Reaction Mechanism of Ca\(^{2+}\)ATPase of Sarcoplasmic Reticulum

EQUILIBRIUM AND TRANSIENT STUDY OF PHOSPHORYLATION WITH Ca-ATP AS SUBSTRATE

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At pH 7.0 and 5 °C, in the absence of potassium and magnesium, the Ca-ATPase of the sarcoplasmic reticulum slowly hydrolyzes the Ca-ATP at a rate of 0.05 s\(^{-1}\). During turnover 4 nmol of phosphoenzyme per mg of total protein accumulate with a \(K_m\) value of 10\(^{-8}\) M.

Combining rapid filtration and rapid acid quenching, we took advantage of the above properties to study the early steps of phosphorylation.

\[
\begin{align*}
\text{Ca}^2+\text{E} + \text{Ca ATP} & \rightleftharpoons \text{Ca}^2+\text{E-ATP} \\
\text{Ca}^2+\text{E-PCa}^2+\text{ADP} & \rightleftharpoons \text{Ca}^2+\text{E-PCa} + \text{ADP}
\end{align*}
\]

Under these conditions, reaction steps leading to the phosphoenzyme formation are too fast to be studied. In particular, ATP binding has never been directly measured in the presence of calcium, and most of the proposed mechanisms for ATP binding are derived from kinetic studies of phosphorylation. In order to elucidate the mechanism of ATP binding, we have chosen conditions that slow down the phosphorylation process. It has been shown that at low temperature and in the absence of magnesium the formation of the covalent phosphoenzyme is slow (Shigekawa et al., 1978; Yamada and Ikemoto, 1980). Under these conditions, the Ca-ATP complex is the substrate (Yamada and Ikemoto, 1980; Shigekawa et al., 1983a, 1983b, 1985), and it is slowly hydrolyzed compared with Mg-ATP. Moreover, competitive inhibition indicates that Ca-ATP and Mg-ATP react with the same catalytic site and lead to the same reaction intermediates (Shigekawa et al., 1985).

Therefore, in the present study we take advantage of this slow turnover in the absence of magnesium to study the early steps of the Ca-ATPase cycle. Combination of rapid filtration and multitasking techniques enable us to measure the rates of Ca-ATP binding and phosphorylation steps. We show that the enzyme has a good affinity for Ca-ATP (\(K_a = 7 \times 10^{-7}\) M) and that the phosphoenzyme slowly accumulates with a high \(K_m\) of 10\(^{-8}\) M. By applying these data to minimal schemes, we have deduced other kinetic constants that could not be directly measured.

MATERIALS AND METHODS

SR vesicles have been prepared and the protein concentration determined as described in Champeil et al. (1985). All experiments have been carried out at 5 °C in a cold room.

The ATPase activity was measured by \([^{32}P]ATP\), released from \([^{32}P]ATP\) in a medium containing 200 mM Mops-Tris, pH 7.0 and at 5 °C, 5 mM CaCl\(_2\), and various ATP concentrations as described in Champeil et al. (1986).

Kinetics of \([^{32}P]ATP\) and \([^{14}C]ATP\) binding were performed with a BIOLOGIC rapid filtration device as already described (Champeil and Guillaud, 1986) except that we used DA 0.65-µm Millipore filter instead of HA 0.45-µm. DA filters allow a higher flow rate for rapid filtration at 5 °C and retain nearly 100% of the protein up to 0.15 mg of ATPase (Lacapere, 1987). Only 20 ms were needed to completely renew the 40 µl of wet volume of the filter; under our conditions, 5 °C
Ca-ATP as Substrate for SR-ATPase

