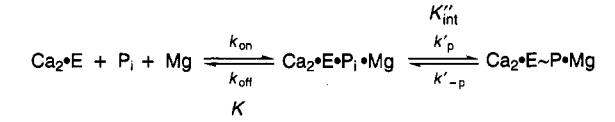
observed ratio of phosphorylated and unphosphorylated enzyme at equilibrium in the presence of a given concentration of P_i and Mg^{2+}, k_{obs} is the observed first-order rate constant for phosphorylation by P_i, and k_f and k_r are the pseudo-first-order rate constants for E−P·Mg formation and breakdown, respectively, under the conditions of the experimental measurements. The constants k_p, k_{-p}, and K are defined in Scheme 1. The observed first-order rate constant for approach to equilibrium is the sum of the pseudo-first-order rate constants k_f for the forward reaction and k_r for the reverse reaction (eq 2), and k_f is given by k_p for E−P·Mg formation multiplied by the fraction of the enzyme that is in the form of $E{\cdot}P_i{\cdot}Mg$, as shown in eq 3 (Fersht, 1985).

A value of $k_{-p} = 38\ s^{-1}$ was calculated for the hydrolysis of E−P·Mg, according to $K'_{obs} = (k_{obs} - k_{-p})/k_{-p}$ and the values of $k_{obs} = 60\ s^{-1}$ and $K'_{obs} = [E{-}P{\cdot}Mg]/([E] + [E{\cdot}P_i{\cdot}Mg]) = [E{-}P{\cdot}Mg]/([E]_{tot} - [E{-}P{\cdot}Mg]) = (0.39\ nmol/mg)/(1.05 - 0.39\ nmol/mg) = 0.6$. A value of $k_p = 26\ s^{-1}$ was calculated for the formation of E−P·Mg from eqs 2 and 3 and values of $k_{obs} = 60\ s^{-1}$, $k_{-p} = 38\ s^{-1}$, $[P_i] = 4$ mM, $[Mg^{2+}] = 20$ mM, and $K = K_{Mg} \times K_{P_i} = 8.7\ mM \times 1.5\ mM = 1.3 \times 10^{-5}\ M^2$. These values of K_{Mg} and K_{P_i} were determined under conditions very similar to those described here (Punzengruber et al., 1978). The value of $k_p = 26\ s^{-1}$ is similar to $k_p = 23\ s^{-1}$, which was calculated from $K'_{int} = [E{-}P{\cdot}Mg]/[E{\cdot}P_i{\cdot}Mg] = k_p/k_{-p}$, $k_{-p} = 38\ s^{-1}$, and $K'_{int} = 0.6$ (Punzengruber et al., 1978). Therefore, the rate constant for the formation of E−P·Mg from bound P_i was set to be $k_p = 25 \pm 2s^{-1}$.

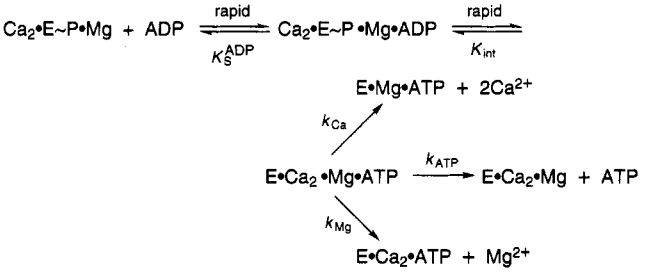
The observed rate constant for phosphorylation of the enzyme by P_i with loaded SRV may be separated into the rate constants k'_p for the formation and k'_{-p} for the hydrolysis of $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, as described above. We have shown previously that lumenal Ca^{2+} binds to the lumenal sites of both E and E−P·Mg, and that both sites are saturated at high concentrations of lumenal Ca^{2+} (Jencks et al., 1993; Myung & Jencks, 1994b). In Scheme 2, we designate enzyme that is saturated with 40 mM Ca^{2+} at the lumenal sites as $Ca_2{\cdot}E$. The simultaneous addition of P_i and Mg^{2+} to $Ca_2{\cdot}E$ results in rapid binding of P_i and Mg^{2+} to form $Ca_2{\cdot}E{\cdot}P_i{\cdot}Mg$, which then undergoes phosphorylation to give $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ (Scheme 2).

