Role of Divalent Cation Bound to Phosphoenzyme Intermediate of Sarcoplasmic Reticulum ATPase*

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Effect of divalent cations bound to the phosphoenzyme intermediate of the ATPase of sarcoplasmic reticulum was investigated at 0 °C and pH 7.0 using the purified ATPase preparations. Our previous study (Shigekawa, M., Watabayashi, S., and Nakamura, H. (1983) J. Biol. Chem. 258, 14157–14161) indicated that 1 mol of the ADP-sensitive phosphoenzyme (E,P) formed from CaATP has 3 mol of high affinity binding sites for Ca2+, of which two are transport sites for calcium while the remainder is the acceptor site for calcium derived from the substrate, CaATP (“substrate site”). When incubated with a chelator of divalent cation, the transport sites were dissociated from the substrate site and one of the divalent cations was released. Evidence was presented indicating that this divalent cation is the acceptor site for the metal component derived from the substrate, the metal-ATP complex, and that Mg2+ binding to this site is essential for rapid hydrolysis of the phosphoenzyme intermediate (6).

It is well known that free Ca2+ is required for activation of the ATPase of sarcoplasmic reticulum (1, 2). Two mol of calcium/each mol of the ATP-dependent phosphorylation site are bound with high affinity by the transport sites of the enzyme, which leads to phosphorylation of the enzyme by ATP (1, 2, 7–9). Calcium bound at the transport sites on the phosphoenzyme is subsequently released before the phosphoenzyme is hydrolyzed (5, 9–12). These processes of binding and release of calcium studied with fragmented membrane preparations or purified enzyme preparations are considered to represent reactions physiologically involved in the transport of calcium across the sarcoplasmic reticulum membranes (1, 2). To describe the minimum requirement of divalent cation for the normal turnover of the ATPase of sarcoplasmic reticulum, a following simple reaction sequence may be proposed (6).

The ATPase of sarcoplasmic reticulum utilizes CaATP as well as MgATP as substrates (1–5). The reaction sequences for ATP hydrolysis with these substrates were found to be basically the same although the rates of the overall and some of the partial reactions catalyzed by the enzyme were much slower with CaATP than with MgATP (5). It was shown previously (5, 6) that during ATP hydrolysis 1 mol of divalent cation remains bound to the all forms of phosphoenzyme intermediate with high affinity until the phosphoenzyme is hydrolyzed. Evidence was presented indicating that this di-

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EXPERIMENTAL PROCEDURES

Sarcoplasmic reticulum vesicles and purified ATPase preparations were obtained from rabbit skeletal muscle as described previously (5). The ATPase preparations were frozen in liquid N₂ and stored at -80 °C. Calcium and magnesium contaminating the purified ATPase preparations were 9.1 to 11.7 nmol of calcium/mg of protein and less than 0.3 nmol of magnesium/mg of protein, respectively, as estimated by atomic absorption spectrometry. As all the calcium in the ATPase preparation was found to be exchangeable (6), calcium contaminating the ATPase preparation was taken into account for calculation of the total Ca²⁺ participating in the reaction. Alkaline metal cations were removed from the ATPase preparations by passage through cation-exchange resins as described previously (5). ATP regeneration was started by addition of [γ-³²P]ATP to the reaction medium and terminated by addition of trichloroacetic acid solution (final concentration, 5% w/v) containing 0.1 M NaCl and 1.0 M AMP. After pelleting the denatured protein, an aliquot was taken from the supernatant for determination of ³²P and [γ-³²P]ATP. The amounts of ³²P and [γ-³²P]ATP were determined as described previously (10). Phosphoenzyme levels were measured as described previously (6). The Ca²⁺-dependent ATPase activity and Ca²⁺-dependent phosphoenzyme levels were estimated by subtracting the ATPase activity and phosphoenzyme levels in 2.5 to 5.0 mM EGTA from those obtained in the presence of Ca²⁺. The reaction mixture for the ATPase reaction contained 0.1 M glucose like that used for the calcium-binding experiment. Glucose at this concentration did not affect the ATPase activity and phosphoenzyme level. Amounts of the ADP-sensitive (E₃P) and ADP-insensitive, K⁺-sensitive (E₅P) components of phosphoenzyme, which are the normal reaction intermediates of ATP hydrolysis, were measured without the use of EGTA or EDTA as described previously (5, 6, 14). The amount of E₃P was estimated by extrapolating the exponential time course of the ³²P-phosphoenzyme decay in excess nonradioactive ATP and excess ADP to the time of addition of ATP and ADP or by subtracting the amount of ³²P liberated during a 5-s period after the addition of nonradioactive ATP and ADP from that of the ³²P-phosphoenzyme which disappeared during the same period.

The active site concentration in the ATPase preparation was estimated from the level of phosphoenzyme obtained 5 to 10 s after the start of phosphorylation in 60 mM imidazole/HCl (pH 7.0), 15 mM MgCl₂, 100 mM CaCl₂, 0.3 mM KCl, and 100 mM [γ-³²P]ATP at 0 °C. The phosphoenzyme level obtained under these conditions can be considered as the active site concentration as shown previously (6).

Calcium binding to the ATPase protein was assayed either by the double membrane filtration method or the single membrane filtration method. In the former method, which was described in detail in Refs. 5 and 6, sets of two membrane filters (upper, GS 0.22-μm Millipore filter; lower, SS 3.0-μm Millipore filter) and the double-labeling technique ([CaCl₂ and D-³¹P]glucose) were used. After filtration of the reaction mixture, the enzyme-bound radioactivity trapped on the filter was counted. The method, which was appropriate for the measurement of the tightly bound calcium, exhibited rather poor time resolution for the fast calcium release but allowed a small amount of the enzyme-bound calcium to be determined accurately.

