Two Alternate Kinetic Routes for the Decomposition of the Phosphorylated Intermediate of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase*

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The decomposition of the phosphorylated intermediate (EP) of sarcoplasmic reticulum ATPase, purified by the method of deoxycholic acid extraction, was studied by first phosphorylating with \([\gamma-32P]\)ATP, then diluting the reaction mixture with 20 volumes of medium containing nonradioactive ATP, and finally quenching serial samples with acid for determination of residual \([32P]\)EP. The time course of \([32P]\)EP decomposition consists of an initial fast phase followed by a slow phase. The two components of EP, EP\(_{fast}\) (1.1 nmol/mg) and EP\(_{slow}\) (2.8 nmol/mg), decomposed with the rate constants of 8 and 0.8 min\(^{-1}\), respectively, in the presence of 0.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), and 90 mM KCl at pH 7.0 and 0°C. The sum of the hydrolytic activities corresponding to the two components accounts for the steady state velocity of the Pi production under the same conditions, indicating that the two components represent simultaneous pathways, rather than sequential steps of EP decomposition.

As the time of phosphorylation with \([\gamma-32P]\)ATP is increased from 2 to 15 s, the fraction of EP\(_{fast}\) decreases in favor of EP\(_{slow}\). This conversion decreases the rate of total Pi production by the enzyme following an initial P\(_i\) burst. Conversion of EP\(_{fast}\) to EP\(_{slow}\) is favored by millimolar concentrations of Ca\(^{2+}\). On the other hand, conversion of EP\(_{slow}\) to EP\(_{fast}\) is obtained by reducing Ca\(^{2+}\) or raising Mg\(^{2+}\) concentration, but is prevented by removal of ADP. The EP\(_{slow}\) fraction decreases in favor of EP\(_{fast}\) as the temperature is increased from 0 to 22°C.

It is now established that in the reaction mechanism of sarcoplasmic reticulum ATPase, the enzyme is first activated by Ca\(^{2+}\) binding to high affinity sites on the cytoplasmic side of the SR vesicles, then a phosphorylated intermediate is formed by the reaction with ATP, and finally the bound Ca\(^{2+}\) is released into the vesicular lumen previous to the decomposition of the EP (1–6). Measurements of EP decomposition provide important information with respect to the mechanism of Ca\(^{2+}\) release inside the vesicle and the rate-limiting steps in the reaction cycle. In this regard, Yamada and Tonomura (7) observed that the steady state turnover of the EP is accelerated by Mg\(^{2+}\) and inhibited by high concentrations of Ca\(^{2+}\). However, the mechanism of inhibition by Ca\(^{2+}\) is not clear. Therefore, we planned to measure directly EP decomposition in the presence of various concentrations of Ca\(^{2+}\) using SR ATPase purified by the method of deoxycholic acid extraction.

The decomposition of \([32P]\)EP formed by incubation of enzyme with \([\gamma-32P]\)ATP in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) can be followed directly after addition of EGTA to chelate Ca\(^{2+}\) and prevent further formation of \([32P]\)EP or after addition of nonradioactive ATP to prevent further formation of \([32P]\)EP. The former procedure prevents studies of EP decomposition in the presence of Ca\(^{2+}\). On the other hand, with the latter procedure, it is difficult to control cation concentrations, especially Mg\(^{2+}\) due to the high affinity of ATP for divalent cations. Therefore, we measured the EP decomposition directly by modifying the latter procedure. The ATPase was phosphorylated by \([\gamma-32P]\)ATP and then further formation of \([32P]\)EP was stopped by diluting the reaction mixture with 20 volumes of reaction medium containing nonradioactive ATP at a concentration identical to that of \([\gamma-32P]\)ATP before dilution. In this manner, we had total control over the concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) before and after formation of \([32P]\)EP.

We found that the time course of EP decomposition at low temperature includes a fast and a slow portion which appear related to two alternate routes for EP decomposition. We then examined the effects of Ca\(^{2+}\), Mg\(^{2+}\), and H\(^+\) and temperature on the alternate routes of mechanism.