The observed first-order rate constant for approach to equilibrium is the sum of the pseudo-first-order rate constants k_f for the forward reaction and k_r for the reverse reaction, as described above for phosphorylation of the enzyme by P_i with empty SRV. Therefore, $k'_p = 25 \pm 1\ s^{-1}$ was calculated for the formation of $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ from bound P_i, from a value of $k_f = 24\ s^{-1}$ that was calculated for the forward reaction according to eq 2 and values of $k_{obs} = 25\ s^{-1}$ and $k'_{-p} \leq 1\ s^{-1}$. The value of k'_{-p} was set to be $\leq 1\ s^{-1}$ because Ca^{2+} inhibits the steady-state activity of the Ca^{2+}-ATPase in leaky SRV by binding to the phosphoenzyme to regenerate $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, which undergoes hydrolysis at a negligible rate under the conditions of these experiments (Souza & de Meis, 1976; Pickart & Jencks, 1982; Stahl & Jencks, 1987; Khananshvili et al., 1990).

Scheme 2



Scheme 3



It should be noted that the rate constants for phosphorylation of the enzyme by bound P_i of $k_p = 25 \pm 2\ s^{-1}$ with empty SRV and $k'_p = 25 \pm 1\ s^{-1}$ with loaded SRV are identical within experimental error, whereas the rate constants for hydrolysis of the phosphoenzyme of $k_{-p} = 38\ s^{-1}$ with empty SRV and $k'_{-p} \leq 1\ s^{-1}$ with loaded SRV are very different. Thus, it appears that the bound lumenal Ca^{2+} does not exert an effect on the rate until the enzyme is phosphorylated to form the phosphoenzyme.

Assay of $Ca_2{\cdot}E{\sim}P{\cdot}Mg$. The rate of formation of ADP-sensitive phosphoenzyme, $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, was measured by the addition of ADP and EDTA to phosphoenzyme that was formed from P_i and loaded SRV, followed in 4 ms by an acid quench. Under the conditions of the experiment, ADP binds rapidly to $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ to form $Ca_2{\cdot}E{\sim}P{\cdot}Mg{\cdot}ADP$; this is followed by rapid phosphoryl transfer to the bound ADP to give $E{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ that is at equilibrium with $Ca_2{\cdot}E{\sim}P{\cdot}Mg{\cdot}ADP$, as shown by K_{int} in Scheme 3 (Pickart & Jencks, 1982; Stahl & Jencks, 1987; Myung & Jencks, 1994a). After ATP is formed at the active site, Ca^{2+}, Mg^{2+}, and the ATP dissociate irreversibly in the presence of EDTA and no added ATP, so that rephosphorylation by bound ATP does not occur. Only the ADP-sensitive phosphoenzyme reacts with ADP to form ATP during the short chase with ADP and EDTA, so that the amount of phosphoenzyme disappearance corresponds to the amount of ATP that is formed; *i.e.*, the formation of ATP is a measure of the formation of ADP-sensitive phosphoenzyme.

Kinetics for the Formation of ADP-Sensitive Phosphoenzyme from P_i with Loaded SRV. Although it is well established that the phosphorylated Ca^{2+}-ATPase with bound Ca^{2+}, $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, is a "high-energy" species that reacts with ADP to form ATP (Knowles & Racker, 1975), the kinetics for its formation from P_i with loaded SRV have not been well characterized. If there is a significant kinetic barrier for the formation of ADP-sensitive phosphoenzyme from the phosphoenzyme that is initially formed upon the addition of P_i to loaded SRV, its formation will be biphasic.