The ATPase preparation was also described in Ref. 5, the reaction mixture containing 4CaCl₂ and the ATPase protein was filtered through a single membrane filter (0.45-μm Millipore filter). The filter was then washed with a solution containing EDTA, and the enzyme-bound radioactivity trapped on the filter was counted. The method, which was used for the measurement of the tightly bound calcium, exhibited rather poor time resolution for the fast calcium release but allowed a small amount of the enzyme-bound calcium to be determined accurately.

When MgCl₂ was added to the reaction mixture without ATPase protein, there were no detectable ³²P-containing cations released from the ATPase preparation. The concentrations of isolated Ca²⁺ and Mg²⁺ in the presence of nucleotides and metal chelators were calculated as described previously (5). Protein concentration was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard. Na₂ATP was purchased from Boehringer Mannheim GmbH, Biochemica. Na₂ADP, Tris, creatine phosphokinase (type I), creatine phosphate (di-Tris salt), pyruvate kinase (type III), and phosphoenolpyruvate (monopotassium salt) were purchased from Sigma. All the other reagents used were of analytical grade. Tris-ATP, Tris-ADP, and [γ-³²P]ATP were prepared as described previously (5). Glassware used for calcium-binding experiment was washed with 1 to 2 N HCl and then rinsed thoroughly with deionized water.

Curve fitting of the experimental data in Figs. 8 and 10 was performed by an iterative least squares method using routines contained in SALS, a computer program which was developed by the Computer Center, The University of Tokyo.

RESULTS

Dissociation of Calcium from Phosphoenzyme Formed from CaATP

It was shown previously (4, 5) that CaATP serves as a substrate for the ATPase in the absence of added Mg²⁺. When CaATP was used as a substrate, high salts such as KCl and Tris-Cl strongly inhibited the E3P to E2P conversion and thus stabilized E3P during the steady state ATP hydrolysis (cf. Table I in Ref. 5). E3P and E2P are defined in Scheme 1 under the Introduction and their levels were measured as described under "Experimental Procedures." In 0.3 mM Tris-Cl, 24 to 104 mM CaCl₂, 0.67 to 5.4 mM [γ-³²P]ATP, and no added KCl at 0 °C and pH 7.0, E3P constituted 82 to 91% of the phosphoenzyme formed in the steady state conditions (cf. Fig. 2). Therefore, E3P could be isolated without the use of KCl. This enabled us to study in some of the following experiments the effect of KCl on the property of the "divalent cation-free" phosphoenzyme which was isolated from E3P formed from CaATP.

In the experiment of Fig. 1, the ATPase was phosphorylated by 65.0 μM ATP in 8.7 mM KCl and 0.3 mM Tris-Cl and in the absence of added Mg²⁺. The steady state level of calcium binding observed in 18 μM ionized Ca²⁺ was about 14.2 nmol/mg of protein while the steady state level of phosphoenzyme was 4.78 nmol/mg of protein with E3P constituting 93% of the phosphoenzyme. The active site concentration in the ATPase preparation used in this experiment was estimated to be 5.2 nmol/mg of protein (see "Experimental Procedures"). If we assume that the major enzyme states accumulating under these conditions were the enzyme-ATP complex (E₃), E₃P and E₂P, the observed level of calcium binding is consistent with our previous conclusion (6) that the stoichiometries of the high affinity calcium binding are 3, 3, and 1 mol/each mol of E₃, E₃P and E₂P, respectively. When EDTA was added to the reaction medium at 30 s after the start of the ATPase reaction, almost all the calcium was released from the enzyme with a half-time of 8 s while the phosphoenzyme level decreased only slightly. Almost completely with the calcium release, the ADP sensitivity of the phosphoenzyme was lost (Fig. 1). The time course of the ADP sensitivity loss was followed by adding excess ADP to the reaction medium at appropriate intervals after the addition of EDTA and by measuring the amount of the phosphoenzyme remaining after the following 5 s. Under the conditions used in this experiment, the phosphoenzyme which disappeared after the addition of ADP reacted with added ADP to form ATP (cf. Figs. 2 and 3). When the same type of experiment was carried out in 0.309 mM KCl, the time courses for the EDTA-induced calcium release and the ADP sensitivity loss of the phosphoenzyme were similar to those observed in Fig. 1 although the phosphoenzyme decay in 0.309 mM KCl proceeded at a rate approximately 6 times greater than that.
observed in Fig. 1 (data not shown) (cf. Fig. 6). When the phosphoenzyme was formed in 0.3 M Tris-Cl and in the absence of added KCl and was then treated with EDTA, the time courses of the calcium release and the ADP sensitivity loss were again similar to those observed in Fig. 1 except that a small amount of calcium remained bound to the enzyme even after long incubation with EDTA. When the enzyme-bound calcium was measured using the single membrane filtration method instead of the double membrane filtration method to improve the precision of the measurement (see "Experimental Procedures"), its amount corresponded to approximately 6% of the phosphoenzyme at 1.5 min after the addition of EDTA (data not shown). This small amount of calcium was probably bound to the residual EP as discussed below. In these experiments, the results were the same whether EGTA or EDTA was used.

