Effect of Temperature on the Ca\(^{2+}\) Transport ATPase of Sarcoplasmic Reticulum*

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The effect of temperature on sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle was studied by measuring the Ca\(^{2+}\) uptake, ATP and ITP hydrolysis, Ca\(^{2+}\)-efflux, ATP synthesis, ATP ↔ P\(_i\) exchange reaction, and phosphorylation of the Ca\(^{2+}\) transport enzyme by either ATP, ITP, or inorganic phosphate. At 0°, the enzyme is phosphorylated by ATP or ITP, but both Ca\(^{2+}\) uptake and P\(_i\) liberation are impaired. The rate of phosphoenzyme hydrolysis increases progressively as the temperature is raised from 0° to 35°. When ITP is used as substrate, the increment of P\(_i\) liberation is accompanied by a decrease of the steady state level of phosphoenzyme. This is not observed when ATP is used as substrate. The phosphoenzyme formed from ATP is more sensitive to ADP at 0° than at 30°.

Synthesis of ATP coupled to calcium efflux and ATP ↔ P\(_i\) exchange reaction are inhibited at 0°. Both the phosphorylation of the enzyme by P\(_i\) and the transfer of phosphate from the phosphoenzyme to ADP are impaired at 0°. The steady state level of enzyme phosphorylated by P\(_i\) increases linearly with temperature up to 20°. When the temperature is raised the fraction of enzyme phosphorylated by ITP decreases while the fraction of enzyme phosphorylated by P\(_i\) increases.

The data presented support the concept of two different conformations of the Ca\(^{2+}\) transport enzyme in equilibrium and indicate that this equilibrium is modified by temperature.

During the process of active Ca\(^{2+}\) transport, the Ca\(^{2+}\)-dependent NTTPase\(^*\) of sarcoplasmic reticulum vesicles is phosphorylated by the NTP at the outer surface of the vesicles (1-5). The phosphoenzyme formed (E-P) undergoes a transition through the membrane which results in the translocation of the externally bound calcium to the inner surface of the vesicles. Evidence has been presented that the calcium is released into the lumen of the vesicles before hydrolysis of the phosphoenzyme (6-8). After hydrolysis of E-P, the enzyme must undergo a new transition to translocate the Ca\(^{2+}\) binding sites back to the outer surface of the vesicles in order to initiate a new cycle (6-10).

It has been shown that at 0° both the calcium uptake and the NTTPase activity are inhibited. However, at this temperature the steady state level of E-P is virtually unaffected (11-16). In this paper the effect of temperature on the Ca\(^{2+}\)-dependent NTTPase was further studied. The aim was to identify the intermediary reactions impaired at low temperature.

In order to assist the reader in following the design and interpretation of the experiments reported, a reaction sequence based on data previously reported (10) is presented first:

\[
\begin{align*}
\text{ATP} & \rightarrow \text{ADP} & \text{(1)} \\
\text{E}^* & \rightarrow \text{E}^* \rightarrow \text{E} \rightarrow \text{E}^* & \text{(2)} \\
\text{E} & \rightarrow \text{E}^* & \text{(3)} \\
\text{E} & \rightarrow \text{E} & \text{(4)} \\
\text{E} & \rightarrow \text{E}^* & \text{(5)} \\
\text{E} & \rightarrow \text{E}^* & \text{(6)}
\end{align*}
\]

This reaction sequence includes two distinct functional states of the enzyme, E and E* (6-10, 17-21). The calcium binding site in the E form faces the outer surface of the vesicles and has an apparent K\(_m\) for calcium in the range of 1 to 3 μM (high affinity). In the E* form the calcium binding site faces the inner surface of the vesicles and has an apparent K\(_m\) for calcium in the range of 1 to 3 mM (low affinity). The E form is phosphorylated by NTP but not by P\(_i\), while the form E* is phosphorylated by P\(_i\), but not by NTP (10, 17, 18). Reaction 6 can be the rate limiting step depending on the NTP used. The conversion of E* into E occurs very slowly in the absence of NTP (22) and increasing concentrations of NTP activate the rate of interconversion, ITP being much less effective than ATP (10, 18, 23). Thus, during ITP hydrolysis, the form E* tends to accumulate and a simultaneous phosphorylation by ITP and P\(_i\) can be measured (10, 18).