Figure 2 shows the rate of formation of phosphoenzyme after the addition of 4 mM $[^{32}P]P_i$ and 20 mM Mg^{2+} to vesicles containing 40 mM Ca^{2+} (●), and the amount of phosphoenzyme that remains 4 ms after a chase with 10 mM

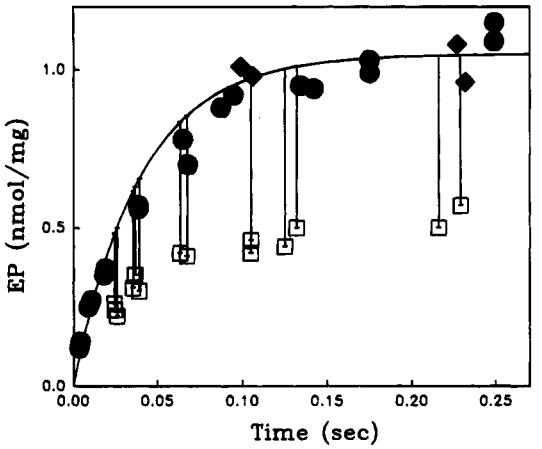


FIGURE 2: Reaction of phosphoenzyme that was formed with P_i and loaded SRV (●, Figure 1) with EDTA (♦) or ADP/EDTA (□). Phosphoenzyme was formed when the solutions from syringes A and B were mixed and allowed to react in an aging tube. The reaction was quenched with the solution from syringe C. Alternatively, the reaction was chased with the solution containing EDTA or ADP/EDTA from syringe C for 4 ms and then quenched with the solution from syringe D. The reaction time after the mixing of solutions from syringes A and B in an aging tube is plotted on the x axis. Syringes A and B contained 40 mM MOPS/Tris buffer, pH 7.0, 0.1 M KCl, and 10 mM EGTA at 25 °C. In addition, syringe A contained 0.96 mg/mL SRV loaded with 40 mM $CaCl_2$. Syringe B contained 8 mM [^{32}P]P_i and 40 mM $MgCl_2$. Syringe C contained 1.5 N HCl and 100 mM P_i (●). Alternatively, syringe C contained 60 mM EDTA (♦) or 30 mM ADP and 60 mM EDTA (□). Syringe D contained 2 N HCl and 100 mM P_i. The solid line was drawn for a rate constant of 25 s^{-1} and an end point of 1.05 nmol/mg. The vertical lines show the amount of phosphoenzyme disappearance in 4 ms after the chase with ADP/EDTA (□).

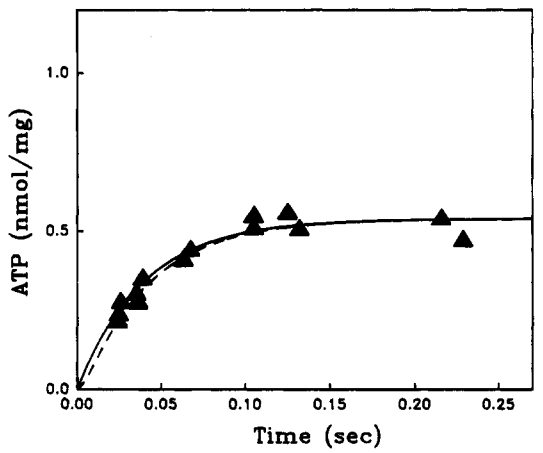


FIGURE 3: Kinetics for the formation of ATP. The amount of ATP that was formed from reaction of Ca_2•E~P•Mg with ADP (▲) was calculated from the amount of phosphoenzyme that disappeared after a chase with 10 mM ADP and 20 mM EDTA for 4 ms. The solid line was drawn for a rate constant of 25 s^{-1} and an end point of 0.54 nmol/mg, while the dashed line was drawn for rate constants of 25 s^{-1} and 250 s^{-1} and the same end point.