In the experiment of Fig. 2, the time course of the EGTA-induced ADP sensitivity loss of the phosphoenzyme formed in 0.3 M Tris-Cl and no added KCl was followed by adding either ADP alone or a mixture of ADP and MgCl₂. The ADP sensitivity loss was checked by the simultaneous measurement of ATP formation and phosphoenzyme disappearance. As shown in the figure, the time course of the ADP sensitivity loss was significantly delayed when the ADP sensitivity was examined by the use of ADP plus MgCl₂, the time required for a half-maximum loss of the ADP-sensitivity (t₅₀) being ~16 s with 0.74 mM ADP and 1.03 mM ionized Mg²⁺ as compared with 4 -- 5 s for 0.74 mM ADP alone. The result indicates that added Mg²⁺ partially restored the ADP reactivi-
FIG. 3 (left). Partial restoration of ADP sensitivity of the phosphoenzyme by added CaCl₂ or added MgCl₂. The ATPase protein (1.05 mg/ml) was phosphorylated at 0°C in 40 mM imidazole/HC1 (pH 7.0), 0.3 M Tris-Cl, 0.1 mM glucose, 33.3 mM CaCl₂, and 0.810 μM [γ-32P]ATP. At 60 s after the start of phosphorylation, EGTA was added to a final concentration of 1.18 mM to induce the ADP sensitivity loss of E,P. Fifteen s thereafter (1), the following additions were made and then time courses of the phosphoenzyme decomposition (open symbols), [γ-32P]ATP formation (filled symbols), and 32P liberation (half-filled symbols) were measured. Additions (final concentrations): α, 0.165 mM ATP and 0.75 mM ADP; β, 0.165 mM ATP, 0.75 mM ADP, and 1.105 mM CaCl₂ (final ionized Ca²⁺ concentration, 1.12 μM); γ, 0.165 mM ATP, 0.75 mM ADP, and 1.364 mM CaCl₂ (final ionized Ca²⁺ concentration, 100 μM); δ, 0.165 mM ATP, 0.75 mM ADP, and 1.5 mM MgCl₂ (final ionized Mg²⁺ concentration, 1.03 mM). For the data shown by the broken lines, KCI was further added to a final concentration of 0.1 M after the additions of ATP, ADP, and either CaCl₂ or MgCl₂ (β), and then the phosphoenzyme disappearance and Pₗ liberation in the presence of 100 μM ionized Ca²⁺ (γ, δ) or 0.1 mM ionized Mg²⁺ (β, δ) were measured. Each point is an average of duplicate determinations. Deviation of these determinations were less than 2% of the average for the levels of 32P-phosphoenzyme and [γ-32P]ATP while it was less than 6% of the average for the level of 32P.

FIG. 4 (center). Displacement with nonradioactive Ca²⁺ of the radioactive calcium bound to E,P formed from Ca₂⁺ATP. The ATPase protein (1.41 mg/ml) was phosphorylated at 0°C in 60 mM imidazole/HC1 (pH 7.0), 0.309 M KCl, 0.1 mM [3H]glucose, 62.1 μM ⁴⁶CaCl₂, 0.1 mM ATP, 0.44 mg/ml of creatine phosphokinase, 5.0 mM creatine phosphate. The phosphoenzyme decomposition exhibited a single exponential time course at least up to 70% of the decay, and then the time course of dissociation of the enzyme-bound ⁴⁶Ca was measured by the double membrane filtration method as described under "Experimental Procedures" (©). 3 mol of ⁴⁶Ca bound/mol of E,P. According to our previous data (6), two-thirds of the ⁴⁶Ca label bound by E,P under these conditions was bound to the transport sites while the remaining one-third of the label was bound to the acceptor site for the metal component derived from the substrate, the metal-ATP complex. When nonradioactive Ca²⁺ was added at 60 s after the start of the reaction, the ⁴⁶Ca label bound to the enzyme disappeared biphasically (Fig. 4). The rapidly disappearing fraction, which was estimated by extrapolating the time course for the slowly disappearing fraction to the time of addition of nonradioactive Ca²⁺, accounted for approximately one-third of the total label bound to the enzyme. In addition, the time required for displacing 50% of the label from the rapidly disappearing fraction was 4–5 s, which was similar to that required for inducing 50% loss of the ADP sensitivity of E,P under similar conditions (see above). The remaining two-thirds of the ⁴⁶Ca label bound to the enzyme disappeared very slowly, an approximate rate constant calculated from its apparently monoeponential time course being 0.16 min⁻¹. Decomposition of the phosphoenzyme was also measured under the same experimental conditions except that a steady state level of phosphoenzyme was reached with 0.32 mg/ml of the ATPase protein in 3.0 mM [γ-32P]ATP and then new formation of ³²P-phosphoenzyme was interrupted by the addition of 0.49 mM nonradioactive ATP which had been pretreated with creatine phosphokinase and creatine phosphate. The phosphoenzyme decomposition exhibited a single exponential time course at least up to 70% of the decay, its rate constant (0.23 min⁻¹) being similar to that for the slow calcium displacement observed above. The results presented in Fig. 4 were obtained with ⁴⁶Ca and similar dissociation time courses were obtained with ⁵⁷Ca. Dissociation of Calcium from Phosphoenzyme Formed from Mg₂⁺ATP—In the experiment of Fig. 5, EGTA-induced calcium dissociation from the ATPase was measured after the phosphoenzyme was formed in 0.12 mM ATP and either in 5 mM MgCl₂ and 0.109 mM KCl or in 20 mM MgCl₂ and 0.309 mM KCl. As in the experiment of Fig. 4, the ATP-regenerating system was included in the reaction mixture to minimize the ADP level so that reversal of enzyme phosphorylation could be prevented. Under the conditions used, 8.9 to 9.5 nmol of calcium/mg of protein was bound by the enzyme in the steady state conditions. Upon addition of excess EGTA, calcium dissociation proceeded almost monoeptunistically with rate...
concentration, constants of 5.71 and 1.81 min\(^{-1}\) in 5 mM MgCl\(_2\) and 0.109 mM KCl, and 20 mM MgCl\(_2\) and 0.309 mM KCl, respectively. Under these conditions, all of the active sites (taken to a concentration, 5.21 nmmol/mg of protein) was phosphorylated and 87 and 97% of the phosphoenzyme were found to be E,P in the former and the latter conditions, respectively. The observed steady state level of calcium binding, therefore, was consistent with the previous findings (6–9, 12, 17) that 2 mol of calcium are bound by 1 mol of E,P in the presence of high Mg\(^{2+}\). Decomposition of the phosphoenzyme was measured under exactly the same conditions as those used for calcium dissociation. Upon addition of EGTA, the phosphoenzymes formed either in 5 mM MgCl\(_2\) and 0.109 mM KCl or in 20 mM MgCl\(_2\) and 0.309 mM KCl decomposed monoexponentially at least up to 80% of their decay time courses with rate constants of 5.1 and 1.76 min\(^{-1}\), respectively. In either condition, the phosphoenzyme decomposition was accompanied by a corresponding amount of Pi liberation (data not shown). Thus, the rate constant for the calcium dissociation agreed with that for the phosphoenzyme hydrolysis under these two different conditions. It should be noted that the rate constants for calcium dissociation and phosphoenzyme hydrolysis obtained in 20 mM MgCl\(_2\) and 0.309 mM KCl were about one third of those obtained in 5 mM MgCl\(_2\) and 0.109 mM KCl. As the rate constants obtained in 5 mM MgCl\(_2\) and 0.309 mM KCl or 20 mM MgCl\(_2\) and 0.109 mM KCl were 65 to 70% of those obtained in 5 mM MgCl\(_2\) and 0.109 mM KCl, high concentrations of both of these agents were required for the pronounced inhibitory effect.

