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Ratio of Hydrolysis and Synthesis of ATP by the Sarcoplasmic Reticulum ATPase in the Absence of a Ca^{2+} Concentration Gradient*

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The reversibility of the reaction catalyzed by purified (Ca^{2+} + Mg^{2+})-dependent ATPase of sarcoplasmic reticulum was studied by measuring the rates of synthesis and hydrolysis of ATP observed during the ATP $\rightleftharpoons$ P_i exchange reaction. The ratio between the velocities of hydrolysis and synthesis of ATP was found to vary between 700 and 1.2 depending on the concentrations of ATP, P_i, Mg^{2+}, and Ca^{2+} in the medium.

Raising the magnesium concentration increases in parallel the calcium concentrations required for half-maximal activation of both the hydrolysis and the synthesis of ATP. The apparent K_m of P_i for the ATP $\rightleftharpoons$ P_i exchange reaction varies, depending on the Mg^{2+} concentration of the medium, being lower with higher Mg^{2+} concentration. The ratio between the velocities of hydrolysis and synthesis of ATP decreases when the Mg^{2+} and P_i concentrations are increased and the ATP concentration is decreased.

The entire sequence of reactions catalyzed by the Ca^{2+} + Mg^{2+}-dependent ATPase from sarcoplasmic reticulum vesicles can flow forward in the direction of ATP hydrolysis, and backward in the direction of synthesis of ATP from ADP and P_i (1-9). When vesicles are incubated in a medium containing ATP, ADP, P_i, Mg^{2+} and Ca^{2+}, ATP is initially hydrolyzed by the enzyme in a process coupled with the transport of calcium from the assay medium to the interior of the vesicles. This leads to the formation of a transmembrane calcium concentration gradient (1, 5-8). Following the initial phase of net calcium accumulation, the rate of ATP hydrolysis decreases due to the increased Ca^{2+} concentration inside the vesicles. When a sufficient transmembrane Ca^{2+} concentration gradient is formed, an exchange between $^{32}P_i$ and the γ-phosphate of ATP pool is observed. This ATP $\rightleftharpoons$ P_i exchange indicates that the enzyme is operating simultaneously forward and backward (3, 9, 10).

When the ATP $\rightleftharpoons$ P_i exchange was described, it was proposed that the energy required for the synthesis of ATP observed during the exchange reaction was derived from the transmembrane calcium concentration gradient (3, 4, 11, 12). More recently, evidence has been presented that the (Ca^{2+} + Mg^{2+})-dependent ATPase can catalyze ATP $\rightleftharpoons$ P_i exchange in the absence of a Ca^{2+} gradient (10, 13-16). This finding indicates that the enzyme must be able to conserve some of the energy released from hydrolysis of ATP in a manner which permits the synthesis of a new ATP molecule from ADP and P_i. In previous reports (9, 10), the ratio between the rates of hydrolysis and synthesis of ATP was used as an index to estimate the degree of energy conservation by the system.

Previous studies of the ATP $\rightleftharpoons$ P_i exchange reaction were performed in the presence of high ATP concentrations (5 to 10 mM). Under these conditions, both the apparent K_m for P_i and the ratio between the velocities of hydrolysis and synthesis of ATP have different values depending on whether or not a Ca^{2+} concentration gradient is formed across the vesicle membrane (9, 10, 13-16). When a gradient is formed, the apparent K_m for P_i is in the range of 3 to 4 mM and the ratio between the velocities of hydrolysis and synthesis of ATP is in the range of 2 to 5. In the absence of a gradient, the K_m for P_i increases to 20 to 50 mM and the ratio hydrolysis:synthesis increases to values higher than 30. These differences can be decreased when the vesicles are treated with silver or when ATP and ADP are replaced by ITP and IDP (9, 10, 14, 16). In this report it is shown that the apparent affinity for P_i and the degree of energy conservation in the absence of a gradient (leaky vesicles) can be increased to values similar to those measured in the presence of a Ca^{2+} gradient simply by varying the ATP and Mg^{2+} concentration of the medium, without the need of treating the vesicles with silver.