MATERIALS AND METHODS

Sarcoplasmic reticulum was prepared by differential centrifugation of muscle homogenate as described by Eletr and Inesi (8). The ATPase was purified by the method of deoxycholic acid extraction described by Meissner et al. (9), frozen by liquid N\(_2\), and stored at −70°C. Creatine kinase from rabbit muscle was purchased from Sigma. \([\gamma-32P]\)ATP was purchased from New England Nuclear and was more than 95% pure as shown by high pressure liquid chromatography using an anion exchange column.

The measurement of EP decomposition was carried out typically as follows. The reaction was started by the addition of 0.2 mM \([\gamma-32P]\)ATP to the enzyme (1 mg/ml) suspended in buffer containing 0.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 4 mM creatine phosphate, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 0°C. After 15 s, the reaction solution was diluted by 20 volumes of the solution containing 0.2 mM nonradioactive ATP and 0.2 mg/ml of creatine kinase in the same buffer as otherwise specified. The dilution solution was prepared at least 30 min before use in order to eliminate the contamination of ADP by enzymatic ATP regeneration. Then, the diluted reaction solution was quenched by 5% trichloroacetic acid, and the denatured \([32P]\)EP was collected by filtration and washing. For this purpose, aliquots of the quenched solutions were applied on Millipore filters (HAWP type, 25-mm diameter, 0.45-μm pore size), which were placed on a vacuum filter.
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filtering manifold, and washed four times with 5 ml of ice-cold 0.25 M perchloric acid containing 20 mM Pi and 10 mM polyphosphoric acid for carriers. Finally, the filters were dissolved by 1 ml of dimethylformamide and counted.

For determination of \[\text{Pi}\] and \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\), the quenched reaction solutions were centrifuged, and 0.5 ml of the supernatant was mixed with 0.5 ml of 4% ammonium molybdate and 1 N HCl. The radioactive Pi and ATP were then separated by the extraction with 1 ml of isobutyl alcohol/benzene (1:1) and counted (18).

RESULTS

Two Routes of Decomposition of EP—Following addition of 0.2 mM \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\) to a medium containing 1 mg/ml of ATPase, 0.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 4 mM creatine phosphate, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 0 °C, \(\[^{32}\text{P}\]\)EP is formed (Fig. 1), rising to a level of 4.4 nmol/mg immediately after starting the reaction and then decreasing slightly to a steady state level of about 3.8 nmol/mg. In the absence of Ca\(^{2+}\), no significant amount of \(\[^{32}\text{P}\]\)EP is formed.

In the presence of Ca\(^{2+}\), the time course of \(\[^{32}\text{P}\]\)EP liberation consists of an initial burst followed by a steady phase as shown in Fig. 1. The size of the P\(_i\) burst, as revealed by the extrapolated value of the steady state slope to zero time, is 3 nmol/mg. The steady state ATPase activity is 10.3 nmol/mg/min. It is well known that SR preparation has the basic ATPase activity which is Ca\(^{2+}\)-independent (5). In the presence of 2 mM EGTA instead of CaCl\(_2\), Pi production proceeds at a constant velocity of 3 nmol/mg/min. Therefore, Ca\(^{2+}\)-dependent ATPase activity is 7.3 nmol/mg/min at steady state.

In parallel experiments, we measured the \(\[^{32}\text{P}\]\)EP decomposition by diluting the reaction solution after a 15 s incubation with \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\). The reaction mixture was diluted with 20 volumes of reaction medium containing 0.2 mM nonradioactive ATP and 0.2 mg/ml of creatine kinase in order to eliminate contaminant ADP. Serial samples were then obtained by acid quenching. We found that at 0 °C, the level of \(\[^{32}\text{P}\]\)EP decreases about 30% within 15 s and then continues to decrease slowly (Fig. 1, inset). The time course of \(\[^{32}\text{P}\]\) liberation after the dilution is coincident with that of \(\[^{32}\text{P}\]\)EP decomposition.