ADP and 20 mM EDTA (□). The amount of phosphoenzyme disappearance, shown as the vertical lines, corresponds to the amount of ATP formation. In contrast, no phosphoenzyme disappears when EDTA alone is added to the phosphoenzyme (♦). We conclude that the disappearance of phosphoenzyme with an ADP/EDTA chase results from the rapid reaction of phosphoenzyme, Ca_2•E~P•Mg, with ADP to form ATP.

Figure 3 shows the observed rate of formation of ATP upon the addition of P_i to loaded SRV. The solid triangles correspond to the vertical lines in Figure 2 and represent the amount of ATP that is formed very rapidly from Ca_2•E~P•Mg and ADP. The solid line shows that the formation of ATP and, therefore, of ADP-sensitive phosphoenzyme proceeds with a rate constant of 25 s^{-1} and no detectable induction period. This rate constant is identical to the rate constant for the formation of phosphoenzyme from the reaction of enzyme in loaded SRV with P_i. For comparison, the dashed line in Figure 3 was calculated for two consecutive irreversible first-order reactions with rate constants of 25 and 250 s^{-1}. We conclude that there is no significant kinetic barrier for the formation of ADP-sensitive phosphoenzyme from the phosphoenzyme that is initially formed from P_i and loaded SRV.

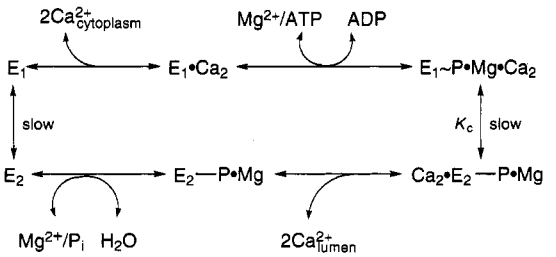
DISCUSSION

Lumenal Ca^{2+} Does Not Inhibit the Rate of Phosphorylation of the Enzyme by P_i. The rate constants for phosphorylation of the enzyme by bound P_i of $k_p = 25 \pm 2$ s^{-1} with empty vesicles and $k_p' = 25 \pm 1$ s^{-1} with loaded vesicles were obtained by treating the reaction as an approach to equilibrium, as described under Results. These identical values of k_p and k_p' show that lumenal Ca^{2+} does not inhibit the rate of phosphorylation of the enzyme by P_i.

This is in contrast to the strong inhibition by cytoplasmic Ca^{2+} of phosphorylation of the enzyme by P_i (Kanazawa & Boyer, 1973). It is well established that a strong interaction exists between the high-affinity cytoplasmic sites for Ca^{2+} and the catalytic site, although these sites are separated by at least 30 Å (Highsmith & Murphy, 1984; Scott, 1985; Toyoshima et al., 1993). The enzyme is phosphorylated by ATP only when cytoplasmic Ca^{2+} is bound to the high-affinity cytoplasmic sites, whereas it is phosphorylated by P_i only in the absence of cytoplasmic Ca^{2+} (Yamamoto & Tonomura, 1967; Makinose, 1969; Kanazawa & Boyer, 1973; Pickart & Jencks, 1984).

Chaloub et al. (1979) have reported that phosphorylation of the enzyme by P_i at pH 6.2 in the absence of KCl proceeds with rate constants of 30 s^{-1} with empty SRV and 2 s^{-1} with SRV that were actively loaded with Ca^{2+} in the presence of ATP, and concluded that lumenal Ca^{2+} inhibits the rate of phosphorylation of the enzyme by P_i. However, their finding that the same concentration of phosphoenzyme is formed at equilibrium from a saturating concentration of P_i with empty and actively loaded SRV is in contrast to the following findings: (1) Phosphorylation of the enzyme by P_i proceeds to different end points of 0.39 nmol/mg with empty SRV and 1.05 nmol/mg with passively loaded SRV (Figure 1). (2) The concentration of phosphoenzyme that is formed at equilibrium from P_i in the presence of 40 or 100 mM KCl increases as the concentration of lumenal Ca^{2+} in passively loaded SRV is increased, because lumenal Ca^{2+} binds to the phosphoenzyme (Yamada et al., 1972; Prager et al., 1979; Myung & Jencks, 1994b). Therefore, their finding shows that only a negligible amount of phosphoenzyme with bound Ca^{2+} was formed in actively loaded SRV in the absence of KCl. This may be a consequence of an increase in the equilibrium constant for dissociation of lumenal Ca^{2+} from the phosphoenzyme in the absence of KCl; Champeil and Guillain (1986) have shown that K^+ decreases the rate constant for dissociation of lumenal Ca^{2+}