EXPERIMENTAL PROCEDURES

Leaky vesicles reconstituted from purified (Ca^{2+} + Mg^{2+})-dependent ATPase were prepared as described by MacLennan *et al.* (17, 18). The specific ATPase activity found in our preparations was 20 to 30 μmol of P_i/mg of protein min^{-1} when tested under the standard conditions described by MacLennan (17). Under the conditions used in this report, there was no measurable ATPase activity in the presence of Mg^{2+} and absence of Ca^{2+}. The reformed vesicles of (Ca^{2+} + Mg^{2+})-dependent ATPase were leaky and unable to accumulate Ca^{2+} under any condition studied (18). The vesicles were stored in 0.2 mM Tris/sucrose buffer (pH 8.0) at -6 °C. Protein was measured by the method of Lowry *et al.* (19). $^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and was purified by extraction as phosphomolybdate with isobutyl alcohol/benzene (v/v), re-extracted to the aqueous phase with ammonium hydroxide solution, and precipitated as the $MgNH_4PO_4$ salt (20). The $^{32}P_i$ was stored in dilute HCl until used. [γ-^{32}P]ATP was prepared according to the method of Glynn and Chapell (21) with small modifications as previously described (22). The ATP concentration was determined spectrophotometrically.

ATPase activity was tested by measuring the amount of $^{32}P_i$ released from [γ-^{32}P]ATP. The assay medium contained, in a final volume of 0.5 ml, 30 mM Tris/maleate buffer (pH 7.0), 100 μM ADP and [γ-^{32}P]ATP, $MgCl_2$, $CaCl_2$, P_i, enzyme, and ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid in the concentrations specified on the figure legends. The reaction time and the enzyme concentration were adjusted so that the maximum hydrolysis of the added ATP was always less than 20%. Under the conditions used, the

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hydrolysis of ATP was linear as a function of time. The reaction was started by the addition of enzyme and stopped by the addition of 0.4 ml of a 30 mM solution of ammonium molybdate in 2 N H_2SO_4. The $^{32}P_i$ produced was extracted from the assay medium as described by de Meis and Carvalho (13) and counted in a liquid scintillation counter.

ATP ⇌ P_i exchange was determined by measuring the appearance of [γ-^{32}P]ATP in the medium. The reaction conditions were the same as described above except that $^{32}P_i$ and nonradioactive ATP were used. Unreacted $^{32}P_i$ was extracted from the assay medium after stopping the reaction with an acid solution of ammonium molybdate by adding 0.3 ml of acetone and mixing the sample vigorously on a Vortex mixer for 40 s with 2 ml of isobutyl alcohol/benzene (v/v). The upper phase containing the organic solvents was discarded, 0.15 ml of 0.02 mM carrier P_i and 0.3 ml of acetone were added to the water phase and re-extracted with isobutyl alcohol/benzene. This was repeated three times. The water phase containing the radioactive ATP formed was counted in a liquid scintillation counter (13).

RESULTS AND DISCUSSION

Effect of Magnesium on the Calcium Binding to the Enzyme—In the absence of a transmembrane Ca^{2+} concentration gradient, the forward and reverse reactions of the (Ca^{2+} + Mg^{2+})-dependent ATPase retain calcium concentration requirements which are similar to those observed on the two sides of the vesicle membrane when a gradient is formed. In the micromolar concentration range, calcium activates the rate of ATP hydrolysis, while in the millimolar range it simultaneously inhibits the rate of ATP hydrolysis and activates the ATP ⇌ P_i exchange reactions (9, 13, 16). This dependence has been attributed to the binding of calcium to high and low affinity binding sites of the enzyme, located, respectively, at the outer and inner surface of the vesicle membrane (9). At a high concentration, Mg^{2+} competes with Ca^{2+} for the high affinity site (23-29). Fig. 1 shows that when the Mg^{2+} concentration of the medium is raised from 1 to 21 mM, in the presence of 0.1 mM ATP and 0.1 mM ADP, there is a parallel shift of the calcium concentrations required for half-maximal ATP hydrolysis (K) and half-maximal ATP ⇌ P_i exchange (K'). For the hydrolysis of ATP, K increases from 3.5 to 25 μM Ca^{2+}, while for the ATP ⇌ P_i exchange, K' increases from 0.2 to 1.3 mM Ca^{2+}. This finding indicates that increasing magnesium concentrations in the medium impairs the access of calcium to both classes of binding sites. In the conditions of Fig. 1, the rates of ATP hydrolysis obtained in the presence of optimal Ca^{2+} concentrations do not vary significantly with the magnesium concentration of the medium. In contrast, the maximal rates of ATP ⇌ P_i exchange increase severalfold when the magnesium concentration is raised from 1 to 21 mM.

