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

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The reversibility of the reaction catalyzed by purified (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-dependent ATPase of sarcoplasmic reticulum was studied by measuring the rates of synthesis and hydrolysis of ATP observed during the ATP \rightleftharpoons \text{ADP} + \text{Pi} exchange reaction. The ratio between the velocities of hydrolysis and synthesis of ATP was found to vary between 0.7 and 1.2 depending on the concentrations of ATP, Mg\textsuperscript{2+}, Mg\textsuperscript{2+} and Ca\textsuperscript{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 \(K_a\) of P\textsubscript{i} for the ATP \rightleftharpoons P\textsubscript{i} exchange reaction varies, depending on the Mg\textsuperscript{2+} concentration of the medium, being lower with higher Mg\textsuperscript{2+} concentration. The ratio between the velocities of hydrolysis and synthesis of ATP decreases when the Mg\textsuperscript{2+} and Pi concentrations are increased and the ATP concentration is decreased.

The entire sequence of reactions catalyzed by the Ca\textsuperscript{2+} + Mg\textsuperscript{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 Pi (1-9). When vesicles are incubated in a medium containing ATP, ADP, P\textsubscript{i}, Mg\textsuperscript{2+} and Ca\textsuperscript{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\textsuperscript{2+} concentration inside the vesicles. When a sufficient transmembrane Ca\textsuperscript{2+} concentration gradient is formed, an exchange between \(32\text{P}\) and the \(\gamma\)-phosphate of ATP pool is observed. This ATP \rightleftharpoons P\textsubscript{i} exchange 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 Pi. 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\textsubscript{i} exchange reaction were performed in the presence of high ATP concentrations (10 mM). Under these conditions, both the apparent \(K_a\) for P\textsubscript{i} and the ratio between the velocities of hydrolysis and synthesis of ATP have different values depending on whether or not a Ca\textsuperscript{2+} concentration gradient is formed across the vesicle membrane (9, 10, 13-16). When a gradient is formed, the apparent \(K_a\) for P\textsubscript{i} is in the range of 3 to 4 mM and the ratio between the velocities of hydrolysis and synthesis of ATP in is in the range of 2 to 5. In the absence of a gradient, the \(K_a\) for P\textsubscript{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 TTP and IDP (9, 10, 14, 16). In this report it is shown that the apparent affinity for P\textsubscript{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\textsuperscript{2+} gradient simply by varying the ATP and Mg\textsuperscript{2+} concentration of the medium, without the need of treating the vesicles with silver.

**EXPERIMENTAL PROCEDURES**

Leaky vesicles reconstituted from purified (Ca\textsuperscript{2+} + Mg\textsuperscript{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 pmol of Pi/mg of protein min\textsuperscript{-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\textsuperscript{2+} and absence of Ca\textsuperscript{2+}. The reformed vesicles of Ca\textsuperscript{2+} + Mg\textsuperscript{2+}-dependent ATPase were leaky and unable to accumulate Ca\textsuperscript{2+} under any condition studied (18). The vesicles were stored in 2.2 mM Tris/sucrose buffer (pH 8.0) at -6 °C. Protein was measured by the method of Lowry et al. (19), \[\text{P}\textsubscript{32}\] 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\textsubscript{4}PO\textsubscript{4} salt (20). The \[\text{P}\textsubscript{32}\] was stored in dilute HCl until used. \[\gamma\text{P}\textsubscript{32}\]ATP was prepared according to the method of Glynn and Chapel (21) with small modifications as previously described (22). The ATP concentration was determined spectrophotometrically.

ATPase activity was tested by measuring the amount of \(\text{P}\textsubscript{32}\), released from \[\gamma\text{P}\textsubscript{32}\]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 \[\gamma\text{P}\textsubscript{32}\]ATP, Mg\textsubscript{35}C\textsubscript{3}, Ca\textsubscript{35}C\textsubscript{3}, enzyme, and ethylene glycol bis(β-aminopropylether)-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
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 H2SO4. The 32Pproduced was extracted from the assay medium as described by de Meis and Carvalho (13) and counted in a liquid scintillation counter.