The biphasic EP decomposition is shown clearly in a semilogarithmic plot (Fig. 2). This biphasic kinetics cannot be explained with a mechanism in which the two components correspond to a sequential reaction, since the rate limitation imposed by the slow reaction is inconsistent with the faster enzyme turnover observed in steady state (Fig. 1). It is rather apparent that the EP consists of two fractions which decompose independently. In fact, the data can be fitted within experimental accuracy (Fig. 2, solid line) with two exponentials corresponding to 2.8 and 1.1 nmol of EP/mg and decomposing with \(k_{\text{slow}} = 0.4\) min\(^{-1}\) and \(k_{\text{fast}} = 6\) min\(^{-1}\), respectively.

Addition of the P\(_i\) production rate from EP\(_{\text{slow}}\) and EP\(_{\text{fast}}\) (2.8 nmol/mg × 0.4 min\(^{-1}\) + 1.1 nmol/mg × 6 min\(^{-1}\) = 7.7 nmol/mg/min) is in satisfactory agreement with the Ca\(^{2+}\)-dependent ATPase velocity (7.3 nmol/mg/min) observed in steady state (Fig. 1).

We then measured the time course of EP distribution in slow and fast fractions by diluting the reaction mixture at various times after starting the reaction with \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\) (Fig. 3). Each of the resulting curves of \(\[^{32}\text{P}\]\)EP decomposition consists of fast and slow phases. The rate constant of the slow phase (\(k_{\text{slow}}\)) is 0.39 min\(^{-1}\), independent of the time of dilution. However, the fraction of EP\(_{\text{slow}}\) estimated by the extrapolation of the slow phase to the time of dilution increases with the reaction time previous to dilution. In fact, the EP\(_{\text{slow}}\) fraction was 1.8 nmol/mg when the mixture was diluted after 2 s of reaction with \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\) and increased to 2.4, 2.8, and 3.1

**Fig. 1.** The time courses of P\(_i\) liberation and EP decomposition. The reaction was started by adding 0.2 mM \(\gamma\text{-\[^{32}\text{P}\]}\text{ATP}\) to 1 mg/ml of ATPase solution in the presence of 0.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 4 mM creatine phosphate, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 0 °C. At the appropriate time, the reaction was quenched by adding 5% trichloroacetic acid, and the amounts of \[^{32}\text{P}\], (C) and \(\[^{32}\text{P}\]\)EP (○) were assayed. In the presence of 2 mM EGTA instead of CaCl\(_2\), the amounts of \[^{32}\text{P}\], (C) and \(\[^{32}\text{P}\]\)EP (□) were also measured. The inset shows \(\[^{32}\text{P}\]\)EP decomposition (△) and \[^{32}\text{P}\] liberation (△) when the reaction solution was diluted at 15 s with the solution containing 0.2 mM nonradioactive ATP, 0.2 mg/ml of creatine kinase, 0.5 mM CaCl\(_2\), 5 mM MgCl\(_2\), 4 mM creatine phosphate, 90 mM KCl, and 50 mM MOPS.

**Fig. 2.** Biphasic decomposition of EP. The time course of EP decomposition was measured under the same conditions as those of Fig. 1. The solid line was calculated by the following equation: \(\ln \text{EP} = 1.1 \times e^{-0.5} + 2.6 \times e^{-0.6} \text{ (s·min)}\).
all the EP decomposes linearly at a very slow rate. At lower Ca\textsuperscript{2+} concentrations, the EP decomposes biphasically. As the Ca\textsuperscript{2+} concentration is reduced, the fraction of EP\textsubscript{fat}, de-

\[ \text{rate constant of Ca}\textsuperscript{2+} \]