Reactivity with Ca\(^{2+}\) and/or K\(^{+}\) of Divalent Cation-Free Phosphoenzyme—As described above, E,P formed from CaATP readily released its bound calcium after the treatment with excess EGTA or EDTA. In the following experiments, the ATPase which was phosphorylated in 0.3 mM Tris-Cl but in the absence of added Mg\(^{2+}\) and added KCl was incubated in excess EGTA or EDTA for longer than 1.5 min (cf. Fig. 1). The phosphoenzyme treated in this way was stable and decomposed at a very slow rate, its rate constant estimated from its apparently single exponential time course being about 0.04 min\(^{-1}\) (Fig. 6). When KC1 at 100 and 200 mM was added to the phosphoenzyme, the rate constant for the phosphoenzyme decay increased to approximately 5- and 10-fold greater values, respectively (Fig. 6). This effect of KC1 was not mimicked by the same concentrations of either choline Cl or Tris-Cl. It was noted that the KC1-induced phosphoenzyme decay was faster in the initial phase than later in the time course (Fig. 6). As the phosphoenzyme decay was accompanied by liberation of a corresponding amount of Pi, the initial phosphoenzyme decay presumably arose from hydrolysis of a small amount of the residual E,P which retained its tightly bound calcium after long incubation in the presence of excess EGTA but in the absence of KCl (see above and Ref. 5). After the 1.5- and 2.5-min incubation with EGTA, such KC1-induced initial drop accounted for about 8 and 4% of the observed phosphoenzyme levels (cf. Fig. 6). Acceleration of hydrolysis of the divalent cation-free phosphoenzyme by KCl is in accord with the present finding that the phosphoenzyme which was formed in 0.309 mM KCl and then treated with excess EGTA for longer than 1.5 min decomposed at a rate approximately 13 times greater than that for the divalent cation-free phosphoenzyme formed in 0.3 mM Tris-Cl.

The divalent cation-free phosphoenzyme formed in 0.3 mM Tris-Cl was made reactive with added ADP to form ATP by addition of high Ca\(^{2+}\) (final ionized concentration, 4.17 mM) but not significantly by addition of low Ca\(^{2+}\) (final ionized concentration, 28.4 \(\mu\)M) (Fig. 7). The ionized Ca\(^{2+}\) up to 100 \(\mu\)M was not effective. Addition of high ionized Mg\(^{2+}\) (5 mM) also did not convert the ADP-insensitive, divalent cation-free phosphoenzyme to the ADP-sensitive form. Upon addition of low Ca\(^{2+}\) (final ionized concentration, 28.4 \(\mu\)M), the phosphoenzyme decomposed to yield P\(_i\) at a rate much greater than that obtained in the absence of added Ca\(^{2+}\). When a mixture of ionized Ca\(^{2+}\) and KCl was added to final concentrations of 30.7 \(\mu\)M and 83.4 \(\mu\)M, respectively, the phosphoenzyme 160 180

**FIG. 7. Effect of added CaCl\(_2\) on the reactivity of the divalent cation-free phosphoenzyme with ADP or KCl.** The ATPase protein (0.13 mg/mg) was phosphorylated at 0 °C in 30 mM imidazole/HCl (pH 7.0), 0.3 M Tris-Cl, 0.1 M glucose, 33.4 mM CaCl\(_2\), and 0.801 \(\mu\)M \([\gamma-32P]\)ATP. At 60 s after the start of phosphorylation, EGTA was added to a final concentration of 1.18 mM to induce calcium dissociation from E,P. At 90 s after the addition of EGTA, a mixture of ADP and nonradioactive ATP was further added to final concentrations of 0.453 and 0.147 mM, respectively (i), to prevent new formation of \(32P\)-phosphoenzyme. At 5 s thereafter (i), the following additions were made and then the time courses of phosphoenzyme decomposition (A), \([\gamma-32P]\)ATP formation (C), and \(32P\) liberation (B) were followed. Additions (final concentrations): O, H\(_2\)O; A, 1.19 mM CaCl\(_2\) (ionized Ca\(^{2+}\), 28.4 \(\mu\)M); \(\Delta\), 5.79 mM CaCl\(_2\) (ionized Ca\(^{2+}\), 4.17 \(\mu\)M); V, 1.19 mM CaCl\(_2\) plus 83.4 mM KCl (ionized Ca\(^{2+}\), 30.7 \(\mu\)M).
Fig. 8. Reactivity of the divalent cation-free phosphoenzyme with added MgCl2 studied in the absence of KCl. The ATPase protein (0.603 mg/ml) was phosphorylated at 0°C in 40 mM imidazole/HCl (pH 7.0), 0.3 M Tris-Cl, 0.1 M glucose, 53.5 μM CaCl2, and 5.23 μM [γ-32P]ATP. At 30 s after the start of phosphorylation, EGTA was added to a final concentration of 2.76 mM to induce calcium dissociation from E·P (I). At 90 s thereafter (I), various concentrations of MgCl2 were added to the reaction mixture to final ionized concentrations indicated, and then the time courses of phosphoenzyme decomposition were followed. The solid lines drawn through the experimental data were the simulated time courses of the phosphoenzyme decay which were calculated using the mathematical equation obtained by solving the differential equations derived from Scheme 2 and following rate constants: k1, 65 m−1 s−1; k−1, 0.0047 s−1; k2, 0.024 s−1; k3, 0.00055 s−1.