MATERIALS AND METHODS

Sarcoplasmic Reticulum Vesicles—These vesicles were obtained from rabbit skeletal muscle as previously described (24). [γ-32P]ATP and [γ-32P]ITP—They were prepared as previously described (15) using 32P\(_i\) obtained from the Brazilian Institute of Atomic Energy. 32P\(_i\) was purified by extraction as phosphomolybdate with isobutyl alcohol/benzene, re-extraction to the aqueous phase.
with ammonia, and precipitation as MgNH₄PO₄ (25).

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NTPase Activity — The activity was assayed by measuring the ³²P release from [γ-³²P]ATP or [γ-³²P]TP. The ³²P, was extracted from the assay medium as phosphomolybdate with isototyl alcohol/benzene (9). In the experiment shown in the Table 1, the ATPase activity was measured by the method of Fiske and Subbarow (26).

Calcium Uptake — Uptake was measured by the filtration method using Millipore filters, average pore diameter 0.45 μ, as previously described (10).

RESULTS

TABLE I

Effect of temperature on Ca²⁺ uptake, ATPase activity, and E-P formation

The assay medium consisted of 20 mM Tris/maleate buffer (pH 7.0), 2 mM [γ-³²P]ATP, 0.2 mM CaCl₂, 0.26 mM EGTA, 4 mM MgCl₂, and 4 mM potassium oxalate. For E-P measurements, potassium oxalate was omitted. The reaction was started by addition of vesicles; other conditions were as described under "Materials and Methods." The values represent the average ± S.E. of four experiments.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>E-P</th>
<th>Ca²⁺ uptake</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g protein</td>
<td>μmol Ca¹⁺/mg protein/min</td>
<td>μmol Pᵢ/mg protein/min</td>
</tr>
<tr>
<td>0°C</td>
<td>2.19 ± 0.11</td>
<td>6.05 ± 0.01</td>
<td>0.026 ± 0.005</td>
</tr>
<tr>
<td>30°C</td>
<td>1.86 ± 0.10</td>
<td>1.84 ± 0.12</td>
<td>1.12 ± 0.10</td>
</tr>
</tbody>
</table>

The data indicate that both conditions, low temperature and low calcium concentration, the PI liberation was inhibited by either the low temperature or saturation of the low affinity calcium binding site. At 30°C and in the presence of low calcium concentration the Pᵢ liberation was maximal and the decrease of the level of E-P' promoted by the addition of ADP, was less pronounced.

The fraction of phosphoprotein sensitive to ADP is greater when the overall reaction was inhibited by either low temperature or saturation of the low affinity calcium binding site. At 30°C and in the presence of low calcium concentration the Pᵢ liberation was maximal and the decrease of the level of E-P' promoted by the addition of ADP, was less pronounced.

These data indicate that both conditions, low temperature and low calcium concentration, the PI liberation was inhibited by either the low temperature or saturation of the low affinity calcium binding site. At 30°C and in the presence of low calcium concentration the Pᵢ liberation was maximal and the decrease of the level of E-P' promoted by the addition of ADP, was less pronounced.