Apparent K_m for P_i—With the use of leaky vesicles and 0.1 mM ATP, raising the magnesium concentrations from 1 to 21 mM leads to a decrease in the apparent K_m of P_i for the ATP ⇌ P_i exchange (Fig. 2*B*). In the presence of 21 mM Mg^{2+}, the apparent K_m for P_i calculated from double reciprocal plots is 2.5 mM (not shown). This value is in the same range as that found when a Ca^{2+} gradient is formed across the vesicle membrane (9). In the presence of 1 mM Mg^{2+}, the apparent K_m for P_i is higher than 20 mM and cannot be determined because the P_i concentrations used are far below saturation (Fig. 2*B*). The P_i concentration on the assay medium cannot be increased above 8 mM due to the formation of insoluble calcium phosphate salt. In Fig. 2*B*, for higher Mg^{2+} concentrations, higher calcium concentrations were used in order to ensure maximal rates of ATP synthesis. The observed increase on the apparent K_m for P_i accounts for the differences in the maximal rates of ATP ⇌ P_i exchange observed in Fig. 1*B* with 2 mM P_i.

In order to determine the ratio between the rates of hydrolysis and synthesis of ATP, in Fig. 2*A* the rate of ATP hydrolysis is measured under the same conditions used to measure the rate of ATP ⇌ P_i exchange. In the presence of millimolar calcium concentrations, the velocity of ATP hydrolysis increases when the magnesium concentration in the medium is raised. However, the activating effect of magnesium for the hydrolysis of ATP (Fig. 2*A*) is smaller than that observed for the synthesis of ATP (Fig. 2*B*).

In the conditions of Fig. 2 and for incubation intervals up to 2 min, both the rates of ATP hydrolysis and ATP ⇌ P_i exchange were found to be linear as a function of time. For incubation intervals longer then 2 min, both rates decreased (data not shown).

Energy Conservation—The calculated ratios between the velocities of hydrolysis and synthesis of ATP obtained under the conditions of Fig. 2 varied between 25 and 2, being lower

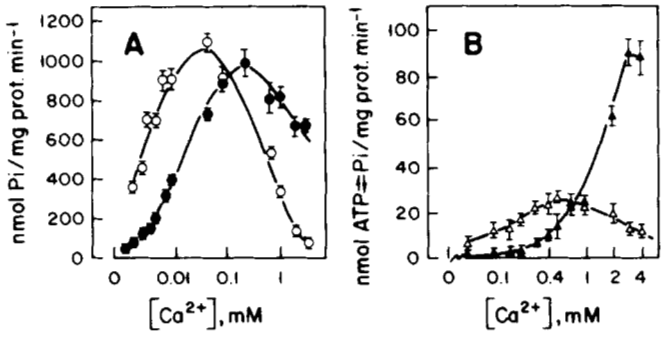


FIG. 1. **Effects of Ca^{2+} and Mg^{2+} on the rates of ATP hydrolysis and of ATP ⇌ P_i exchange.** *A*, ATP hydrolysis: the reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP, 0.1 mM; P_i, 2 mM; [γ-^{32}]ATP, 0.1 mM; EGTA, 5 mM; different $CaCl_2$ concentrations to give the final free calcium concentrations shown in the figures and 1 mM $MgCl_2$ (○) or 21 mM $MgCl_2$ (●). *B*, ATP synthesis same experimental conditions as described in *A* except that nonradioactive ATP and $^{32}P_i$ were used. △, 1 mM $MgCl_2$; ▲, 21 mM $MgCl_2$. The reaction was started by the addition of leaky vesicles, total of 15 μg/ml, and arrested after 1 min of incubation at 36.5 °C. *Symbols* and *bars* represent the average ± S.E. of four experiments.