RESULTS

Ca-ATP as a Substrate at Steady State: Hydrolysis and Enzyme Phosphorylation—High calcium concentrations have been used to ensure that all the ATP is present in the form of its calcium complex, minimizing any effect of contaminant magnesium. Fig. 1 shows that at 5 mM CaCl₂ and in the absence of magnesium, Ca-ATP hydrolysis by native SRV is biphasic and depends on ATP concentration. Supposing that the stoichiometry of the active site is 3–5 nmol/mg protein (Yamada and Ikemoto, 1980; Shigekawa et al., 1983a; and Fig. 2 herein), the 5–7 nmol of P, liberated in the fast phase in Fig. 1 correspond to one or two turnovers. This fast phase is followed by a slow Ca-ATP hydrolysis, and this slowing down is probably due to inhibition of the SR-ATPase by increasing calcium concentration inside the vesicles. Normally, an ionophore would prevent this calcium accumulation and thereby extend the fast hydrolysis phase, but the presence of 5 mM CaCl₂ makes the ionophore counterproductive. For an ATP concentration between 0.25 and 10 µM, the initial rate of Ca-ATP hydrolysis is 0.05 nmol/mg-s, i.e. 15 times lower than in the presence of 5 mM MgCl₂ and 0.1 mM CaCl₂ (data not shown). The activation observed in Fig. 1 for ATP concentrations higher than 10 µM has been already discussed by Champeil and Guillain (1986) for Mg-ATP and will be discussed in a forthcoming paper for Ca-ATP (Lacapbre, 1987).

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Despite the low ATPase activity in the absence of magnesium, Ca-ATP is a good substrate for phosphorylation (Yamada and Ikemoto, 1980). Fig. 2 shows that taking 4 nmol/mg for the maximal level of phosphorylation, half-maximal phosphorylation is reached with 0.01 µM ATP (Kₚ₅) in the presence of 5 mM CaCl₂. It is worth mentioning that such a low value for Kₚ₅ necessitates experimental precautions, as described under "Materials and Methods," to maintain a constant excess of Ca-ATP relative to the stoichiometry of maximal phosphorylation.

Phosphorylation Kinetics with Ca-ATP: Multimixing Experiments—The time course of phosphorylation was measured at pH 7.0 and at 5 °C by the fast quench technique after mixing equal volumes of SRV (preincubated in 0.05 mM CaCl₂) with 0.004 mM ATP and 10 mM calcium (Fig. 3). Preincubation conditions were chosen to avoid high internal calcium concentration before phosphorylation, which could...
slow down the first turnover. During phosphorylation, the amount of ATP was kept almost constant by choosing an initial [ATP]/[active site] ratio of 10. Fig. 3 shows that under these conditions phosphoenzyme formation has an initial rate of 2.2 nmol/mg-s, thereafter following a single exponential with 0.6 s⁻¹ as the observed rate constant (kobs) and 3.7 nmol/mg for maximal phosphorylation level; these values are comparable with those obtained by Yamada and Ikemoto (1980) and Shigokawa et al. (1983a).

**Kinetics of Ca·ATP Binding: Rapid Filtration Experiment**—Since phosphorylation is fast in the presence of magnesium, it has not previously been possible to measure directly ATP binding before phosphorylation. The present experimental conditions, i.e. slow phosphorylation combined with the rapid filtration technique, make measurement of the transient E-ATP complex possible (Lacapère et al., 1986). As shown in Fig. 4. In this experiment, SRV were preincubated in 0.05 mM CaCl₂ (as for the phosphorylation experiment), were layered on filters, and were washed for various times with a mixture of [U-³²P]ATP and [³²P]ATP (0.002 mM total ATP) in 5 mM CaCl₂. Fig. 4A shows that during the first 200 ms the amounts of bound [³²P] and [¹⁴C] cannot be distinguished but that thereafter the amount of [³²P] remains constant while the amount of [¹⁴C] decreases; this effect is shown on a longer scale in Fig. 4B. With reference to Scheme II, the amount of [¹⁴C] represents the sum of ADP and ATP bound to the enzyme and the amount of [³²P] represents the sum of bound ATP and covalent phosphoenzyme.