Scheme 4



from the phosphoenzyme. Furthermore, Ca^{2+} may have been lost from the actively loaded SRV because they were subjected to centrifugation and resuspension after loading of the vesicles. Their finding that the rates of hydrolysis of phosphoenzyme with empty and actively loaded SRV were similar (Chaloub et al., 1979) suggests that the actively loaded SRV were not tightly sealed, because the hydrolysis of Ca_2•E~P•Mg in passively loaded vesicles is very slow. This suggestion is also supported by the report that the concentration of phosphoenzyme that was formed from P_i with actively loaded SRV decreased by 40% when the concentration of KCl was increased from 0 to 300 mM (Chaloub & de Meis, 1980), whereas it was stable in the same concentration range of KCl when the phosphoenzyme was formed with passively loaded SRV (Punzengruber et al., 1978).

Galina and de Meis (1991) have suggested that Ca^{2+} can bind to lumenal sites of the unphosphorylated enzyme and that this binding inhibits the phosphorylation of the enzyme by P_i by decreasing the concentration of the enzyme species that is reactive toward P_i. However, we have shown that lumenal Ca^{2+} does not inhibit the rate of phosphorylation of the enzyme by P_i (see Results); the enzyme species with lumenal Ca^{2+} bound, Ca_2•E, in Scheme 2, is reactive toward P_i and is phosphorylated by P_i with the same rate constant as the free enzyme species, E, in Scheme 1.

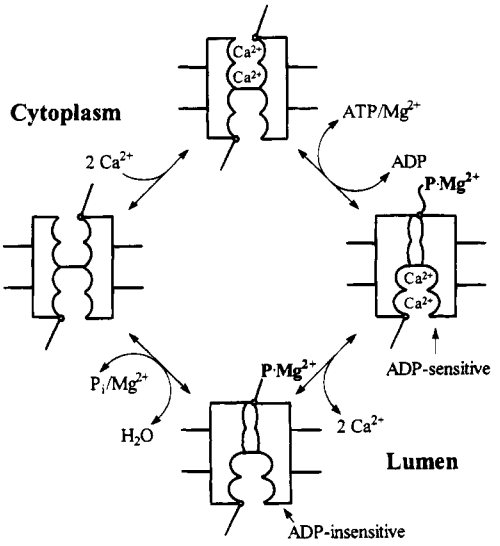
Phosphoenzyme Becomes ADP-Sensitive as Soon as It Is Formed from P_i with Loaded Vesicles. The active vectorial transport of Ca^{2+} ions catalyzed by the Ca^{2+}-ATPase has usually been described according to a model with two major conformational states of the enzyme, such as E and E*, E and E′, or E_1 and E_2 (de Meis & Vianna, 1979; de Meis, 1981, 1988; MacLennan, 1990); a typical E_1-E_2 model (Green et al., 1986) is shown in Scheme 4. According to several E_1-E_2 and related two-state models, one conformation, E_1, has cytoplasmic binding sites with a high affinity for Ca^{2+} and is reactive toward ATP, whereas another conformation, E_2, has lumenal binding sites with a low affinity for Ca^{2+} and is reactive toward P_i. Translocation of two Ca^{2+} ions occurs as the result of a rate-limiting conformational change from an ADP-sensitive E_1~P•Mg•Ca_2 to an ADP-insensitive Ca_2•E_2—P•Mg species (Watanabe et al., 1981; Green et al., 1986; Brandl et al., 1986; Fernandez-Belda & Inesi, 1986). A slow conformational change in the reverse direction is thought to be responsible for passive Ca^{2+} efflux (Inao & Kanazawa, 1986).