**Fig. 1.** Effect of temperature and different Ca²⁺ concentrations on E-P formation and ITTase activity of leaky sarcoplasmic reticulum vesicles. The assay medium consisted of 40 mM Tris/maleate buffer (pH 7.0), 10 mM MgCl₂, 1 mM [γ-³²P]ITP and 0.3 mM CaCl₂ (Δ, Δ) or 20 mM CaCl₂ (□, □). The reaction was started by adding leaky vesicles to a final protein concentration of 0.66 mg/ml and stopped after 10 s using perchloric acid as described under "Materials and Methods." Essentially the same results were obtained in four different vesicle preparations tested. Full lines, E-P formation; dashed lines, ITTase activity.
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FIG. 2. E-P sensitivity to ADP under different conditions. The assay medium consisted of 20 mM Tris/maleate buffer (pH 7.0), 1 mM [γ\textsuperscript{32}P]ATP, 1 mM MgCl\textsubscript{2}, 0.1 or 10 mM CaCl\textsubscript{2}, and different concentrations of ADP. In the tubes without ADP, 3 mM phosphoenolpyruvate and 150 μg/ml of pyruvate kinase (EC 2.7.1.40) were added. The reaction was carried out at different temperatures and started by addition of leaky vesicles to a final protein concentration of 0.66 mg/ml. The reaction was stopped after 10 s by adding perchloric acid and the E-P level (left) or ATPase activity (right) were assayed as described under "Materials and Methods." The data in the left figure were plotted considering the E-P level in the absence of ADP as 100%. Each point represent the average ± S.E. of three experiments. ○, 0.1 mM CaCl\textsubscript{2} at 30°; □, 0.1 mM CaCl\textsubscript{2} at 0°; ○, 10 mM CaCl\textsubscript{2} at 30°; and ■, 10 mM CaCl\textsubscript{2} at 0°.

FIG. 3. Effect of temperature on calcium efflux and ATP synthesis. The assay medium consisted of 20 mM Tris/maleate buffer (pH 7.0), 10 mM MgCl\textsubscript{2}, 5 mM EGTA, 2 mM ADP, 8 mM \textsuperscript{32}P, for ATP synthesis and 8 mM nonradioactive P\textsubscript{i} for Ca\textsuperscript{2+} efflux, 50 mM glucose, and 6 units/ml of hexokinase. The reactions were started by addition of vesicles loaded with calcium oxalate. The values of Ca\textsuperscript{2+} efflux were correct for the passive Ca\textsuperscript{2+} efflux measured as described under "Materials and Methods." The values represent the average ± S.E. of three experiments. ○, 0.1 mM CaCl\textsubscript{2} at 30°; □, 0.1 mM CaCl\textsubscript{2} at 0°; ○, 10 mM CaCl\textsubscript{2} at 30°; and ■, 10 mM CaCl\textsubscript{2} at 0°.

FIG. 4. Effect of temperature on the ATP→P\textsubscript{i} exchange reaction. The assay medium consisted of 20 mM Tris/maleate buffer (pH 7.0), 5 mM ATP, 20 mM MgCl\textsubscript{2}, 5 mM CaCl\textsubscript{2}, 8 mM \textsuperscript{32}P. The reaction was started by addition of leaky vesicles to a final protein concentration of 0.4 mg/ml. All other procedures were as described under "Materials and Methods." The values represent the average ± S.E. of four experiments. ○, 30°; □, 0°.

FIG. 5. Temperature dependence of E-P formation from P\textsubscript{i}. The assay medium consisted of 20 mM Tris/maleate buffer (pH 6.0), 10 mM MgCl\textsubscript{2}, 1 mM EGTA, and 4 mM \textsuperscript{32}P. The reaction was carried out at different temperatures for 15 s (○) or 8 min (□). The final protein concentration was 0.66 mg/ml. All other procedures were as described under "Materials and Methods." The values represent the average ± S.E. of four experiments.

Temperature Effect on Phosphoprotein Formation from \textsuperscript{32}P\textsubscript{i}.—Although the data of Figs. 3 and 4 show that the reversal of the Ca\textsuperscript{2+} pump is inhibited at low temperature, they do not greatly limit the number of steps at which the low temperature might act. The synthesis of ATP is initiated by the phosphorylation of the enzyme by P\textsubscript{i} (22, 27, 30, 31). Using empty vesicles at pH 6.0 (17, 27), the steady state level of enzyme phosphorylated by \textsuperscript{32}P\textsubscript{i} increases linearly with temperature up to 20° (Fig. 5). Above this temperature no further increase of E-P is observed. The same results were obtained using vesicles loaded with calcium oxalate (data not shown). The enzyme affinity for \textsuperscript{32}P\textsubscript{i} does not change at different temperatures (Fig. 6). However, the maximal phosphorylation level increases as the temperature increases (Figs. 5 and 6) suggesting that at higher temperature more sites are available to be phosphorylated by \textsuperscript{32}P\textsubscript{i}.