zyme was hydrolyzed at a rate 7.4-fold as great as that obtained in low Ca2+ alone. In these experiments a mixture of nonradioactive ATP and ADP was added 5 s before the addition of divalent cations to prevent new formation of 32P-phosphoenzyme due to added Ca2+. When nonradioactive ATP and ADP were added together with divalent cation, the results were almost the same as in Fig. 7.

Reactivity with Mg2+ of Divalent Cation-Free Phosphoenzyme—The phosphoenzyme which was formed in 0.3 M Tris-Cl and then made divalent cation-free by excess EGTA decomposed at a faster rate when MgCl2 was added to the reaction medium (Fig. 8). The phosphoenzyme decomposition was accompanied by a corresponding amount of P1 liberation as in the case of the phosphoenzyme decomposition induced by addition of KCl or low Ca2+ (cf. Fig. 7). The Mg2+-induced phosphoenzyme decomposition exhibited an initial induction period. The time courses of the phosphoenzyme decomposition after addition of various concentrations of MgCl2 were simulated reasonably well using following reaction sequence where EP is the divalent cation-free phosphoenzyme, and a following set of rate constants: k1 = 65 m−1 s−1, k−1 = 0.0047 s−1, k2 = 0.024 s−1, k3 = 0.00055 s−1 (Fig. 8).

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\begin{align*}
EP + Mg^{2+} &\xrightarrow{k_1} EP Mg^{2+} \\
&\xrightarrow{k_2} E + Mg^{2+} + P1
\end{align*}
\]

**Scheme 2**

k3 was determined experimentally from the time course for decomposition of the divalent cation-free phosphoenzyme observed in the absence of added Mg2+. The values of k1, k−1, and k2 were estimated by curve fitting using an iterative least squares method as described under “Experimental Procedures.” The estimated value for k3 should be correct because at high added Mg2+ (>1.5 mM), the k5 value significantly different from this value gave a poor fit to the experimental data. The small values of k3, k−1, and k2 indicate that Mg2+ binding to and dissociation from the divalent cation-free phosphoenzyme as well as hydrolysis of the magnesium-phosphoenzyme complex were very slow. Slow dissociation of Mg2+ from the magnesium-phosphoenzyme complex was also suggested in the experiment of Fig. 9. In this experiment, the divalent cation-free phosphoenzyme was allowed to react with added Mg2+ (final ionized concentration, 1.92 mM) for 20 s and then excess EDTA was added to chelate free Mg2+. The phosphoenzyme treated in this way decomposed at a rate much faster than that for the divalent cation-free phosphoenzyme, indicating that Mg2+ remained bound to the phosphoenzyme even in the presence of excess EDTA. When KCl was added to a final concentration of 0.1 M at appropriate intervals after the addition of EDTA, the phosphoenzyme decomposed rapidly and then leveled off at a much slower rate. The amount of slowly decomposing fraction of the phosphoenzyme was estimated by extrapolating its decay time course to the time of addition of KCl. The amounts of rapidly decomposing fractions were estimated by subtracting the amount of slowly decomposing fractions from the total amounts of the phosphoenzyme present at the time that KCl was added. The amount of the rapidly decomposing fraction decreased with time whereas the amount of the slowly decomposing fraction almost remained constant up to 100 s after the additions of EDTA (Fig. 9). When the logarithm of the amount of the rapidly decomposing fraction was plotted against the time after addition of EDTA, the plot was found to be linear and the rate constant of 1.3 min−1 was calculated from the slope of the plot. This value was similar to the value of 1.4 min−1.
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with the experimental data obtained in the presence of various concentrations of ionized Mg\textsuperscript{2+} (Fig. 10). The value of \( k_0 \) used in this simulation was similar to those obtained from the apparently monoexponential time courses of the initial portion of the rapid phosphoenzyme decay observed in the presence of high ionized Mg\textsuperscript{2+} (2.51 mM) and 0.1 mM KCl regardless of whether MgCl\textsubscript{2} was added 20 s before KCl addition (cf. Fig. 9) or KCl was added 30 s before MgCl\textsubscript{2} addition (cf. Fig. 10). Comparison of the results of Figs. 8 and 10 shows that KCl markedly increased all the values of the rate constants, i.e. the rate constants for Mg\textsuperscript{2+} binding and dissociation from the phosphoenzyme and hydrolysis of the magnesium-phosphoenzyme complex as well as hydrolysis of the divalent cation-free phosphoenzyme. It should be added that the apparent rate of the phosphoenzyme decay induced by the addition of MgCl\textsubscript{2} (final concentration, 1.5 mM) was the same whether the phosphoenzyme was formed in 0.3 mM Tris-Cl and then KCl was added 30 s before the addition of MgCl\textsubscript{2} as in Fig. 10 or whether the phosphoenzyme was formed in 0.1 mM KCl and 0.3 mM Tris-Cl and then made divalent cation-free with excess EGTA.

