A Calorimetric Study of Ca$^{2+}$ Binding by Wheat Germ Calmodulin

REGULATORY STEPS DRIVEN BY ENTROPY*

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A hypothesis has been proposed for Ca$^{2+}$-binding proteins that a regulatory Ca$^{2+}$ binding step is endothermic and is driven solely by entropy in the absence of Mg$^{2+}$ (Imaiizumi, M., Tanokura, M., and Yamada, K. (1987) J. Biol. Chem. 262, 7963-7966). To confirm this idea, microcalorimetric titrations of wheat germ calmodulin, with Ca$^{2+}$ in the presence and absence of Mg$^{2+}$ and with Mg$^{2+}$ in the absence of Ca$^{2+}$, have been carried out at 25°C and at pH 7.0. The results indicate that the four binding sites in each molecule are thermodynamically equivalent to one another for both Ca$^{2+}$ and Mg$^{2+}$ binding and that the reaction is endothermic for Ca$^{2+}$ binding in the absence of Mg$^{2+}$. As compared with bovine brain calmodulin on Ca$^{2+}$ binding in the absence of Mg$^{2+}$, Ca$^{2+}$ binding in both proteins is driven solely by a large favorable entropy change despite unfavorable enthalpy change. Therefore, the above idea seems plausible.

A family of the Ca$^{2+}$-binding proteins with the EF-hand structure includes calmodulin, troponin C, parvalbumin, and so on (1). Some of them, including calmodulin and troponin C, are known to play important regulatory roles in various biological processes by interacting with enzymes or proteins. However, others including parvalbumin remain unclear in the physiological functions or express their physiological roles without interaction with other proteins. Their conformational changes on Ca$^{2+}$ binding were studied by various physicochemical and biochemical methods (1-7).

Among the physicochemical techniques, calorimetry can provide unique information on conformational changes on the basis of thermodynamic properties. It has been used to examine possible conformational changes associated with Ca$^{2+}$ binding to the Ca$^{2+}$-binding proteins: troponin C, calmodulin, calmodulin-trifluoperazine complex, and parvalbumin (8-18). Through these studies, we reached the conclusion that the regulatory Ca$^{2+}$ binding step is endothermic and is driven by entropy, where Ca$^{2+}$ binding to a protein bears the physiological role by interacting with other proteins (17). Because Ca$^{2+}$ binding to every site of bovine brain calmodulin is endothermic (12), calmodulin from other species is chosen for testing this idea. For this purpose, wheat germ calmodulin may be suitable because it shows a distinct difference in amino acid sequence from bovine brain calmodulin (19).

Calorimetric titrations of wheat germ calmodulin were carried out so that, by comparing the results with those of bovine brain calmodulin (12, 13), species differences in the thermodynamic properties among calmodulins could be elucidated. The results indicate that, like brain calmodulin, the Ca$^{2+}$ binding to the four sites were all endothermic despite the equivalence of Ca$^{2+}$ affinity, a different property than brain calmodulin.

**MATERIALS AND METHODS**

Calmodulin was extracted from wheat germ and purified by column chromatography according to the method reported in earlier studies (10-21). Isolated calmodulin was dialyzed against 1 mM NaHCO$_3$ and 0.2 mM dithiothreitol. The purity was confirmed by polyacrylamide gel electrophoresis with SDS (22). The protein thus prepared was in the calcium-free form after trichloroacetic acid treatment (12). The amount of calcium contamination in the protein solutions was less than 0.2 mol/mol of protein as determined with an atomic absorption spectrometer (Seiko SAS 727). Protein concentrations were determined by the biuret method with the coefficient of bovine serum albumin (13).

Calorimetric titrations were carried out at 25°C in an LKB batch microcalorimeter equipped with twin gold cells. Titrations with Ca$^{2+}$ or Mg$^{2+}$ were performed by the successive addition of a small amount (4.2 μl) of 25 mM CaCl$_2$ or 50 mM MgCl$_2$ solutions. The calorimeter cell contained 5 ml of 170-180 μM wheat germ calmodulin, 100 mM KCl, and 20 mM Pipes-NaOH (pH 7.0). For titrations with Ca$^{2+}$ in the presence of Mg$^{2+}$, the solution contained 10 mM MgCl$_2$ and 70 mM KCl instead of 100 mM KCl to keep the ionic strength constant. Further details of the calorimetric titrations are given in published papers (12, 23, 24).