increases. Since the ratio of the EP\textsubscript{fast} and EP\textsubscript{slow} fractions varies with the Ca\textsuperscript{2+} concentration, we examined the possible conversion between the two fractions by changing the Ca\textsuperscript{2+} concentration during the reaction. Fig. 5A shows the conversion of EP\textsubscript{slow} to EP\textsubscript{fat}. The reaction was started by adding 0.2 mM [\gamma-\textsuperscript{32P}] ATP to the enzyme solution containing 5 mM CaCl\textsubscript{2} and other components (Fig. 1). Fifteen seconds after the addition of [\gamma-\textsuperscript{32P}]ATP, 4.9 mM EGTA was added (0.2 mM free Ca\textsuperscript{2+}), and 5 s thereafter (20th s of reaction), the mixture was diluted with 20 volumes of medium containing nonradioactive ATP, creatine kinase, and approximately 0.2 mM free Ca\textsuperscript{2+} (5 mM CaCl\textsubscript{2} + 4.9 mM EGTA). In this case, the time course of [\textsuperscript{32P}] EP decomposition (Fig. 5A, \( \Delta \)) was biphasic, and approximately 60% of EP decomposed slowly at a rate of 0.8 min\textsuperscript{-1}. This time course is almost identical to that observed at 0.2 mM CaCl\textsubscript{2} before and after dilution (Fig. 4, \( \Delta \)). Therefore, the slow component, which is dominant at 5 mM CaCl\textsubscript{2} (Fig. 4, \( \bigcirc \)), was converted into the fast component by reducing the Ca\textsuperscript{2+} concentration 5 s before dilution with nonradioactive ATP. On the other hand, when creatine kinase (2 mg/ml) was added together with EGTA (Fig. 5A, \( \square \)) 5 s before the dilution, almost all [\textsuperscript{32P}] EP retained slow decomposition kinetics, in spite of the low Ca\textsuperscript{2+} concentration. The apparent rate constant of the slow phase was 0.8 min\textsuperscript{-1}. These results indicate that either conversion of EP\textsubscript{slow} to EP\textsubscript{fast} or a step in the fast pathway requires ADP. We also observed no conversion when the Ca\textsuperscript{2+} concentration was reduced at the same time (20th s of reaction) as the dilution with nonradioactive ATP (Fig. 5A, \( \bigcirc \)). It is likely that the lack of conversion in this condition is due to ADP dilution.

Fig. 5B shows conversion of EP\textsubscript{fast} to EP\textsubscript{slow}. In the presence of 0.05 mM CaCl\textsubscript{2}, approximately 50% of EP decays with slow
kinetics (Fig. 5B, O; see also Fig. 4, O). On the other hand, when 5 mM CaCl₂ was added 5 s before the dilution with nonradioactive ATP, EPₚ was converted into EPslow and the entire phosphoenzyme pool decomposed very slowly. This conversion occurred by increasing the Ca²⁺ concentration, irrespective of the presence or absence (Fig. 5B, O and Δ, respectively) of creatine kinase. When we changed the Ca²⁺ concentration from 0.05 to 5 mM at the same time as the dilution with nonradioactive ATP, about half of EPₚ decomposed rapidly and the rest of EPₚ was converted into EPslow. These results suggest that the rate of the conversion of EPₚ into EPslow is nearly equal to that of EPₚ decomposition.

We then examined the ADP sensitivity of EPₚ and EPslow (Fig. 6). For this purpose, the ATPase (2 mg/ml) was phosphorylated by the addition of 50 μM [γ-³²P]ATP in the presence of 0.5 mM CaCl₂, 5 mM MgCl₂, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 0 °C. Fifteen seconds after the addition of [γ-³²P]ATP, the reaction was diluted with 20 volumes of medium containing 0.1 mM ADP and 50 μM nonradioactive ATP. We found that the level of [³²P]EP decreased rapidly from 4 nmol/mg to zero within 15 s, and the amounts of [γ-³²P]ATP increased immediately from 18.5 to 22.5 nmol/mg and kept almost constant. It is then apparent that both of EPₚ and EPslow can react with ADP to form ATP.