**DISCUSSION**

This paper investigated the effect of the bound divalent cation on the property of the phosphoenzyme formed in the ATPase of sarcoplasmic reticulum. When the ATPase was phosphorylated with Ca\textsubscript{2+}ATP in the presence of high salts, a high level of calcium binding (14 ~ 16 mmol/mg of protein) was observed and most of the active site (85 ~ 95%) was found to be in the form of E\textsubscript{1}P in the steady state conditions (Figs. 1 and 4). Our previous study (6) indicates that each mol of E\textsubscript{1}P and E\textsubscript{2}P formed from Ca\textsubscript{2+}ATP has 3 and 1 mol of high affinity binding sites for calcium, respectively. Two of the calcium-binding sites on E\textsubscript{1}P are transport sites for calcium while the remaining one is the acceptor site for calcium derived from the substrate, Ca\textsubscript{2+}ATP (6), the latter being referred to as "substrate site" in the following discussion.

As shown in Fig. 1, calcium bound at both the transport sites and the substrate site on E\textsubscript{1}P was released upon addition of excess EDTA, which resulted in the loss of ADP sensitivity of the phosphoenzyme. The EDTA- or EGTA-induced loss of the ADP sensitivity of E\textsubscript{1}P formed in the absence of added Mg\textsuperscript{2+} was already reported by several workers (4, 8, 18, 19). In most of these previous studies, the loss of the ADP sensitivity was considered to be due to dissociation of Ca\textsuperscript{2+} from the transport sites on E\textsubscript{1}P. In contrast, Dupont (8) suggested that it is caused by dissociation of calcium from the substrate site. He observed that the ADP-insensitive phosphoenzyme, which was formed by treatment of E\textsubscript{1}P with EDTA, decayed rapidly in the presence of ADP after treatment with high Mg\textsuperscript{2+}. His result, however, does not appear to be conclusive because ATP formation was not measured in his experiment and because under his experimental conditions (0.1 mM KCl and 5 mM MgCl\textsubscript{2}) a significant portion of the ADP-insensitive phosphoenzyme could have been hydrolyzed as suggested in the experiment of Fig. 10 of the present study. In addition, Takakuwa and Kanazawa (20) have recently reported that the ADP-insensitive phosphoenzyme could not be rendered ADP-sensitive by addition of EGTA plus MgCl\textsubscript{2} under otherwise the same conditions as those used in Dupont's experiment above. At present, therefore, there is no consensus as to how E\textsubscript{1}P formed from Ca\textsubscript{2+}ATP becomes ADP-insensitive. For this reason, this problem was reinvestigated in the present study in an unambiguous way. As seen in Fig. 2, E\textsubscript{1}P formed from Ca\textsubscript{2+}ATP in 0.3 mM Tris-Cl gradually lost its reactivity with added ADP in the presence of EGTA. The time course of the
ADP sensitivity loss ($t_{1/2}$, 4 ~ 5 s) was similar to those reported previously by others (4, 8, 18). The ADP sensitivity loss was significantly delayed ($t_{1/2}$, ~16 s) when it was tested by successive additions of ADP and MgCl₂, indicating that the ADP release of Mg$^{2+}$ of the phosphoenzyme was partially restored by added Mg$^{2+}$. The sensitivity of the phosphoenzyme to ADP and MgCl₂, however, was eventually lost after a long incubation with EGTA (Figs. 2 and 3). It should be noted that the time course of this delayed loss of the ADP sensitivity was similar to the late phase of the time course for the calcium release from E₂P observed in the presence of EDTA (Fig. 1). Both Mg$^{2+}$ and Ca$^{2+}$ were effective in causing the partial recovery of the ADP reactivity (Fig. 3). Low ionized Ca$^{2+}$ (11.2 to 100 μM) was as effective as high ionized Mg$^{2+}$ (1.03 mM) although the observed effect of Mg$^{2+}$ could have been underestimated to some extent because EGTA-induced calcium release would continue even after addition of ADP plus MgCl₂. It should be noted that ATP formation leveled off rather rapidly after addition of ADP and either low Ca$^{2+}$ or high Mg$^{2+}$, no further increase in the ATP level being observed later in the time course (Fig. 3). This finding was interpreted as indicating that almost all of the reactive phosphoenzyme completed its reaction with both added divalent cation and added ADP in a relatively short time presumably because the concentrations of these agents were sufficiently high relative to the phosphoenzyme level. The ADP-unreactive phosphoenzyme obtained in the presence of Ca$^{2+}$ or Mg$^{2+}$ appears to be of E₁P type because it was not ADP-sensitive in 100 μM ionized Ca$^{2+}$ but was hydrolyzed in the presence of KCl (Fig. 3).