To obtain the heat attributable to Ca$^{2+}$ or Mg$^{2+}$ binding, the observed heat must be corrected for the heat caused by the interaction between the Pipes buffer and protons released when Ca$^{2+}$ or Mg$^{2+}$ binds. The latter was determined by measuring the amount of NaOH required for maintaining the pH at 7.0 when a Ca$^{2+}$ or Mg$^{2+}$ solution was mixed with a calmodulin solution without Pipes buffer (10, 12, 13). Enthalpy titration curves were obtained by plotting the cumulative values of the heat produced (ΔH) against the molar ratio of added Ca$^{2+}$ or Mg$^{2+}$ to wheat germ calmodulin. Because Ca$^{2+}$ binding to calmodulin is reversible (25), the titration profiles can be analyzed as an equilibrium system. By assuming that the wheat germ calmodulin molecule has four independent Ca$^{2+}$- or Mg$^{2+}$-binding sites, the observed enthalpy titration curves were analyzed by the least squares method to estimate the most probable values of the intrinsic binding constant in M$^{-1}$ (k), the enthalpy change in kJ (mol site)$^{-1}$ (ΔH), and the apparent mole fraction (f) of its binding sites (10, 13, 14, 26). The observed heat in kJ (mol protein)$^{-1}$ (Q) has the opposite sign from the enthalpy change and can be expressed as follows with a molar concentration of free Ca$^{2+}$ or Mg$^{2+}$ ([M]), an arbitrary constant to compensate for any error in estimating the contamination of Ca$^{2+}$ or Mg$^{2+}$ (C), and the molar

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‡ The abbreviation used is: Pipes, 1,4-piperazinediethanesulfonic acid.
ratio of metal ion contamination to protein \( (n_0) \).

\[
Q = - \sum a_i (f_i - f_0) \Delta H a_i + C \quad \text{(Eq. 1)}
\]

where

\[
a_i = k_i [M]/(1 + k_i [M]) \quad \text{(Eq. 2)}
\]

\[
f_0 = n_0a_0/\left( \sum a_i \right) \quad \text{(Eq. 3)}
\]

\( [M] \) is obtained from the total concentrations of calcium or magnesium \( ([M]_T) \) and protein \( ([CaM]_T) \) by the equation.

\[
[M]_T = [M] + [CaM]_T \sum f_i a_i \quad \text{(Eq. 4)}
\]

RESULTS AND DISCUSSION

When a wheat germ calmodulin solution was titrated with \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \), protons were released; 0.10, 0.05, and 0.08 mol of protons/mol of binding site were released on the titrations with \( \text{Ca}^{2+} \) in the absence of \( \text{Mg}^{2+} \), with \( \text{Ca}^{2+} \) in the presence of 10 mM \( \text{Mg}^{2+} \), and with \( \text{Mg}^{2+} \) in the absence of \( \text{Ca}^{2+} \), respectively. Because the enthalpy change in the protonation of Pipes is \(-11.5\) kJ/mol at 25°C (27), the observed enthalpy changes were corrected by the amounts of protons released.

In Fig. 1, cumulative values of the heat produced (\( \Delta H \)) are plotted against the molar ratio of added \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \) to wheat germ calmodulin. No significant enthalpy changes were observed on the titration with \( \text{Mg}^{2+} \) in the presence of 0.4 mM CaCl\(_2\) (not shown). \( \text{Ca}^{2+} \) binding to wheat germ calmodulin is an endothermic reaction in the absence of \( \text{Mg}^{2+} \).