Effects of Ca²⁺ and Mg²⁺—The steady state Pi production and EP levels were measured in the presence of various concentrations of CaCl₂ in order to evaluate the ATPase turnover in the light of the EP decomposition (Fig. 7). The activity of Ca²⁺-dependent Pi production was 16 nmol/mg/min in the presence of 0.05 mM CaCl₂ and 5 mM MgCl₂ at pH 7.0 and 0 °C. The activity decreased as the Ca²⁺ concentration was raised to approximately 0.2 nmol/mg/min in the presence of 5 mM CaCl₂. It should be pointed out that the time course of Pi liberation consisted of a burst phase followed by a steady state activity as shown in Fig. 1. The size of Pi burst decreased from 15 to 2 nmol/mg as the Ca²⁺ concentration was raised from 0.05 to 5 mM (data not shown). The steady state levels of EPₚ remained almost constant (3.5 nmol/mg) within the 0.05–0.5 mM Ca²⁺ range. At higher Ca²⁺ concentrations, the levels of EPₚ increased to about 4.5 nmol/mg at 5 mM CaCl₂. The data described above are compared in Fig. 7 with the levels of EPslow and the rate constants of EPslow decomposition (kslow) which were obtained in the experiments illustrated in Fig. 4. The plot indicates that the levels of EPslow increase from 1.8 to 4.5 nmol/mg and kslow decrease from 1.27 to 0.08 min⁻¹ as the CaCl₂ concentration is raised from 0.05 to 5 mM. The plot of reciprocal values of kslow against the CaCl₂ concentration yields a straight line, and the apparent constant for Ca²⁺ inhibition is approximately 150 μM (not shown).

The Mg²⁺ concentration dependence of the steady state Pi production, the levels of EPₚ and EPslow, and the rate constants of EPslow decomposition in the presence of 0.1 mM CaCl₂ at pH 7.0 and 0 °C are shown in Fig. 8. The activity of Ca²⁺-dependent Pi production was enhanced by Mg²⁺ up to 5 mM and inhibited by a further rise in Mg²⁺ concentration. The level of EPₚ at steady state increased as the MgCl₂ concentration was raised up to a maximum of 5.2 nmol/mg in the presence of 20 mM MgCl₂. We also measured the time courses of [³²P]EP decomposition at various concentrations of MgCl₂ by the dilution method used in the experiments shown in Fig. 4 and obtained the levels of EPslow and the rate constants of EPslow decomposition (kslow) (Fig. 8). We found that the fractional level of EPslow (EPslow/EPₚ) decreased considerably as the MgCl₂ concentration was raised. On the other hand, kslow was only slightly increased by raising the MgCl₂ concentration up to 20 mM.

Effects of pH and Temperature—Maximal velocity of Ca²⁺-dependent Pi production in the presence of 0.2 mM CaCl₂ and 5 mM MgCl₂ at 0 °C was obtained at pH 7.0. The level of EPₚ at steady state was approximately 4 nmol/mg at pH 7.0 and increased to approximately 5 nmol/mg at pH 8.8. The
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**FIG. 8.** MgCl₂ concentration dependence of Ca²⁺-ATPase activity, amounts of EP_total and EP_slow, and k_slow. The amount of EP_total (A) and Ca²⁺-dependent ATPase activity (●) were measured in the presence of 0.1 mM CaCl₂ under the same conditions as those of Fig. 7 except for the concentration of CaCl₂ and MgCl₂. The amount of EP_slow (△) and the rate constant of EP_slow decomposition (k_slow) (○) were calculated from similar experiments to Fig. 4.

**FIG. 9.** pH dependence of Ca²⁺-ATPase activity, amounts of EP_total and EP_slow, and k_slow. The amount of EP_total (A) and Ca²⁺-dependent ATPase activity (●) were measured in the presence of 0.2 mM CaCl₂ and 5 mM MgCl₂ under the same conditions as those of Fig. 7 except for CaCl₂ concentration and pH buffer (100 mM MES (pH 5.9-7.0) or 100 mM Tris-Cl (pH 7.3-8.8)). The amount of EP_slow (△) and the rate constant of EP_slow decomposition (k_slow) (○) were calculated from similar experiments to Fig. 4.