Thus, these first 200 ms represent ATP binding before any cleavage of the ATP molecule. Thereafter, the rate of [¹⁴C] decrease is similar to the rate of covalent phosphoenzyme formation measured in Fig. 3; this indicates that the dissociation of ADP following phosphoenzyme formation is fast. Also, the data indicate that an appreciable amount of the enzyme remains in Ca₂⁺⁻E-CaATP after 5 s. This bound ATP is directly measured by [³²C] which reaches a minimum of 0.5 nmol/mg after 10 s. Also, comparison of the 4 nmol/mg [³²P] bound after 10 s measured by rapid filtration (Fig. 4B) with the 3.5 nmol/mg phosphoenzyme measured by multimixing (Fig. 3) confirms that 0.5 nmol of bound ATP are not hydrolyzed. The discrepancy results because multimixing measures only covalent phosphoenzyme while rapid filtration measures covalent plus noncovalent [³²P].

Returning to the initial 200 ms in Fig. 4A, the corresponding initial velocity of ATP binding is 40 nmol/mg-s at 0.002 mM ATP, which is 20 times higher than the corresponding initial velocity of covalent phosphoenzyme formation (Fig. 3). Therefore, during these first 200 ms when no phosphoenzyme has formed, it is possible to measure directly the initial velocity of Ca·ATP binding. Fig. 5A shows that this initial velocity (V₀) is proportional to the Ca·ATP concentration and Fig. 5B shows the corresponding observed rate constants (kobs), deduced by fitting a single exponential to the first 200 ms. Values of kobs and V₀ give 3.5 and 5 x 10⁶ M⁻¹ s⁻¹, respectively, for the rate constant for Ca·ATP binding (k₀) assuming 4 nmol/mg for maximal active site stoichiometry. However, direct estimation of initial velocities for Ca·ATP binding from filtration experiments requires precise knowledge of time 0. Since the reaction actually starts only after flushing the filter volume occupied by the vesicles (see “Materials and Methods”), a small uncertainty is introduced in determining initial velocity of Ca·ATP binding, particularly for high substrate concentration when this initial velocity is high. We therefore favor the 3.5 x 10⁶ M⁻¹ s⁻¹ deduced from the slope of the curve in Fig. 5B. By extrapolating kobs in Fig. 5B to [Ca·ATP] = 0 an apparent off rate constant (kₜₐ₉) of 2.5 s⁻¹ is obtained. It is the intention of the following experiments to confirm that this off rate constant effectively corresponds to the actual dissociation step of Ca·ATP.

**ADP-induced Phosphoenzyme Decomposition: Multimixing Experiment**—In order to measure the rate of Ca·ATP dissociation from the enzyme the reaction can be run in reverse by adding ADP to the phosphoenzyme. However, to directly measure this rate, ADP binding to Ca₂⁺⁻E-PCa and the phosphate transfer to ADP must be fast in comparison with Ca·ATP dissociation. In other words, addition of ADP should induce a transient accumulation of an appreciable amount of Ca₂⁺⁻E-CaATP in order to measure the true dissociation rate constant.

Fig. 6 shows that the decomposition of the [³²P]-phosphoenzyme by ADP is fast and gives an initial velocity of dephosphorylation of 30-40 nmol/mg-s and an observed rate constant of 15 s⁻¹ when fitted by a single exponential. Only 2 nmol/mg of the original 3 nmol/mg phosphoenzyme follow this fast dephosphorylation process. Since the rate constant is strongly coupled to the maximal level of dephosphorylation, we have to verify that under these conditions (i.e. in the absence of magnesium and potassium) only ½ of the phosphoenzyme is ADP-sensitive.

In order to establish that part of the phosphoenzyme, formed with Ca·ATP as substrate, is ADP-insensitive we used TNP-ATP. Indeed, it has been shown by Andersen et al. (1985) that in the presence of magnesium the transition from the ADP-sensitive to the ADP-insensitive phosphoenzyme is
FIG. 5. Concentration dependence of the initial rate \( (V_o, A) \) and of the observed rate constant \( (k_{obs}, B) \) of Ca-ATP binding. This experiment was similar to the one in Fig. 4, but the amount of Ca-ATP perfused was varied between 1 and 3 \( \mu \)M, and the amount of protein was kept constant (0.05 mg). The first 200 ms were then fit with a single exponential to determine \( k_{obs} \).