For the recovery of the ADP reactivity, the transport sites and the substrate site on the phosphoenzyme must be occupied by Ca$^{2+}$ and either Ca$^{2+}$ or Mg$^{2+}$, respectively, because of the principle of microreversibility. Therefore, added Mg$^{2+}$ restored the ADP reactivity by binding to the substrate site on the phosphoenzyme whose transport sites were still occupied by Ca$^{2+}$. Low added Ca$^{2+}$ also appeared to bind only to the substrate site of the phosphoenzyme because the observed restoration of the ADP reactivity was similar to that with high Mg$^{2+}$. All of these results, therefore, indicate that calcium dissociation from the substrate site on E₂P formed from CaATP is significantly faster than that from the transport sites on the same phosphoenzyme. The EDTA- or EGTA-induced loss of the ADP sensitivity of E₂P formed in the absence of added Mg$^{2+}$, therefore, is primarily due to dissociation of Ca$^{2+}$ from the substrate site. This conclusion is consistent with that by Dupont described above, and also provides an explanation for the apparently puzzling result obtained by Hasselbach et al. (21) that Mg$^{2+}$ is required for dephosphorylation of phosphoenzyme by GDP. Under their experimental conditions (use of GTP etc.), Ca$^{2+}$ could have been dissociated rapidly from the substrate site of the phosphoenzyme when enzyme phosphorylation was interrupted by the addition of excess EGTA. The present conclusion is also consistent with the finding that only one-third of the radioactive calcium bound to E₂P formed from CaATP was displaced rapidly with nonradioactive Ca$^{2+}$ (Fig. 4). The rate of disappearance of the $^{40}$Ca label in the rapidly disappearing fraction was found to be similar to that for the ADP sensitivity loss of E₂P. The result, therefore, shows that calcium bound at the substrate site on E₂P can readily equilibrate with free Ca$^{2+}$ in the reaction medium. It appears that the result provides a mechanism for the well known phenomenon that the rate of hydrolysis of the phosphoenzyme formed in the absence of added Mg$^{2+}$ is stimulated by added Mg$^{2+}$ after a short lag (8, 22). Added Mg$^{2+}$ also may exchange readily with calcium bound at the substrate site on the phosphoenzyme. In this context, it is interesting to note that calcium which is bound to the substrate site on E₁P formed from CaATP in the absence of added KCl neither dissociates readily in the presence of excess EDTA nor exchanges with free Mg$^{2+}$ in the reaction medium (5). These present and previous findings indicate that an extensive conformational change is induced in the substrate site when the enzyme undergoes transition from E₂P to E₁P. This conclusion is in good agreement with the recent report by Dupont and Pouton (23) that a large decrease in the polarity in the catalytic site of the ATPase is induced upon transition from E₂P to E₁P. It should be pointed out that the loose and tight binding of Ca$^{2+}$ to the ADP-sensitive and ADP-insensitive phosphoenzymes, respectively, were observed in the (Na⁺, K⁺)-ATPase of the plasma membrane (24, 25). Although the binding site for this calcium has not been defined unambiguously in the (Na⁺, K⁺)-ATPase, the above findings may further support the previous suggestion (26) that the reaction mechanisms of Ca$^{2+}$-ATPase of sarcoplasmic reticulum and that of (Na⁺, K⁺)-ATPase are similar.

The phosphoenzyme formed from CaATP in 0.3 M Tris-Cl without added KCl and then treated with EGTA for 1.5 min showed evidence of a slight inhomogeneity. As shown above (see Fig. 6 and "Results"), a small fraction (6 ~ 8%) of the phosphoenzyme was E₁P which still retained its bound calcium at the substrate site. However, most of the phosphoenzyme isolated after the treatment with EGTA was divalent cation-free both at the substrate site and the transport sites as indicated by the calcium-binding experiment (see Fig. 1 and "Results"). The divalent cation-free phosphoenzyme did not react with added ADP to form ATP both in the presence and absence of low Ca$^{2+}$ (up to 100 μM) (Fig. 7 and "Results") while its hydrolysis was accelerated significantly by high added KCl although the accelerated rate itself was still very slow (Fig. 6). The hydrolysis of the divalent cation-free phosphoenzyme was also accelerated by added Mg$^{2+}$ or low added Ca$^{2+}$ (Fig. 7). KCl addition in the presence of Ca$^{2+}$ and Mg$^{2+}$ further increased the rate of the phosphoenzyme hydrolysis markedly (Figs. 7, 9, and 10). In addition, magnesium once bound to the divalent cation-free phosphoenzyme did not dissociate readily in the absence of KCl so that the phosphoenzyme treated with Mg$^{2+}$ and then with excess EDTA decomposed at a rate comparable to that obtained in the absence of EDTA (Fig. 9). Finally, the divalent cation-free phosphoenzyme was rendered ADP-reactive when it was incubated with high ionized Ca$^{2+}$ (4.17 mM) (Fig. 7). These results suggest that the divalent cation-free phosphoenzyme, which is originally formed from E₂P, is not of E₁P type but could be of E₁P type because it was ADP-insensitive in 100 μM Ca$^{2+}$ and its hydrolysis was accelerated by added KCl. Low Ca$^{2+}$ or Mg$^{2+}$ bound only to the substrate site of the divalent cation-free phosphoenzyme to form a phosphoenzyme whose properties were almost the same as those of E₂P which is the normal reaction intermediate of ATP hydrolysis (5, 13). It was thus concluded that the divalent cation bound at the substrate site is essential for the normal reactivity of E₂P. It should be noted that the rate constant for binding of Mg$^{2+}$ to the divalent cation-free phosphoenzyme was small (Figs. 8 and 10). This finding suggests that Mg$^{2+}$ binding is not a simple encounter process but involves a rate-limiting isomerization of the complex of the phosphoenzyme with Mg$^{2+}$. It should also be noted that the kinetics of Mg$^{2+}$ binding was affected greatly by the treatment of the divalent cation-free phosphoenzyme with high KCl as shown by very large increases in the values of association and dissociation rate.
constants (Figs. 8 and 10, and “Results”). These effects of KCl, however, were not investigated further in the present study.

