The Calcium Binding Sites Involved in the Regulation of the Purified Adenosine Triphosphatase of the Sarcoplasmic Reticulum*

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SUMMARY

The role of the interaction of Ca\(^{2+}\) with the purified Ca\(^{2+}\)-dependent ATPase in the regulation of enzyme activity has been investigated. It appears that the sensitivity to Ca\(^{2+}\) of the sarcoplasmic reticulum, resulting in activation and inhibition of ATPase activity, is intrinsic to the ATPase moiety of the membrane. Three types of Ca\(^{2+}\) binding sites have been found in equilibrium dialysis studies. In the absence of ATP there is approximately one of each per 10\(^6\) daltons; the binding constants are 4 \times 10^4 (\(\alpha\) site), 4 \times 10^3 (\(\beta\) site), and 1 \times 10^3 (\(\gamma\) site). Addition of 1.5 mM ATP slightly increases the affinity of all sites and reduces the apparent capacity of the \(\alpha\) and \(\beta\) sites. Study of ATP hydrolysis in parallel with calcium binding has shown that Ca\(^{2+}\) binding at the \(\alpha\) site activates it and binding to the \(\gamma\) site inhibits it, while binding of Ca\(^{2+}\) to the \(\beta\) site appears not to be involved in the enzymatic regulation.

The sarcoplasmic reticulum plays its role in the regulation of muscle function by accumulating Ca\(^{2+}\) and reducing the concentration of Ca\(^{2+}\) in the cytoplasm below 10\(^{-7}\) M, thereby producing relaxation. The lowering of the concentration of Ca\(^{2+}\) is achieved by the transport of Ca\(^{2+}\) across the membrane through a process tightly coupled to ATP hydrolysis catalyzed by an enzyme in the membrane (1, 3). At [Ca\(^{2+}\)] of 10\(^{-8}\) - 10\(^{-6}\) M, the transport of Ca\(^{2+}\) is accompanied by increased hydrolysis of ATP and the formation of a phosphorylated protein intermediate takes place (1, 5, 7-11). The increase of [Ca\(^{2+}\)] in the vesicles of fragmented sarcoplasmic reticulum leads to a decrease in ATPase activity and a leveling off of Ca\(^{2+}\) uptake (5, 12, 13).

This paper describes results of a simultaneous study of binding of Ca\(^{2+}\), ATP hydrolysis by the isolated ATPase enzyme of the sarcoplasmic reticulum. As will be shown, one can distinguish among various Ca\(^{2+}\) binding sites of the ATPase enzyme and their roles in the regulation of the enzyme activity.