![Graph](image)

**Fig. 1.** Enthalpy titration curves of wheat germ calmodulin at 25°C and at pH 7.0. Titrations are: \( \Delta \), with \( \text{Ca}^{2+} \) in the absence of \( \text{Mg}^{2+} \); \( \bullet \), with \( \text{Ca}^{2+} \) in the presence of 10 mM \( \text{Mg}^{2+} \); and \( \blacksquare \), with \( \text{Mg}^{2+} \) in the absence of \( \text{Ca}^{2+} \). The abscissa indicates the molar ratio of \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \) to protein, and the ordinates indicate the heat produced in kJ/mol of protein. Solid lines are the theoretical curves calculated according to Equations 1–4 described under “Materials and Methods” by inserting the values listed in Table I.

**TABLE I**

<table>
<thead>
<tr>
<th>Site</th>
<th>( f )</th>
<th>( \log k )</th>
<th>( \Delta H )</th>
<th>( \Delta G^0 )</th>
<th>( \Delta S^0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ca}^{2+} ) binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Mg}^{2+} ) free</td>
<td>1–4</td>
<td>0.8</td>
<td>-2.3</td>
<td>-29.7</td>
<td>112</td>
</tr>
<tr>
<td>10 mM ( \text{Mg}^{2+} )</td>
<td>1–4</td>
<td>0.7</td>
<td>4.3</td>
<td>-11.0</td>
<td>-24.5</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} ) binding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Ca}^{2+} ) free</td>
<td>1–4</td>
<td>0.8</td>
<td>2.9</td>
<td>16.5</td>
<td>111</td>
</tr>
</tbody>
</table>

The titration curves were monophasic and were fitted well to the theoretical curves calculated by assuming that the four binding sites are equivalent to each other (Fig. 1). The most probable values of the binding parameters accounting for the enthalpy titration curves of Fig. 1 are summarized in Table I. The apparent mole fraction \( (f) \) is 0.7–0.8 for every binding site, which may indicate that the coefficient used in the biuret method is slightly smaller than the true value for wheat germ calmodulin. The binding constants are consistent with the values determined for bovine brain calmodulin (7, 28).

The enthalpy change associated with \( \text{Mg}^{2+} \) binding is 16.5 kJ/mol of site and that with \( \text{Ca}^{2+} \) binding in the presence of 10 mM \( \text{Mg}^{2+} \) is \(-11.0\) kJ/mol of site. The sum (5.5 kJ/mol of site) is equal to the enthalpy change associated with \( \text{Ca}^{2+} \) binding to calmodulin (3.7 kJ/mol of site) within experimental error. This indicates that \( \text{Ca}^{2+} \) displaces \( \text{Mg}^{2+} \) at each of the four binding sites of wheat germ calmodulin (\( \text{Ca}^{2+}-\text{Mg}^{2+} \) sites).

Associated with \( \text{Ca}^{2+} \) binding to wheat germ calmodulin in the absence of \( \text{Mg}^{2+} \), the reaction is endothermic with the positive enthalpy change of 3.7 kJ/mol of site. This indicates that \( \text{Ca}^{2+} \) binding to individual sites is driven solely by a favorable positive entropy change (112 J/mol of site/K). This characteristic is the same as bovine brain calmodulin (12, 13) and is markedly different than parvalbumins from carp, bullfrog, and toad skeletal muscles (9, 14, 16, 23, 24). For troponin C, endothermic reactions are \( \text{Ca}^{2+} \) binding to the second site of rabbit skeletal muscle troponin C (10) and the third site of bullfrog skeletal muscle troponin C (17, 18), and exothermic reactions are \( \text{Ca}^{2+} \) binding to the other sites including that to the first two sites of bovine cardiac troponin C (11). The third site of cardiac troponin C was silent in calorimetric titrations (11), which may be caused by the small enthalpy change associated with \( \text{Ca}^{2+} \) binding.

The present results favor the previous conclusion that unfavorable positive enthalpy and favorable positive entropy changes are characteristic of the regulatory reactions of \( \text{Ca}^{2+} \)-binding proteins, that is the regulatory reactions are driven by large positive entropy changes (18). This could mean that, in the regulatory reactions, \( \text{Ca}^{2+} \)-binding proteins increase their mobility and become more flexible to be allowed to interact with the target proteins or enzymes in a specific way, and that the bound water molecules are released from calmodulin upon \( \text{Ca}^{2+} \) binding (13, 15, 17).

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REFERENCES

Calorimetry of Wheat Germ Calmodulin