FIG. 6. Time course of ADP-induced SR-ATPase dephosphorylation measured by acid quench flow. Medium was 200 mM Mops-Tris, pH 7.0 at 5 °C. Syringe 1 of the Durrum multimixer contained SRV (0.2 mg/ml) in 0.05 mM CaCl\(_2\); syringe 2 contained 2 mM ADP and 2.5 mM EGTA-Tris. The reaction was stopped in the collection syringe by 240 mM perchloric acid (ETA) and 30 mM Pi. The inset is a schematic representation of the experiment. The different symbols correspond to different experiments.

FIG. 7. Mg-ATP and Ca-ATP enhancement of TNP-ATP fluorescence. Medium was 200 mM Mops-Tris, pH 7.0, 0.1 mg/ml SRV, 50 \( \mu \)M ATP, 2 \( \mu \)M TNP-ATP at 5 °C with either 5 mM MgCl\(_2\), 0.1 mM CaCl\(_2\) in the case of Mg-ATP or 5 mM CaCl\(_2\) in the case of Ca-ATP.

FIG. 8. Time course of ATP dissociation after dephosphorylation and ATP synthesis induced either by Ca-ADP (open symbols) or by free ADP (closed symbols). Enzyme phosphorylation was performed for 5 s directly on the filter in 200 mM Mops-Tris, pH 7.0, 1 mM \([H]^+\)sucrose, 5 mM CaCl\(_2\), 3 \( \mu \)M \([32P]ATP, 0.1 mg of SRV at 5 °C (see “Materials and Methods”). The immobilized phosphoenzyme was perfused with either 1 mM ADP and 5 mM CaCl\(_2\) (open symbols) or 1 mM ADP and 0.05 mM CaCl\(_2\) (closed symbols).

accompanied by an increase in the fluorescence of TNP-ATP. The first trace in Fig. 7 depicts an experiment performed in the presence of 5 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\) when the phosphoenzyme is predominantly in the ADP-insensitive form (Wakabayashi et al., 1986). As expected, under these conditions, ATP addition induces a 250% increase in TNP-ATP fluorescence (Dupont and Pougeois, 1983; Nakamoto and Inesi, 1984). The second trace shows that in the absence of magnesium but in the presence of 5 mM CaCl\(_2\), addition of ATP induces a 50% increase. Therefore, TNP-ATP experiment with Ca-ATP as the substrate shows that 1 nmol/mg is ADP-insensitive assuming 4 nmol/mg for the maximal level of phosphoenzyme. This verifies our fit of the data in Fig. 6 and is consistent with previous studies under similar conditions (Shigekawa and Dougherty, 1978; Wakabayashi and Shigekawa, 1987; Wakabayashi et al., 1986). Specifically, this verifies 15 s\(^{-1}\) as the rate of dephosphorylation, which is fast relative to the extrapolated rate of Ca-ATP dissociation (2.5 s\(^{-1}\) in Fig. 5B).

Rate of Ca-ATP Dissociation after ATP Synthesis Induced by Ca-ADP and Free ADP: Rapid Filtration Experiments—We have previously shown that rapid filtration experiments measure both covalent and non-covalent \(^{32}\)P. Since we have observed that dephosphorylation is fast compared with extrapolated Ca-ATP dissociation, we expect that ADP-induced dephosphorylation will give rise to a transient accumulation of a non-covalent E-CaATP complex whose dissociation could be followed by rapid filtration. Indeed, Fig. 8 shows that when covalent phosphoenzyme is layered on a filter and is perfused by an ADP-containing solution (see “Materials and Methods”), bound \(^{32}\)P disappears slowly. The initial velocity is six times slower than that observed for dephosphorylation (Fig. 6). Fig. 8 shows that ATP dissociates with a rate constant of 2 s\(^{-1}\) when 1 mM metal-free ADP is perfused (Fig. 8, solid squares), whereas the dissociation rate constant is only 0.3 s\(^{-1}\) when 1 mM Ca-ADP is perfused (Fig. 8, open triangles). Perfusion of 0.1 mM ADP (data not shown) gives the same dissociation rate constants, 2 and 0.3 s\(^{-1}\) in the absence and in the presence of 5 mM CaCl\(_2\), respectively. This confirms that metal-free ADP is the actual substrate for ATP synthesis (Yamada and Ikemoto, 1980) and that the affinity of ADP for the ADP-sensitive phosphoenzyme is better than 0.1 mM (Shigekawa and Kanazawa, 1982, Wang, 1986). When the enzyme was phosphorylated by \([14C]ATP, perfusion with ADP caused a decrease of the amount of bound \([14C]\) nucleotide at the same rate as with \([32P]ATP. This \([14C]\) signal corresponds to the Ca- E-CaATP complex still present after 5 s of phosphorylation, as discussed above in reference to Fig. 4B. Therefore, the initial 4 nmol/mg of \([32P]\) bound in Fig. 8 represents...
the E-CaATP complex (0.6 nmol/mg, see Fig. 4B), plus the actual ADP-sensitive phosphoenzyme (2 nmol/mg, see Fig. 6), plus the ADP-insensitive phosphoenzyme (1 nmol/mg, see Figs. 6 and 7). The dephosphorylation by metal-free ADP (square symbols in Fig. 8) is biphasic; thus, from the slower component of this biphasic decrease, we have estimated the rate of hydrolysis of the 1 nmol/mg of ADP-insensitive (E2-P species of Scheme I) to be 0.08 ± 0.02 s⁻¹. Both the slow level and the slow rate of hydrolysis of E₂-CaP agree with the initial rate of phosphate liberation measured at steady state (Fig. 1).