**FIG. 10.** Time courses of EP decomposition at various temperatures. The reaction was started by adding 0.2 mM [γ-³²P]ATP to 1 mg/ml of ATPase solution in the presence of 0.5 mM CaCl₂, 5 mM MgCl₂, 4 mM creatine phosphate, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 5 (□), 10 (○), or 22 (△) °C. After 5 s, the reaction solution was diluted with 20 volumes of the solution containing 0.2 mM nonradioactive ATP, and the amounts of [³²P]EP were measured.

level of EP_slow was 1.7 nmol/mg at pH 7.0, 2.5 nmol/mg at pH 6.3, and 5 nmol/mg at pH 8.3. The rate constant of EP_slow decomposition (k_slow) decreased from 1 to 0.2 min⁻¹ as the pH was raised from 5.9 to 8.8 (Fig. 9).

We also measured the time courses of EP decomposition at various temperatures in the presence of 0.5 mM CaCl₂ and 5 mM MgCl₂ at pH 7.0 (Fig. 10). As already shown in Fig. 2, the fractional level of EP_slow (EP_slow/EP_total) was 0.7 and the rate constant of EP_slow decomposition was 0.4 min⁻¹ at 0 °C. We found that the fractional level of EP_slow was 0.61, 0.55, and 0.33 at 5, 10, and 22 °C, respectively. On the other hand, k_slow increased from 1.4 to 4.2 and 8.4 min⁻¹ as the temperature was raised from 5 to 10 and 22 °C, respectively.

**DISCUSSION**

One of the most interesting findings obtained during our experimentation is that the time course of EP decomposition consists of an initial fast phase followed by a slow phase especially in the presence of high concentrations of Ca²⁺ at low temperatures. This was demonstrated with the aid of a dilution technique which changes the specific radioactivity of ATP but leaves the composition of the medium unchanged. Therefore, these measurements were obtained without disturbing the reaction turnover. The simplest explanation for the observed kinetics of EP decomposition is that EP consists of two components (EP_fast and EP_slow) decomposing independently with different rate constants. In fact, assuming sequential hydrolysis of EP_slow and EP_fast, neither the rate constant for hydrolysis of EP_fast nor that of EP_slow, multiplied by the number of phosphorylation sites in either components, could alone account for the overall turnover of the ATPase reaction. On the other hand, a satisfying agreement with the overall turnover was obtained when the decomposition of the two components was considered to be simultaneous (Figs. 1 and 2). It should be also noted that the time course of [³²P]EP liberation after the dilution was coincident with that of [³²P]EP decomposition. This indicates that no significant reverse reaction from EP + ADP to E + ATP occurs upon dilution.

As shown in Fig. 3, EP_slow/EP_total increases gradually after maximal levels of ET_total are reached. This indicates that EP_fast is converted into ET_slow during the ATPase reaction under certain conditions. This mechanism explains the initial burst of P_i production following addition of ATP. Thus, most of the P_i is produced from ET_fast during the initial phase and from both EP_fast and EP_slow at steady state. Several investigators have previously described the occurrence of an initial burst of...
P, upon addition of ATP to SR vesicles, Kanazawa et al. (10) observed a P, burst occurring within a few seconds after starting the reaction at pH 7.0 and 15 °C. Froehlich and Taylor (11, 12) measured P, bursts within 100 ms after starting the reaction at pH 6.8 and 20 °C in the presence and absence of Ca2+. From the experimental point of view, the P, burst observed in our present experiments appears to be the same phenomenon as that reported by Kanazawa et al. (10), which was attributed by Sumida et al. (13) to a time-dependent transition of the apparent rate constant of EP hydrolysis. Our direct measurements of EP decomposition demonstrate that the P, burst is actually caused by conversion of a fraction of EPfast to EPslow which decomposes independently.

We also demonstrate that the two components of the phosphorylated intermediate can be converted into each other by changing the Ca2+ concentration (Fig. 5). EPfast is converted to EP, slow by increasing the Ca2+ concentration, and EPslow is converted to EPfast by decreasing the Ca2+ concentration. It is of interest that the latter conversion is not accompanied by the expected rapid EP decomposition when ADP is removed, suggesting that ADP binding plays a role in some step of the fast pathway.

![Scheme 1](image)

The mechanism is essentially the same as those proposed for the (Na+,K+)-ATPase reaction by Yamaguchi and Tonomura (14) and for the p-nitrophenylphosphatase reaction of SR by Nakamura and Tonomura (15). A multiple pathway for ATP utilization by solubilized SR ATPase was also suggested by Yamamoto and Tonomura (16).