EXPERIMENTAL PROCEDURE

Fragmented sarcoplasmic reticulum was prepared from rabbit skeletal muscle as described previously (14). The most active ATPase fraction, which sedimented at 36,500 \(\times g\) (15) after solubilization of the fragmented sarcoplasmic reticulum with Triton X-100 followed by chromatography on Sepharose 4B, was used for this study. The conventional dialysis method for Ca\(^{2+}\) binding was modified in order to shorten the time required for establishing equilibrium to about 60 min, thereby maintaining the enzyme activity intact and permitting a simultaneous assay of ATPase. A sample (0.4 ml) of the purified ATPase enzyme solution (3.0 mg of protein per ml) was placed in narrow dialysis tubing (0.22-inch diameter, inflated, Fisher Scientific, Pittsburgh, Pa.) into which a round-ended glass rod, 112 mm in length and 6 mm in diameter, was subsequently inserted. This procedure led to a thin enzyme layer (0.12 mm average thickness). Prior to equilibrium dialysis, contaminating Ca\(^{2+}\) was removed by dialysis against 2 liters of a medium containing 0.1 M KCl, 0.3 mM sucrose, 50 \(\mu\)M EGTA, and 10 mM Tris-maleate, pH 7.0, for 10 hours at 0\(^\circ\). The amount of calcium in the preparation was reduced to 6 nmoles per mg of protein, as determined by atomic absorption spectrometry. Equilibrium dialysis of each sample was carried out against 20 ml of either Medium A (0.1 M KCl, 5 mM MgCl\(_2\), 5 \times 10\(^{-4}\) M EGTA, and 10 mM Tris-maleate, pH 7.0) or Medium B (0.1 M KCl, 5 mM MgCl\(_2\), and 10 mM Tris-maleate, pH 7.0) containing various concentrations of Ca\(^{2+}\) and amounts of \(^{45}\)Ca to produce about 20,000 cpm per 0.2 ml regardless of the Ca\(^{2+}\) concentration. Simultaneous assays of Ca\(^{2+}\) binding and ATPase activity were carried out by dialysis at 0\(^\circ\) versus Medium A or B containing 1.5 mM ATP which had been treated with Dowex 50 to eliminate contaminating Ca\(^{2+}\) (16). Solutions containing 0.1 M KCl and 5 mM MgCl\(_2\), an optimal ionic milieu for ATPase assay, were contaminated with 2.5 \times 10\(^{-4}\) M Ca\(^{2+}\) as determined by atomic absorption spectrometry. This was taken into account in the calculation of Ca\(^{2+}\) concentration with the use of a computer program (17). Constants used for the calculation are listed in the legend to Fig. 1. For the Ca\(^{2+}\) binding assay, 0.2-ml portions, taken from the dialysis medium at 0 time and at 3 hours and from the sample inside the dialysis tubing at 3 hours, were dried on filter paper strips and subjected to scintillation counting (21). For ATPase assay, portions were taken from the dialysis medium at 1, 2, and 3 hours and \(P_i\) was determined according to Fiske and SubbaRow (22). The time course of \(P_i\) liberation measured outside the dialysis tubing at 3 hours, were dried on filter paper strips and subjected to scintillation counting (21). For ATPase assay, portions were taken from the dialysis medium at 1, 2, and 3 hours and \(P_i\) was determined according to Fiske and SubbaRow (22). The time course of \(P_i\) liberation measured outside the dialysis tubing at 3 hours, were dried on filter paper strips and subjected to scintillation counting (21).

1 The abbreviations used are: EGTA, ethyleneglycol bis(\(\beta\)-aminoethyl ether)-N\(_2\), N\(_4\)-tetraacetic acid; SDS, sodium dodecyl sulfate.

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FIG. 1. Binding of Ca\(^{2+}\) to the isolated ATPase enzyme of the fragmented sarcoplasmic reticulum as a function of concentration of free Ca\(^{2+}\) in the absence (0) and presence (0) of ATP. Ca\(^{2+}\) binding was measured as described under "Experimental Procedure." Concentrations of free Ca\(^{2+}\) in the range of 1.1 \(\times 10^{-6}\) to \(10^{-4}\) M were produced by varying the added Ca\(^{2+}\) in Medium A and in the range of 2.2 \(\times 10^{-6}\) to \(4.7 \times 10^{-3}\) M by varying the added Ca\(^{2+}\) in Medium B. Logarithms of the association constants of metals and H\(^{+}\) to various ligands used in the calculation of [Ca\(^{2+}\)] were as follows. H\(^{+}\) to ATP\(^{-}\), 6.5; HATP\(^{3}\), 4.0; EGTA\(^{-}\), 9.46; HEGTA\(^{-}\), 8.85; H\(_2\)EGTA\(^{-}\), 2.68; and H\(_2\)EGTA\(^{-}\), 2.68. Mg\(^{2+}\) to ATP\(^{-}\), 3.6; HATP\(^{3}\), 4.5; EGTA\(^{-}\), 11.0; and HEGTA\(^{-}\), 5.33. Mn\(^{2+}\) to ATP\(^{-}\), 4.0; HATP\(^{3}\), 4.5; EGTA\(^{-}\), 5.21; and HEGTA\(^{-}\), 3.37 (U-20). The curves in the figures were calculated using a least squares method on the assumption that the binding to the enzyme had taken place in three classes of sites according to the formula

\[
\sum n_iK_i[Ca^{2+}] / 1 + K_i[Ca^{2+}] 
\]

\((i = 1, 2, 3\) refers to \(\alpha, \beta, \gamma\) respectively; \(K\), association constant; \(n\), capacity of binding).