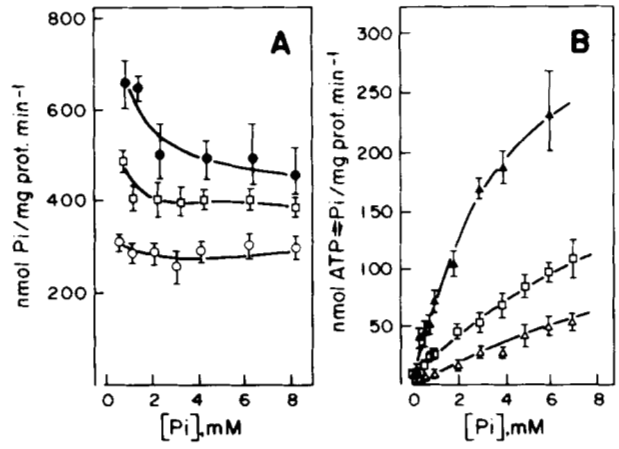


FIG. 2. **Effects of P_i and Mg^{2+} on the rates of ATP hydrolysis and ATP ⇌ P_i exchange.** *A*, ATP hydrolysis: the reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP 0.1 mM; [γ-^{32}P]ATP, 0.1 mM; P_i in the indicated concentrations; and 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (○); 2 mM $CaCl_2$ plus 5 mM $MgCl_2$ (□), or 3 mM $CaCl_2$ plus 21 mM $MgCl_2$ (●). *B*, ATP ⇌ P_i exchange: same experimental conditions as described in *A* except that nonradioactive ATP and $^{32}P_i$ were used. △, 1 mM $CaCl_2$ plus 1 mM $MgCl_2$; □, 2 mM $CaCl_2$ plus 5 mM $MgCl_2$; ▲, 3 mM $CaCl_2$ plus 21 mM $MgCl_2$. The reaction was started by the addition of leaky vesicles, total of 15 μg protein/ml, and arrested after 1 min at 36.5 °C. *Symbols* and *bars* represent the average ± S.E. of three experiments.

when the P_i and Mg^{2+} concentrations in the medium were higher (Fig. 3*A*) ATP antagonizes the effect of P_i and Mg^{2+} (Fig. 3*B*). In the presence of optimal Ca^{2+} concentrations, and for each given combination of P_i and Mg^{2+} concentrations, higher ratios are obtained with higher ATP concentrations in the medium. Table I summarizes the variation on the ratios between the velocities of hydrolysis and synthesis of ATP measured in the presence of different ATP, P_i, and $MgCl_2$ concentrations. The observed values are higher the higher the ATP and the lower the P_i and $MgCl_2$ concentrations used, varying between 700 and 1.2. These results favor the proposition that the simple binding of ions and substrates to the enzyme can regulate the rates of the reactions of ATP hydrolysis and synthesis independently of the presence of a transmembrane calcium gradient.

In this report it is shown that ratios between the rates of hydrolysis and synthesis of ATP even lower than those measured in presence of transmembrane Ca^{2+} gradient can be measured with the use of leaky vesicles simply by varying the Ca^{2+}, Mg^{2+}, P_i, and ATP concentrations in the medium.

The hydrolysis of ATP by the (Ca^{2+} + Mg^{2+})-dependent ATPase shows a complex substrate concentration dependence that cannot be fitted with a single straight line on a double reciprocal plot (9, 30–36). At concentrations above 0.1 mM, ATP binds both to the catalytic and to a regulatory site of the enzyme. Saturation of the regulatory site accelerates the rate of ATP hydrolysis (9, 30–36). Evidence that the sarcoplasmic reticulum ATPase undergoes a conformational change during the hydrolysis of ATP has been presented by different authors (28, 37–42).

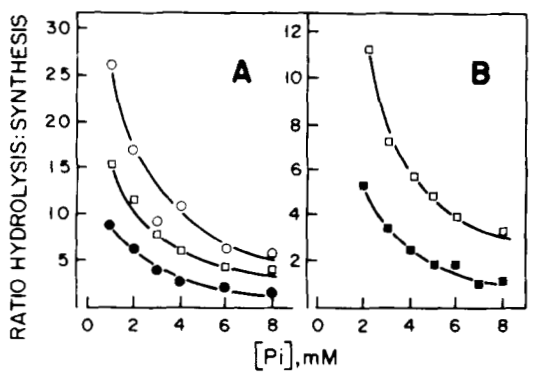


FIG. 3. **Effect of Mg^{2+} and ATP on the ratio between the velocities of hydrolysis and synthesis of ATP.** *A*, the reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP, 0.1 mM; [γ-^{32}P]ATP or ATP, 0.1 mM; $^{32}P_i$ or P_i in the indicated concentrations; and 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (○), 2 mM $CaCl_2$ plus 5 mM $MgCl_2$ (□); or 3 mM $CaCl_2$ plus 21 mM $MgCl_2$ (●). *B*, the reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP, 0.1 mM; $CaCl_2$, 2 mM; $MgCl_2$, 5 mM; $^{32}P_i$ or P_i in the indicated concentrations; and ATP or [γ-^{32}P]ATP, 0.1 mM (□) or 0.01 mM (■). The reaction was started by the addition of leaky vesicles, total of either 15 μg (0.1 mM ATP) or 5 μg (0.01 mM ATP) of protein/ml, and arrested after a 1-min incubation at 36.5 °C.