ATP \( \rightleftharpoons \) P, exchange was determined by measuring the appearance of \( ^{32} \text{PATP} \) in the medium. The reaction conditions were the same as described above except that \( ^{32} \text{P} \) and nonradioactive ATP were used. Unreacted \( ^{32} \text{P} \), was extracted from the assay medium after stopping the reaction with an acid solution of ammonium molybdate by adding 0.3 ml of acetic acid 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, and 0.3 ml of acetic acid 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 Ca2+ concentration gradient, the forward and reverse reactions of the (Ca2+ + Mg2+) -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 \( \rightleftharpoons \) P, 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, Mg2+ competes with Ca2+ for the high affinity site (23–29). Fig. 1 shows that when the Mg2+ 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 \( \rightleftharpoons \) P, exchange (K'). For the hydrolysis of ATP, K increases from 3.5 to 25 mM Ca2+, while for the ATP \( \rightleftharpoons \) P, exchange, K' increases from 0.2 to 1.3 mM Ca2+. 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 Ca2+ concentrations do not vary significantly with the magnesium concentration of the medium. In contrast, the maximal rates of ATP \( \rightleftharpoons \) P, exchange increase severalfold when the magnesium concentration is raised from 1 to 21 mM.

Apparent Ks for P,—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 Ks of P, for the ATP \( \rightleftharpoons \) P, exchange (Fig. 2B). In the presence of 21 mM Mg2+, the apparent Ks for P, calculated from double reciprocal plots is 2.5 mM (not shown). This value is in the same range as that found when a Ca2+ gradient is formed across the vesicle membrane (9). In the presence of 1 mM Mg2+, the apparent Ks for P, is higher than 20 mM and cannot be determined because the P, concentrations used are far below saturation (Fig. 2B). The P, concentration on the assay medium cannot be increased above 8 mM due to the formation of insoluble calcium phosphate salt. In Fig. 2B, for higher Mg2+ concentrations, higher calcium concentrations were used in order to ensure maximal rates of ATP synthesis. The observed increase on the apparent Ks for P, accounts for the differences in the maximal rates of ATP \( \rightleftharpoons \) P, exchange observed in Fig. 1B with 2 mM P,.

In order to determine the ratio between the rates of hydrolysis and synthesis of ATP, in Fig. 2A the rate of ATP hydrolysis is measured under the same conditions used to measure the rate of ATP \( \rightleftharpoons \) P, 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. 2A) is smaller than that observed for the synthesis of ATP (Fig. 2D).

In the conditions of Fig. 2, the ATP synthesis was maintained for incubation intervals up to 5 min, both the rates of ATP hydrolysis and ATP \( \rightleftharpoons \) P, exchange were found to be linear as a function of time. For incubation intervals longer than 5 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
when the Pi and Mg\(^{2+}\) concentrations in the medium were higher (Fig. 3A) ATP antagonizes the effect of Pi, and Mg\(^{2+}\) (Fig. 3B). In the presence of optimal Ca\(^{2+}\) concentrations, and for each given combination of Pi, 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, Pi, and MgCl\(_2\) concentrations. The observed values are higher the higher the ATP and the lower the Pi 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\(^{2+}\), Pi, 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 reticular ATPase undergoes a conformational change during the hydrolysis of ATP has been presented by different authors (25, 37–42).

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):

![Diagram of ATP Hydrolysis and Synthesis](image)

**Table I**

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

The reaction medium contained Tris/maleate buffer, 30 mM (pH 7.0); ADP, 0.1 mM; [P\(_{\text{ATP}}\)]ATP or ATP, 0.1 mM; 25\(^{32}\)P or 25\(^3\)P, in the indicated concentrations; and 1 mM CaCl\(_2\), plus 1 mM MgCl\(_2\) (C), 2 mM CaCl\(_2\) plus 5 mM MgCl\(_2\) (C); or 3 mM CaCl\(_2\) plus 21 mM MgCl\(_2\) (C). 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; 25\(^{32}\)P or 25\(^3\)P, in the indicated concentrations; and ATP or [P\(_{\text{ATP}}\)]ATP, 0.1 mM C or 0.01 mM (E). The reaction was started by the addition of leaky vesicles, total of either 15 \(\mu\)g (0.1 mM ATP) or 5 \(\mu\)g (0.01 mM ATP) of protein/ml, and arrested after a 1-min incubation at 36.5 °C.

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 Pi.

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 Pi (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 Pi 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 ~ P. In previous reports (13, 14, 16, 42, 45–48) it was shown that the phosphoenzyme *E ~ P is not able to transfer its phosphate to ADP (low energy). In the sequence, only the phosphoenzyme form Ca\(^{2+}\)E ~ 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 Ca\(^{2+}\)E ~ P is spontaneously converted into Ca\(^{2+}\)E ~ P. When the Ca\(^{2+}\) and P, 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\(^{2+}\), Pi (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 Ca\(^{2+}\)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, 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 CaE instead of being driven in the reverse direction through phosphorylation by P.

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 1).

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Mr. Isaltino R. Soares, Valdecir A. Suzano, and Antonio Carlos M. da Silva.

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