**DISCUSSION**

It has previously been shown that SR ATPase hydrolyzes the Ca·ATP complex slowly (Shigekawa et al., 1978, 1983a; Yamada and Ikemoto, 1980). In addition to confirming this observation, Fig. 1 shows that high concentrations of ATP activate the ATPase activity in a way similar to Mg·ATP which is the physiological substrate (Vigna, 1975).

At 5 °C in the presence of 5 mM external calcium, no internal calcium and no added magnesium, the complex Ca·ATP phosphorylates the SR-ATPase with an observed rate constant of 0.6 s⁻¹ (Fig. 3 and Shigekawa et al., 1983a). The maximal level of phosphoenzyme is 4 nmol/mg and half-phosphorylation is achieved with 0.01 μM Ca·ATP. Such a high apparent affinity is indicative of an almost irreversible step; this step is likely to be ADP release, which occurs very quickly after the formation of the covalent phosphoenzyme.

ADP-induced dephosphorylation of the E-CaATP complex (0.6 nmol/mg, see Fig. 4B), plus the actual ADP-sensitive phosphoenzyme (2 nmol/mg, see Fig. 6), plus the ADP-insensitive phosphoenzyme (1 nmol/mg, see Figs. 6 and 7). The dephosphorylation by metal-free ADP (square symbols in Fig. 8) is biphasic; thus, from the slower component of this biphasic decrease, we have estimated the rate of hydrolysis of the 1 nmol/mg of ADP-insensitive (E₂-P species of Scheme I) to be 0.08 ± 0.02 s⁻¹. Both the slow level and the slow rate of hydrolysis of E₂-CaP agree with the initial rate of phosphate liberation measured at steady state (Fig. 1).

**ADP-induced dephosphorylation of the ADP-sensitive phosphoenzyme (E₁-P) generating ATP synthesis and the slow phase to the ADP-insensitive phosphoenzyme (E₂-P) resulting only in P₁ release. The presence of a significant amount of the E₂-P indicates that the rate of calcium release (E₁-P → E₂-P) and the rate of E₂-P dephosphorylation are of the same order of magnitude. More original are our filtration experiments. Indeed, perfusion of a mixture of [³²P]ATP and [¹⁴C]ATP allows estimates of covalently and non-covalently bound phosphate. Furthermore, comparison with data obtained by acid quenching, which only measure covalently bound phosphate, makes the evaluation of transient, non-covalent complexes possible. Thus, the present work shows that Ca·ATP binds to the SR-ATPase with a velocity proportional to the substrate concentration with a rate constant of 3.5 × 10⁶ M⁻¹ s⁻¹. The next measurable step is a slow, covariant phosphorylation which is rapidly followed by ADP dissociation; in fact, with the methods used here, the observed rate of ADP dissociation cannot be distinguished from the rate of the phosphorylation itself.