We evaluated our findings in the light of a reaction mechanism including two sequential forms of EP: a first form which is ADP-sensitive and a second form which is ADP-insensitive and/or Mg++-sensitive (17-19). In our experiments, the entire EP pool, including EPfast and EPslow, reacted with ADP to form ATP in the presence of relatively high concentrations of Ca2+ at neutral pH (Fig. 6). This suggests that the ADP-insensitive EP is not present in significant amounts under our conditions. The ADP-insensitive EP follows EPslow in the reaction sequence, because the ADP-insensitive EP has been observed only at low temperature and/or high pH (17-19), under which conditions the slow pathway is dominant (see Figs. 9 and 10).

![Scheme 2](image)

Measurements of overall turnover at steady state ([E] or [E] of [32P]E decomposition after preventing [32P]E formation by the addition of EGTA have shown that the decomposition of EP is accelerated by Mg++ and inhibited by Ca2+ (7, 10). Our measurements of [32P]EP decomposition using the dilution technique in the presence of various concentrations of Ca2+ and Mg+ provide a more detailed understanding of the effect of these divalent cations on the EP decomposition. We found that the rate constant of EPslow decomposition (kslow) is not significantly affected by Mg++, however, the ratio EPslow/EPfast is maintained in favor of EPfast as the concentration of Mg++ is raised. Under appropriate experimental conditions, the enhancement of total ATPase activity by Mg++ is due to maintenance of EP in the fast pathway, rather than to an increase in kslow.

On the other hand, we found two kinds of Ca2+ effect on the kinetics of EP decomposition. One is observed at relatively low concentrations (~0.2 mM). The rate constant, kslow, is reduced as the Ca2+ concentrations is increased, and the plots of reciprocal of kslow against Ca2+ concentration yield a straight line. This inhibition might be due to the binding of Ca2+ to the internal transport sites, and it suggests that Ca2+ release from the sites is not cooperative. The other is observed at higher concentrations of Ca2+ (~1 mM). The fraction of EPfast is reduced (by decreasing Mg++ concentrations as mentioned above) as the Ca2+ concentration is increased. It is possible that this effect of Ca2+ is produced by the binding of Ca2+ to the Mg++ site. This kind of EP, which contains two Ca2+ at transport sites and an additional Ca2+ at the Mg++ site, has been proposed and discussed (19, 20). These divalent cation effects are summarized in Scheme 3, in which the Ca2+ transport sites and the Mg++ site are designated on upper left and lower left of each enzyme species, respectively.

![Scheme 3](image)

We postulate that 2 mol of Ca2+ are transported through both, fast and slow, pathways in Scheme 3 because it is well established that the coupling ratio of Ca2+ transport to ATP hydrolysis is 2:1 in the initial phase of the reaction as well as in the steady state (1, 21) in spite of the decrease in the contribution of EPfast to the reaction turnover with increasing reaction time. On the basis of above kinetic considerations, we suggest that SR ATPase includes two enzyme populations, Epfast and Epslow, which form EPfast and EPslow by reaction with ATP, respectively. The equilibrium between these populations is influenced by Ca2+, Mg++, and H+, temperature, and enzyme phosphorylation. Re-equilibration between Epfast and Epslow then induces the turnover change and P, burst which are observed in the early part of the reaction following addition of ATP.

Additional and interesting results were obtained when we changed the reaction temperature. Since Inesi et al. (22) reported a transition at about 20 °C on Arrhenius plots of ATPase activity, many investigators have confirmed this finding and argued about the mechanism for the temperature transition (see review in Ref. 6). We have now observed temperature-dependent changes not only in the rate constant of EP decomposition but also in the distribution of EP in the fast and slow components. This suggests that two routes of EP decomposition, as well as the conversion between the two components of EP, have different activation energies and that a different pathway is dominant at different temperatures. From the experimental point of view, it is important to realize that comparison of kinetic data obtained at different temper-
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atures and Ca²⁺ concentrations must take into account this complexity.

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