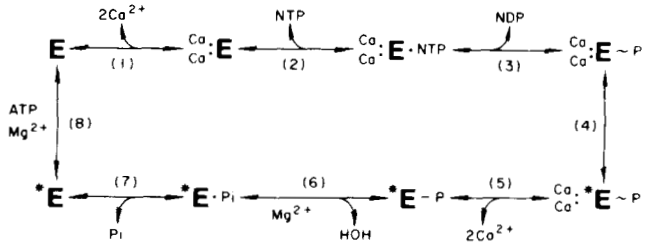
TABLE I

Variation of the ratio between the velocities of ATP hydrolysis and synthesis as a function of the relative concentrations of P_i, Mg^{2+}, and ATP in the reaction medium

The reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP, 0.1 mM; ATP, P_i, and $MgCl_2$ concentrations as indicated in the table. The $CaCl_2$ concentrations were 1 mM, 2 mM, and 3 mM for the magnesium concentrations 1 mM, 5 mM, and 21 mM, respectively. The experimental conditions were as described in Figs. 1 to 3. The values shown in the table were calculated using the average values of the rates of hydrolysis and synthesis of ATP measured in 3 to 4 different experiments.

$MgCl_2$	P_i (2 mM)			P_i (8 mM)		
	ATP (5 mM)	ATP (100 μM)	ATP (10 μM)	ATP (5 mM)	ATP (100 μM)	ATP (10 μM)
1 mM		15			6	
5 mM	700	11.4	7.9		3.5	1.2
21 mM	34	7.6		30	1.8	

The intermediary steps involved in the catalytic cycle of the (Ca^{2+} + Mg^{2+})-dependent ATPase can be tentatively interpreted according to the following reaction sequence (10):



This sequence includes two distinct functional states of the enzyme E and $*E$ which differ by their respective calcium affinities. When in the E conformation, the site which translocates calcium through the membrane faces the outer surface of the vesicle membrane and has a high affinity for Ca^{2+}. E can be phosphorylated by ATP but not by P_i.

In previous reports, it was shown that during the hydrolysis of nucleotide measured in presence of Ca^{2+}, an enzyme form is generated which is phosphorylated by P_i (10, 43, 44) and is able to catalyze a rapid phosphate-oxygen exchange (44). In the reaction sequence, this enzyme form is represented by $*E$. Phosphorylation by P_i is accounted for by reversal of reactions 6 and 7 and the phosphate-oxygen exchange by these two reactions flowing forward and backward during steady state. Both the steady state level of phosphoenzyme formed from P_i as well as the rate of phosphate-oxygen exchange are decreased when the ATP concentration in the medium is raised from the micromolar to the millimolar range (10, 43, 44). In the reaction sequence this is accounted for by reaction 8. High ATP concentrations accelerate the rate of reaction 8 forward leading to a decrease of the steady state level of $*E$ and $*E \sim P$. In previous reports (13, 14, 16, 42, 45–48) it was shown that the phosphoenzyme $*E \sim P$ is not able to transfer its phosphate to ADP (low energy). In the sequence, only the phosphoenzyme form $\overset{Ca}{Ca}E \sim P$ is able to transfer its phosphate to ADP (high energy). With the use of leaky vesicles, the phosphoenzyme of low energy is converted into high energy when the Ca^{2+} concentration in the medium is raised to the millimolar range (13, 14, 16, 42, 45–48). In this condition, Ca^{2+} binds to the low affinity site of the enzyme (reaction 5) and the phosphoenzyme $\overset{Ca}{Ca}*E \sim P$ is spontaneously converted into $\overset{Ca}{Ca}E \sim P$. When the Ca^{2+} and P_i concentrations are not sufficient to bind to the enzyme form $*E$, reactions 7, 6, and 5 become irreversible and the enzyme catalyzes only the hydrolysis of ATP (Figs. 1 and 2). In the presence of saturating concentrations of ADP, Mg^{2+}, P_i (Fig. 2), and Ca^{2+} (Fig. 1), the ratio between the rates of hydrolysis and synthesis depends on the rate of conversion of $*E$ into $\overset{Ca}{Ca}E$ (reactions 8 and 1). This ratio approaches unity when the catalytic cycle is confined between reactions 2 and 7. For each ATP molecule hydrolyzed, one molecule of $*E$ becomes available to react with P_i and participate in the reverse reaction leading to the synthesis of a new ATP molecule. The ratio of hydrolysis to synthesis is higher than 1 to the extent that some of the

enzyme units $^*E$ are converted into $\overset{Ca}{Ca}E$ instead of being drived in the reverse direction through phosphorylation by P_i. Therefore, the higher the ATP concentration in the medium, the faster the forward rate of reactions 8 and 1, and the higher the ratio between the rates of ATP hydrolysis and synthesis (Table I).

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