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CIRCULAR DICHLROISM AND FLUORESCENCE STUDIES ON INTERACTION OF CALMODULIN (CaM) WITH PURIFIED (Ca$^{2+}$-Mg$^{2+}$)ATPase OF ERYTHROCYTE GHOSTS

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It was found, using circular dichroism spectroscopy, that CaM, in the presence of Ca$^{2+}$, decreases the α-helix content of (Ca$^{2+}$-Mg$^{2+}$)ATPase of porcine erythrocytes from 66% to 55%. In the absence of Ca$^{2+}$ the enzyme showed 46% of α-helix. Moreover, quenching of the ATPase intrinsic fluorescence by acrylamide indicated that, depending on the enzyme conformational status, the accessibility of its tryptophan residues is influenced by direct interaction with CaM at micromolar Ca$^{2+}$ concentration. This was also confirmed by the observation that fluorescence energy transfer occurred from tryptophan residues of (Ca$^{2+}$-Mg$^{2+}$)ATPase to dansylated CaM. The presented results may indicate that binding of CaM gives rise to a novel conformational state of the enzyme, distinct from E1 and E2 forms of the Ca$^{2+}$ pump.

Erythrocyte plasma membrane (Ca$^{2+}$-Mg$^{2+}$)ATPase (EC 3. 6. 1. 38), activity of which is stimulated by CaM, belongs to the class of P-type ATPases, undergoing E1-E2 conformational transitions during the catalytic cycle [1]. However, the mechanism of activation of the ATPase by CaM remains unclear. Therefore, to study this problem we have employed the circular dichroism technique for detection of conformational changes of the ATPase caused by CaM. In addition, quenching of intrinsic fluorescence of tryptophan residues of purified enzyme in either E1 or E2 states, and in the presence or absence of CaM, was assessed. To study direct interaction between both proteins a method of fluorescence energy transfer was used.

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MATERIALS AND METHODS

Purification of (Ca$^{2+}$-Mg$^{2+}$)ATPase of porcine erythrocytes was performed as in [2]. CaM of bovine brain was dansylated according to [3]. Circular dichroism spectra were recorded as in [4] and the $\alpha$-helix content of purified ATPase was evaluated on the basis of ellipticity measurements following the calculations described in [5]. Quenching of tryptophan intrinsic fluorescence of the enzyme, fluorescence energy transfer between tryptophan residues of the ATPase and dansylated CaM, as well as its activity, free Ca$^{2+}$ and protein concentrations were determined as in [4].

RESULTS AND DISCUSSION

Using circular dichroism spectroscopy we detected conformational changes which occurred upon interaction of CaM with (Ca$^{2+}$-Mg$^{2+}$)ATPase. Our observations summarized in Table 1 corroborate the results reported in [6] that E$_1$ form of the enzyme is characterized by a higher content of $\alpha$-helix then E$_2$ form. Furthermore, the evaluation of spectroscopic data revealed that in the presence of Ca$^{2+}$ the $\alpha$-helix content of the ATPase decreased from 66% to 55%, upon addition of CaM, which may suggest that the enzyme attains a novel conformational state. This suggestion was confirmed experimentally by showing that the intensity of intrinsic fluorescence of (Ca$^{2+}$-Mg$^{2+}$)ATPase tryptophan residues was significantly reduced by acrylamide, with no effect of the quencher on the ATPase activity. The calculated values of the Stern-Volmer constant ($K_{SV}$) for quenching of tryptophan fluorescence indicated that, in addition to the difference in the accessibility of tryptophan residues of the enzyme for acrylamide observed between E$_1$ and E$_2$ forms of (Ca$^{2+}$-Mg$^{2+}$)ATPase, the rate of quenching was also affected by CaM. The latter phenomenon may be due to the diminution of the accessibility for quencher of a single tryptophan residue located within CaM-binding domain of the enzyme [7]. Direct interaction of purified ATPase with CaM was demonstrated by transfer of fluorescence energy from tryptophan residues of the enzyme to dansylated CaM. It was found that the transfer occurs either in the presence or absence of Ca$^{2+}$, presumably because even in the latter case, CaM is located closely to CaM-binding domain of the ATPase. Ca$^{2+}$ further promotes this interaction. In summary, parallel circular dichroism and steady state fluorescence studies have shown that interaction of the enzyme with CaM induces a novel conformational form of (Ca$^{2+}$-Mg$^{2+}$)ATPase, which differs in respect of secondary structure from E$_1$ and E$_2$ states.
Table 1

Effect of CaM on conformational status of purified (Ca$^{2+}$-Mg$^{2+}$)ATPase of erythrocyte ghost membranes. Evidence of fluorescence energy transfer between tryptophan residues of the enzyme and dansylated CaM

The circular dichroism and steady state fluorescence measurements were performed in the basic medium containing: 50 mM (for circular dichroism) or 100 mM KCl, 5 mM (for circular dichroism) or 10 mM Hepes/Tris, pH 7.4, 1 mM MgCl$_2$, 0.25 mg phosphatidylcholine/ml, 0.1 mg polydacyanol/ml, 0.4 mM dithiothreitol and 0.024 mg protein/ml (0.25 mg protein/ml for circular dichroism). The basic medium was supplemented with 0.5 mM (a) or 0.1 mM (b) EGTA, 10 μM free Ca$^{2+}$ (c), 1.8 μM (d) or 0.18 μM (e) CaM, and 0.18 μM dansylated (f) CaM. 0.1 mM phenylmethylsulfonyl fluoride was present throughout as a protease inhibitor, except for the medium for circular dichroism spectra measurements. Quenching of intrinsic fluorescence of tryptophan residues of purified enzyme was performed in the presence of 0-100 mM acrylamide. The ATPase activity was determined using coupled enzyme assay system with the effectors listed in the table. Mean values ± S.E. and number of measurements (given in parentheses) are shown.

<table>
<thead>
<tr>
<th>Additions to the basic medium</th>
<th>x-Helix content of the enzyme (%)</th>
<th>$K_{sv}$ for quenching of fluorescence (M$^{-1}$)</th>
<th>Relative fluorescence intensities of tryptophan residues at:</th>
<th>Enzyme activity assayed at 37°C (μmol P$_i$/mg per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA (E$_2$ state)</td>
<td>46.0 ± 1.8$^{a}(3)$</td>
<td>7.0 ± 0.1$^{b}(5)$</td>
<td>100$^{b}(3)$</td>
<td>0$^{b}(3)$</td>
</tr>
<tr>
<td>EGTA + CaM</td>
<td>45.3 ± 1.9$^{a}(3)$</td>
<td>7.2 ± 0.2$^{b}(3)$</td>
<td>92.5 ± 2.5$^{b}(3)$</td>
<td>3.8 ± 0.2$^{b}(3)$</td>
</tr>
<tr>
<td>Ca$^{2+}$ (E$_1$ state)</td>
<td>66.0 ± 3.0$^{c}(3)$</td>
<td>10.0 ± 0.3$^{c}(3)$</td>
<td>105.6 ± 3.0$^{c}(3)$</td>
<td>0$^{b}(3)$</td>
</tr>
<tr>
<td>Ca$^{2+}$ + CaM</td>
<td>55.0 ± 2.0$^{c}(3)$</td>
<td>8.5 ± 0.2$^{c}(3)$</td>
<td>83.9 ± 2.0$^{c}(3)$</td>
<td>7.0 ± 0.3$^{c}(3)$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.5 ± 0.5$^{c}(10)$</td>
</tr>
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REFERENCES