**RESULTS**

Solubilization of the fragmented sarcoplasmic reticulum with Triton X-100 leads to a considerable increase in ATPase activity (15) while the dependence of ATPase activity on the Ca\(^{2+}\) concentration, both activation and inactivation, remains unchanged (13). Even with the purified ATPase enzyme, the activity of which is about 3 times that of the ATPase of unfractionated sarcoplasmic reticulum activated by Triton X-100 (15), the Ca\(^{2+}\) dependence is identical with that of intact fragmented sarcoplasmic reticulum. A similar Ca\(^{2+}\) dependence has been reported for the ATPase enzyme purified from fragmented sarcoplasmic reticulum solubilized with deoxycholate (23, 24). These results indicate that the mechanism by which ATPase is regulated as a function of Ca\(^{2+}\) concentration is intrinsic to the ATPase enzyme moiety of the membrane, and does not depend on the interaction of the enzyme with other membrane components.

Fig. 1 depicts the results of Ca\(^{2+}\) binding studies in the absence and presence of ATP. The binding data can be fitted by a least squares method with the use of a computer assuming that the binding of Ca\(^{2+}\) to the ATPase enzyme takes place to three noninteracting classes of sites, \(\alpha, \beta, \gamma\) in Table I. Each class has a binding capacity of approximately 1 mole per 10^5 g.

### Table 1

<table>
<thead>
<tr>
<th>Sites</th>
<th>(K)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)</td>
<td>3.8 (\times 10^4)</td>
<td>1.0</td>
</tr>
<tr>
<td>(\beta)</td>
<td>6.0 (\times 10^4)</td>
<td>1.5</td>
</tr>
<tr>
<td>(\gamma)</td>
<td>1.0 (\times 10^6)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

The results in the table were obtained by computer least squares fit of the formula shown in the legend to Fig. 1 to the binding data shown in Figs. 1 and 2.

**DISCUSSION**

The results presented here have an important bearing on the mechanism of the regulation of calcium transport in the sarcoplasmic reticulum. The association constant of the \(\alpha\) site in the presence of ATP agrees well with the reported \(K_a\) values for ATPase and phosphorylation of the fragmented sarcoplasmic reticulum (8, 10, 12, 27). Thus, it seems that the \(\alpha\) site is the
one involved in the activation of the ATPase enzyme, and presumably in Ca\textsuperscript{2+} transport across the membrane. The second type of site (β site) has little or no involvement in the regulation of ATPase. Since the Ca\textsuperscript{2+} dependence of the phosphorylated intermediate formation shows a plateau (10), as does ATP hydrolysis, in the concentration range of Ca\textsuperscript{2+} of 10\textsuperscript{-4} – 10\textsuperscript{-1} M, the β site seems not to be involved in the regulation of phosphorylation either. Binding of Ca\textsuperscript{2+} to the third, γ site appears to be involved in the inhibition of ATPase. It is known that if the Ca\textsuperscript{2+} concentration within the vesicles increases to about 1 mM as a result of ATP-dependent transport (28-30), ATPase and Ca\textsuperscript{2+} uptake are inhibited (12, 13). At that concentration, binding of Ca\textsuperscript{2+} to the γ site could take place, which in turn would produce inhibition of ATPase and, in the intact system, presumably of the Ca\textsuperscript{2+} transport. It is worth noting that in the range of Ca\textsuperscript{2+} concentrations where binding takes place to the γ site there is little dependence on Ca\textsuperscript{2+} of the formation of the phosphorylated intermediate (31). This would indicate that binding of Ca\textsuperscript{2+} to the γ site primarily affects the breakdown or transformation of the E ~ P complex.

A Ca\textsuperscript{2+} binding site which has an association constant of 5 × 10\textsuperscript{5} – 1.5 × 10\textsuperscript{6} M\textsuperscript{-1} has been reported in the fragmented sarcoplasmic reticulum (32, 33) and in the "calcium pump" protein (34, 35).

A puzzling feature of the present results is the apparent reduction in the presence of ATP of the binding capacity of the α and β sites and a higher value for the γ site, particularly since the values are nonintegral in terms of the estimated molecular weight of 100,000. A tentative interpretation suggests that in the presence of ATP a dimer of subunits, each of which has a molecular weight of 100,000, is involved, in which only one of the two α sites or of the two β sites would be able to bind Ca\textsuperscript{2+}. Changes in conformation, probably caused by subunit interaction, are also suggested by the changes in the Ca\textsuperscript{2+} binding constants brought about by ATP.

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