If a conformational change between an ADP-sensitive and an ADP-insensitive phosphoenzyme with bound Ca^{2+} is kinetically significant, as predicted by some E_1-E_2 and related two-state models, there will be a significant kinetic barrier for the formation of ADP-sensitive E_1~P•Mg•Ca_2 from the ADP-insensitive Ca_2•E_2—P•Mg species that is formed from the reaction of E_2 with P_i in the presence of lumenal Ca^{2+} (Scheme 4). In Figures 2 and 3, however, we have shown that with loaded vesicles the formation of phosphoenzyme and of ADP-sensitive phosphoenzyme from P_i proceeds with the same rate constant of 25 s^{-1} and no detectable induction period; *i.e.*, the phosphoenzyme becomes ADP-sensitive as soon as it is formed from P_i. This result shows that there is no significant kinetic barrier for the formation of ADP-sensitive phosphoenzyme from the phosphoenzyme that is initially formed from P_i with loaded SRV. This is not consistent with most E_1-E_2 and related two-state models.

Several investigators have shown that there is a biphasic reaction, with a rapid burst followed by a slow first-order disappearance of phosphoenzyme when ADP is added to phosphoenzyme that was formed from ATP in the presence of cytoplasmic Ca^{2+} (Pickart & Jencks, 1982; Froehlich & Heller, 1985; Wang, 1986; Stahl & Jencks, 1987). However, this biphasic reaction is not consistent with a rapid reaction of E_1~P•Mg•Ca_2 with ADP, followed by a slow conformational change that converts Ca_2•E_2—P•Mg to E_1~P•Mg•Ca_2, as shown in Scheme 4, because both the size of the initial burst and the rate constant of the slow phase increase proportionally as the concentration of ADP is increased (Pickart & Jencks, 1982). Furthermore, these increases follow a hyperbolic dependence on the concentration of ADP, and the dependence on ADP concentration is identical for the size of the burst and for the rate constant of the slow phase. This is not expected for the E_1-E_2 model because the concentration of an ADP-insensitive Ca_2•E_2—P•Mg species initially present, which would represent the slow phase of the reaction, should be independent of the concentration of added ADP. However, this hyperbolic dependence on the concentration of ADP is expected if the burst represents very rapid formation of enzyme-bound ATP that is at equilibrium with Ca_2•E~P•Mg and ADP; this is followed by dissociation of ATP or Ca^{2+} in a slow first-order reaction as shown in Scheme 3. Therefore, these experiments also give no indication for a conformational change between an ADP-sensitive E_1~P•Mg•Ca_2 and an ADP-insensitive Ca_2•E_2—P•Mg species that is kinetically significant (Pickart & Jencks, 1982; Stahl & Jencks, 1987).

We would like to understand whether there are two stable phosphoenzyme intermediates with bound Ca^{2+}, such as an ADP-sensitive E_1~P•Mg•Ca_2 and an ADP-insensitive Ca_2•E_2—P•Mg species, that can equilibrate rapidly. The dashed line in Figure 3 was calculated for phosphorylation through two consecutive irreversible first-order reactions with rate constants of 25 and 250 s^{-1}. If the rate constant of 25 s^{-1} is assigned to the formation of an ADP-insensitive Ca_2•E_2—P•Mg species from P_i with loaded SRV, then the rate constant of 250 s^{-1} would represent a conformational change from Ca_2•E_2—P•Mg to an ADP-sensitive E_1~P•Mg•Ca_2 species. The rate constant for the conformational change in the reverse direction can be set to be 30 s^{-1}, which is the rate constant for loss of ADP-sensitivity upon the addition of EGTA to phosphoenzyme formed from ATP and cytoplasmic Ca^{2+} in leaky vesicles (Hanel & Jencks, 1991). The value of K_c = [Ca_2•E_2—P•Mg]/[E_1~P•Mg•Ca_2] = 30 s^{-1}/250 s^{-1} would then be 0.12, and the reaction would favor the ADP-sensitive phosphoenzyme; *i.e.*, ~90% of the phosphoenzyme would be ADP-sensitive at equilibrium and would disappear rapidly upon the addition of excess ADP. This is not