In the ATP synthesis direction of the reaction, comparison of the data for covalent (Fig. 6) and the non-covalent (Fig. 8) binding shows that ADP-induced dephosphorylation is fast compared with ATP dissociation. Therefore, the transfer of phosphate from the phosphoenzyme to ADP is fast (observed rate constant of 15 s⁻¹), whereas dissociation of ATP is slow and, as we will see, may comprise several steps.

Scheme II is the minimal scheme required to fit the present experimental data and Table I summarizes assignment of rate constants. Assignment for the two first steps is a direct interpretation of data both for the binding and dissociation of ATP and for the phosphorylation and dephosphorylation. In the third step, k₃ must be greater than 50 s⁻¹ to explain the observed similarity between rates of [³²P] covalent binding (Fig. 3) and [¹⁴C] dissociation (Fig. 4B). Thus, the implication of Scheme II is that the dissociation constant for Ca·ATP is 0.7 μM and that ATP binding is followed by an unfavorable phosphorylation step (Kₐₐₚ = k₄/k₋₃ = 0.067), which is likely to be driven by ADP release when ATP concentration is low. However, it has been recently proposed by Petithory and Jencks (1986) that the rate-limiting step of the phosphorylation by Mg·ATP is a conformational change of the enzyme·Mg·ATP complex before phosphorylation. In light of their proposal, we analyzed our data according to Scheme III, which includes a slow conformational change in step 2 followed by a fast phosphorylation in step 3.

At first sight there are several possible combinations of rate

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**Table I**

<table>
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<th>Step</th>
<th>Reaction</th>
<th>Constants</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>E·Ca₄ + Ca·ATP → Ca₂·E·CaATP</td>
<td>kᵢ = 3.5 × 10⁶ M⁻¹ s⁻¹</td>
<td>Fig. 6</td>
</tr>
<tr>
<td>2.</td>
<td>Ca₂·E·CaATP → Ca₂·E-PCa·ADP</td>
<td>kᵣ = 2.5 s⁻¹</td>
<td>Figs. 5 and 8</td>
</tr>
<tr>
<td>3.</td>
<td>Ca₂·E-PCa·ADP → Ca₂·E·PCa + ADP</td>
<td>k₃ = 1 s⁻¹</td>
<td>Fig. 5</td>
</tr>
<tr>
<td>4.</td>
<td>Ca₂·E·PCa → E·PCa</td>
<td>k₄ = 15 s⁻¹</td>
<td>Fig. 6</td>
</tr>
<tr>
<td>5.</td>
<td>E·PCa (E₁ + P₁Ca) + 2Ca → E·Ca₂</td>
<td>k₅ = 0.05 s⁻¹</td>
<td>Figs. 1 and 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rate Constants Ref.</th>
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<tbody>
<tr>
<td>kᵢ = 3.5 × 10⁶ M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>kᵣ = 2.5 s⁻¹</td>
</tr>
<tr>
<td>k₃ = 1 s⁻¹</td>
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<tr>
<td>k₄ = 15 s⁻¹</td>
</tr>
<tr>
<td>k₅ = 0.05 s⁻¹</td>
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**Scheme III**

Ca·ATP

Ca₂·E·PCa·ADP → Ca₂·E-PCa + ADP

ADP

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**Figures and Symbols**

1. E·Ca₄ + Ca·ATP → Ca₂·E·CaATP
2. Ca₂·E·CaATP → Ca₂·E-PCa·ADP
3. Ca₂·E-PCa·ADP → Ca₂·E·PCa + ADP
4. Ca₂·E·PCa → E·PCa
5. E·PCa (E₁ + P₁Ca) + 2Ca → E·Ca₂
Table II

Summary of the rate constants used for the simulation of the reaction steps of Scheme III