As shown in Fig. 4, calcium bound at the transport sites on E\textsuperscript{P} formed from Ca\textsuperscript{ATP} was displaced with nonradioactive calcium only at a very slow rate which was almost the same as that for the phosphoenzyme hydrolysis observed under the similar conditions. This result shows that calcium bound at the transport sites on E\textsuperscript{P} formed from Ca\textsuperscript{ATP} does not exchange with free Ca\textsuperscript{2+} in the reaction medium. When Mg\textsuperscript{ATP} was used as a substrate, calcium dissociation from E\textsuperscript{P} and the phosphoenzyme hydrolysis also proceeded at the same rate in the presence of EGTA and either in 5 mM MgCl\textsubscript{2} and 0.109 M KCl or in 20 mM MgCl\textsubscript{2} and 0.309 M KCl although the rates of these processes were approximately 3 times greater under the former than the latter conditions (Fig. 5 and “Results”). As hydrolysis of E\textsuperscript{P} formed either from Ca\textsuperscript{ATP} or Mg\textsuperscript{ATP} is very rapid in high KCl (5, 13), these results indicate that calcium dissociation from the transport sites on E\textsuperscript{P} and the apparent conversion from E\textsuperscript{P} to E\textsuperscript{2P} occur simultaneously. The simultaneous occurrence of these two processes may be interpreted as indicating that the calcium bound at the transport sites on E\textsuperscript{P} is dissociated only after the phosphoenzyme changes its conformation from E\textsuperscript{P} to E\textsuperscript{P}. Occlusion of calcium bound at the transport sites on E\textsuperscript{P} formed from Mg\textsuperscript{ATP} was suggested previously by Dupont (8) and later by others (12, 17). In this context, it should be noted that displacement of calcium from the transport sites on E\textsuperscript{P} formed from Ca\textsuperscript{ATP} was much faster in the presence of excess chelator than in the presence of nonradioactive calcium (Figs. 1 and 4). This observation may be explained by the present finding that, in the presence of a chelating agent, calcium was released at a faster rate from the substrate site on E\textsuperscript{P} than from its transport sites. As the divalent cation-free phosphoenzyme exhibited properties similar to some of those of E\textsuperscript{2P} (see above), it may be that the chelator-induced calcium dissociation from the substrate site facilitates the conformational transition of the phosphoenzyme from an E\textsuperscript{P} type to an E\textsuperscript{2P} type, thereby allowing calcium at the transport sites to be released from the phosphoenzyme.

The results described in the preceding paragraphs may be summarized by the following scheme.

\begin{center}
\textbf{SCHEME 3}
\end{center}

\textit{Me} denotes the metal moiety of the metal-ATP complex. E\textsuperscript{P} is the divalent cation-free phosphoenzyme. The upper part of the Scheme 3, which is the same as Scheme 1, describes the reaction sequence for the normal ATPase turnover either with Mg\textsuperscript{ATP} as a substrate (27) or with Ca\textsuperscript{ATP} as a substrate (5). This portion of Scheme 3 also describes the minimum requirement of divalent cation for the normal ATPase turnover as briefly discussed under the Introduction. Our previous results (5, 6) indicate that the metal moiety of the metal-ATP complex determines affinity of the metal-ATP complex to the enzyme and the catalytic rate of subsequent reaction steps. The maximal rate of enzyme phosphorylation and the \textit{K}_{\text{m}} value for the phosphorylation, for example, are 8 to 10 times greater for Mg\textsuperscript{ATP} than for Ca\textsuperscript{ATP} (5). In addition, the apparent rate of transition from E\textsuperscript{P} to E\textsuperscript{2P} is more than 10-fold as fast when E\textsuperscript{P} is formed from Mg\textsuperscript{ATP} as when E\textsuperscript{P} is formed from Ca\textsuperscript{ATP} (Ref. 5; Figs. 4 and 5, and “Results” of this study). During the normal turnover of the ATPase, 2 mol of calcium are released from the transport sites when E\textsuperscript{P} is transformed into E\textsuperscript{2P} (step 5) because the latter has low affinity for Ca\textsuperscript{2+} (6, 9, 12).

When Ca\textsuperscript{ATP} is used as a substrate, calcium bound at the substrate site and the transport sites on E\textsuperscript{P} is successively released from the phosphoenzyme upon addition of a chelator of divalent cation as shown in the lower part of Scheme 2 (steps 1 and 2). This is because the chelator-induced calcium release from the substrate site on E\textsuperscript{P} (step 1) is much faster than the forward reaction of step 5 (cf. Figs. 1 and 4). The sequential release of calcium from E\textsuperscript{P} formed from Ca\textsuperscript{ATP} (steps 1 and 2) is consistent with the findings (a) that the chelator-induced calcium release is significantly faster from the substrate site on E\textsuperscript{P} than from its transport sites (Figs. 2 and 3); (b) that added divalent cation can bind to the empty substrate site of the phosphoenzyme whose transport sites are still occupied by calcium, to reconstitute E\textsuperscript{P} (Figs. 2 and 3); and (c) that calcium bound at the substrate site on E\textsuperscript{P} equilibrates readily with free Ca\textsuperscript{2+} in the reaction medium while calcium bound at the transport sites on the same phosphoenzyme is not exchangeable (Fig. 4). The divalent cation-free phosphoenzyme, which is hydrolyzed at a very slow rate (step 3), is not of E\textsuperscript{P} type but exhibits properties similar to some of those of E\textsuperscript{2P} as discussed above. Upon addition of low Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, a phosphoenzyme whose properties are almost the same as those of E\textsuperscript{P} can be successfully reconstituted from the divalent cation-free phosphoenzyme (step 4) (see above). The ADP-sensitive phosphoenzyme can also be reconstituted from the divalent cation-free phosphoenzyme and high added Ca\textsuperscript{2+} (Fig. 7). E\textsuperscript{P} could be formed from the divalent cation-free phosphoenzyme and high Ca\textsuperscript{2+} either by the reversal of steps 2 and 1 or via step 4 and then by the
reversal of step 5.

When MgATP is used as a substrate, release of magnesium bound at the substrate site on E,P appears to be significantly slower in the presence of excess EDTA than that of calcium bound at the same site (cf. Ref. 8 and Fig. 1 of this study) although the rate of magnesium release itself cannot be estimated accurately because of the simultaneous occurrence of the fast E,P to E2P transition (step 5). It is not clear at present, however, whether this tighter binding of magnesium at the substrate site is causally related to the fast turnover of E,P formed from MgATP.

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REFERENCES
Role of divalent cation bound to phosphoenzyme intermediate of sarcoplasmic reticulum ATPase.

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