Scheme 5



observed. Pickart and Jencks (1982) have shown that only 70% of the phosphoenzyme reacts rapidly with excess ADP to give ATP. The 3-fold difference between 30% and 10% phosphoenzyme remaining after the ADP chase is far larger than the experimental uncertainty of the data. Furthermore, the accumulation of an ADP-sensitive $E_1{\sim}P{\cdot}Mg{\cdot}Ca_2$ species would result in a thermodynamic barrier in the reaction cycle that would prevent turnover of the enzyme at a useful rate. Moreover, rapid equilibration between $E_1{\sim}P{\cdot}Mg{\cdot}Ca_2$ and $Ca_2{\cdot}E_2{-}P{\cdot}Mg$ would result in rapid leakage of Ca^{2+} ions from SRV through the phosphoenzyme intermediate because in principle Ca^{2+} can dissociate from cytoplasmic Ca^{2+} binding sites in the E_1 conformation. The Ca^{2+}-ATPase would then not be able to pump Ca^{2+} ions into the SR vesicles.

We conclude that there is no evidence for the existence of more than one species of phosphoenzyme with bound Ca^{2+} that has a significant lifetime, such as $E_1{\sim}P{\cdot}Mg{\cdot}Ca_2$ and $Ca_2{\cdot}E_2{-}P{\cdot}Mg$. All of the phosphoenzyme with bound Ca^{2+}, $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, is ADP-sensitive phosphoenzyme that loses its ADP-sensitivity only when Ca^{2+} dissociates from it into the lumen of the vesicles to form $E{-}P{\cdot}Mg$, which is ADP-insensitive. When ADP is added to $Ca_2{\cdot}E{\sim}P{\cdot}Mg$, it reacts with the phosphoenzyme at a rate that is too fast to measure, to give an equilibrium mixture of $E{\cdot}Ca_2{\cdot}Mg{\cdot}ATP$ and $Ca_2{\cdot}E{\sim}P{\cdot}Mg{\cdot}ADP$ as noted above (Pickart & Jencks, 1982).

The model shown in Scheme 5 describes a simple mechanism for catalysis of the transport of two Ca^{2+} ions coupled to the hydrolysis of ATP (Jencks et al., 1993; Myung & Jencks, 1994b). The first step in this mechanism is the binding of two Ca^{2+} ions to the high-affinity cytoplasmic sites, which activates the enzyme for phosphorylation by ATP. The second step is the formation of ADP-sensitive phosphoenzyme from ATP, which is coupled to the thermodynamically unfavorable translocation of the two Ca^{2+} ions from the high-affinity to the low-affinity sites. Translocation of Ca^{2+} ions in the reverse direction occurs only when ADP is phosphorylated by the ADP-sensitive phosphoenzyme to give ATP. The third step is dissociation of two Ca^{2+} ions from the ADP-sensitive phosphoenzyme into the lumen of the sarcoplasmic reticulum to give ADP-insensitive phosphoenzyme, which is the rate-limiting step of the overall reaction in the presence of saturating concentrations of ATP and cytoplasmic Ca^{2+} at pH 7. The final step is hydrolysis of the ADP-insensitive phosphoenzyme, which completes the transport cycle.