The experiment of Table II was designed in order to ascertain whether the synthesis of ATP is inhibited by low temperature only at the phosphorylation step or also at the calcium translocation step (Reaction 3) as suggested by the study of the NTP hydrolysis (Table 1 and Fig. 2). To analyze this specific point, vesicles loaded with calcium oxalate were
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**Figure 6.** Effect of temperature on the maximal phosphorylation level. The assay medium consisted of 20 mM Tris/maleate buffer (pH 6.0), 10 mM MgCl\textsubscript{2}, 1 mM EGTA, and 1, 1.5, 2.0, or 7.0 mM \textsuperscript{32}P. The reaction was started by the addition of vesicles and stopped after 15 s by perchloric acid. 0, 4°; 0, 8°; or A, 37°. The values represent the average ± S.E. of four experiments.

**Table II**

ATP synthesis

<table>
<thead>
<tr>
<th>Temperature</th>
<th>E-P</th>
<th>ATP synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol E-P/μg protein</td>
<td>μmol ATP/μg protein/min</td>
</tr>
<tr>
<td>0°</td>
<td>0.23 ± 0.04</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>30°</td>
<td>0.19 ± 0.1</td>
<td>20.3 ± 1.6</td>
</tr>
</tbody>
</table>

Fig. 7. Phosphorylation of the enzyme by ATP and P\textsubscript{i}. The assay medium consisted of 40 mM Tris/maleate buffer (pH 6.4), 0.4 mM ATP, 5 mM P\textsubscript{i}, 20 mM MgCl\textsubscript{2}, and 20 mM CaCl\textsubscript{2}. The reaction was carried out at different temperatures and started by adding leaky vesicles to a final protein concentration of 0.66 mg/ml. The reaction was stopped after 1 min as described under "Materials and Methods." The values represent the average ± S.E. of four experiments.

The data presented in this report support the assumption that the Ca\textsuperscript{2+} transport ATPase of sarcoplasmic reticulum vesicles can assume two different conformations E and E* (6–10, 17–21). They also indicate that the interconversion of the two enzymatic forms is temperature-sensitive where low temperatures lead to the accumulation of the enzymatic forms E and 2CaE-P. The following data support this conclusion:

1. At 0°, the phosphoenzyme formed from either ATP or ITP is poorly hydrolyzed (Figs. 1 and 2 and Table I) and is very sensitive to the addition of ADP (Fig. 2). On the other hand, at 0° the phosphoenzyme formed from P\textsubscript{i} transfers its phosphate to ADP slowly (Table II). This indicates that at low temperatures, the rate of interconversion of the enzymatic forms 2CaE-P → CaE-E-P (Reaction 3) decreases.

2. At 0°, the phosphorylation of the enzyme by P\textsubscript{i} is impaired (Figs. 5 to 7) while the phosphorylation by NTP is maximal (Figs. 1 and 7 and Table I). As the temperature is raised, the fraction of enzyme phosphorylated by \textsuperscript{32}P, increases (Figs. 5 to 7) while the fraction of enzyme phosphorylated by ITP decreases (Figs. 1 and 7). These data indicate that at 0° the interconversion of the enzymatic forms E → E* (Reaction 6) is modified leading to the accumulation of the enzymatic forms E (Figs. 5 and 6) and 2CaE-P (Figs. 1, 2, and 7) to the detriment of the enzymatic forms E* and 2CaE-E-P.

The simultaneous effect of temperature on Reactions 3 and 6 can account for the inhibition of the overall reaction both in the forward direction (Ca\textsuperscript{2+} uptake and ATP hydrolysis) and in the reverse direction (Ca\textsuperscript{2+} efflux and ATP synthesis). The effect of temperature described in this paper can be interpreted as a modification of the lipid environment of the enzyme. Several reports have presented evidences that the fluidity of the sarcoplasmic reticulum vesicle membrane increases in the temperature range of 0° to 20° (1, 16, 32, 33).

Finally, the low level of enzyme phosphorylation by \textsuperscript{32}P, and the absence of a break at 20° reported by Kanazawa (34) can be attributed to the use of solubilized enzyme.

**REFERENCES**

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