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Constants</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ca₂⁺-E + Ca-ATP → Ca₂⁺-E.Ca⁺⁺²</td>
<td>(k_1 = 4 \times 10^5 \text{ M}^{-1} \text{s}^{-1})</td>
<td>Fig. 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(k_2 = 2.5 \text{ s}^{-1})</td>
<td>Figs. 5 and 8</td>
</tr>
<tr>
<td>2.</td>
<td>Ca₂⁺-E.CaATP → Ca₂⁺-E'-Ca⁺⁺²</td>
<td>(k_3 = 30 \text{ s}^{-1})</td>
<td>Hypothesis (Fig. 3)</td>
</tr>
<tr>
<td>3.</td>
<td>Ca₂⁺-E'-CaATP → Ca⁺⁺²-E'-PCa⁺⁺²-ADP</td>
<td>(k_4 = 100 \text{ s}^{-1})</td>
<td>See text</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(k_5 = 100 \text{ s}^{-1})</td>
<td>See text</td>
</tr>
<tr>
<td>4.</td>
<td>Ca⁺⁺²-E'-PCa⁺⁺²-ADP → Ca⁺⁺²-E'-PCa⁺⁺² + ADP</td>
<td>(k_6 = 0.05 \text{ s}^{-1})</td>
<td>Figs. 5 and 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(k_7 = 0.05 \text{ s}^{-1})</td>
<td>Figs. 5 and 6</td>
</tr>
<tr>
<td>5.</td>
<td>Ca⁺⁺²-E'-PCa⁺⁺² + ADP</td>
<td>(k_8 = 0 \text{ s}^{-1})</td>
<td>There is no P in the medium</td>
</tr>
<tr>
<td>6.</td>
<td>E'-PCa⁺⁺² (E' + P₅Ca) + 2Ca → E-Ca⁺⁺²</td>
<td>(k_9 = 100 \text{ s}^{-1})</td>
<td>See text (Fig. 7)</td>
</tr>
</tbody>
</table>

Fig. 9. Simulation of the reaction according to Schemes II and III. Panel A shows binding and phosphorylation with 2 μM Ca-ATP, and panel B shows dephosphorylation and ATP synthesis following addition of excess free ADP to the phosphoenzymes. The different species described are: Ca⁺⁺²-E, [1]; Ca⁺⁺²-E.CaATP, [2]; Ca⁺⁺²-E'-PCa⁺⁺²-ADP, [3]; Ca⁺⁺²-E'-PCa⁺⁺²; E'-PCa⁺⁺² and the dotted line represents the Ca⁺⁺²-E.CaATP of Scheme III, [5].

is faster and more favorable. Since our data obtained with Ca-ATP as substrate can be fit equally well by using either scheme, these data alone cannot confirm the existence of this conformational change. Nevertheless, we have recently measured a change in tryptophan fluorescence upon addition of ATP in the absence of calcium, which may correspond to a conformational change (Lacapère et al., 1990). However, this conformational change is very fast (Fernandez-Belda et al., 1984; Lacapère, 1987) and therefore probably does not correspond to the slow conformational change proposed as the rate-limiting step for phosphorylation.

In conclusion, we would like to emphasize that comparison of Ca-ATP and Mg-ATP complexes shows that Ca-ATP has a better affinity for the SR-ATPase (Yamada and Ikemoto, 1980; Shigekawa et al., 1987; Yamada et al., 1986; Ogawa et al., 1986; Lacapère, 1987) and a lower \(K_m\) value for phosphorylation. Therefore, under conditions generally used to study the sarcoplasmic reticulum Ca-ATPase (pH 7.0, 0.1 mM CaCl₂, 1-5 mM MgCl₂, 1 mM ATP with or without KCl), Ca-ATP may represent a significant proportion of the substrate for phosphorylation, and, during the ATPase cycle, the calcium ion bound in the catalytic site via Ca-ATP binding is probably exchanged for magnesium some time after phosphorylation (Shigekawa et al., 1983b; Lund and Moller, 1988; Orlowaksi et al., 1988).

Acknowledgment-We gratefully acknowledge David L. Stokes for his critical reading of the manuscript.

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More information about the choice of rate constants is available on request.
Reaction mechanism of Ca\textsuperscript{2+} ATPase of sarcoplasmic reticulum. Equilibrium and transient study of phosphorylation with Ca.ATP as substrate.
J J Lacapere and F Guillain


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