Why Is $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ a "High-Energy" Species? It has been proposed that $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ is a "high-energy" species that reats rapidly with ADP to form ATP as the result of mutual destabilization between Ca^{2+} bound at the transport site and the covalently bound phosphate group of the phosphoenzyme; this destabilization is relieved when the phosphate is transfered to ADP and the Ca^{2+} is stabilized by its much stronger binding to E in $E{\cdot}Ca_2$ (Jencks, 1980, 1989b, 1990; Pickart & Jencks, 1984). It is not possible to synthesize ATP from reaction of ADP with $E{-}P{\cdot}Mg$, the phosphoenzyme without bound Ca^{2+} that is formed at equilibrium from P_i and Mg^{2+} with leaky or empty SRV (Beil et al., 1977). We would like to understand the mechanism of this apparent destabilization of $Ca_2{\cdot}E{\sim}P{\cdot}Mg$. It is not likely that there is direct physical contact between the phosphoryl group and the Ca^{2+} ions because several measurements indicate that the phosphorylation and transport sites are separated by at least 30 Å (Highsmith & Murphy, 1984; Scott, 1985; Toyoshima et al., 1993). Several investigators have proposed that there is a long-range interaction between the phosphorylation site and the transport site for the two Ca^{2+} ions and that phosphorylation of the enzyme brings about a conformational change, which perturbs the transport site and decreases its affinity for Ca^{2+} (Clarke et al., 1989; Inesi & Kirtley, 1990; Inesi et al., 1990).

Recently, however, we have concluded that the change in the affinity for Ca^{2+} upon phosphorylation of the enzyme is brought about by movement of the two Ca^{2+} ions from the high-affinity cytoplasmic sites of $E{\cdot}Ca_2$ to a different pair of binding sites with a low affinity for Ca^{2+} in $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ (Jencks et al., 1993; Myung & Jencks, 1994b). Measurements of phosphoenzyme formation at equilibrium in the presence of different concentrations of P_i, Mg^{2+}, and lumenal Ca^{2+} have provided evidence that the Ca^{2+}-ATPase has two pairs of Ca^{2+} binding sites, and that two Ca^{2+} ions are forced to move from a pair of high-affinity cytoplasmic sites to a different pair of low-affinity lumenal sites when the enzyme is phosphorylated by ATP. In the reverse direction, the movement of two Ca^{2+} ions from the low-affinity sites, with millimolar dissociation constants, to the high-affinity sites, with micromolar dissociation constants, results in a favorable change in $\Delta G^{\circ\prime}$ of approximately -8 kcal/mol. This movement of two Ca^{2+} ions from low-affinity to high-affinity sites provides a large driving force, which accounts for the high-energy nature of $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ and allows it to react with ADP to give ATP and $E{\cdot}Ca_2$.

The notion that a Ca^{2+} gradient across the membrane of SR vesicles is required for the formation of ATP by the Ca^{2+}-ATPase could be misleading, because a significant amount of ATP is formed when ADP and millimolar Ca^{2+} are added to phosphoenzyme that was formed at equilibrium from leaky SRV and P_i (Knowles & Racker, 1975). Furthermore, Stahl and Jencks (1987) have shown that the burst of ATP formation has the same size upon the addition to $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ of 1.1 mM ADP and six different concentrations of free Ca^{2+} in the range of $0.01{-}50\ \mu M$. This shows that the same concentration of ATP is formed in the presence of different Ca^{2+} gradients across the membrane of the SR vesicles. We conclude that the formation of ATP from $Ca_2{\cdot}E{\sim}P{\cdot}Mg$ and ADP is accounted for by the driving force that is provided by the movement of two Ca^{2+} ions from the low-affinity

lumenal Ca^{2+} binding sites, with millimolar dissociation constants, to the high-affinity cytoplasmic Ca^{2+} binding sites, with micromolar dissociation constants, as described above. It is still not clear just how this driving force is utilized to bring about ATP synthesis at a rate that is too fast to measure, when ADP is added to phosphoenzyme in Ca^{2+}-loaded vesicles.

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