Equilibrium Dialysis Measurements of the Ca\(^{2+}\)-Binding Properties of Recombinant Radish Vacuolar Ca\(^{2+}\)-Binding Protein Expressed in Escherichia coli

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Vacuoles of radish (Raphanus sativus) contained a Ca\(^{2+}\)-binding protein (RVCaB) of 43 kDa. We investigated the Ca\(^{2+}\)-binding properties of the protein. RVCaB was expressed in Escherichia coli and was purified from an extract by ion-exchange chromatography, nitrocellulose membrane filtration, and gel-filtration column chromatography. Ca\(^{2+}\)-binding properties of the recombinant protein were examined by equilibrium dialysis with \(^{45}\)Ca\(^{2+}\) and small dialysis buttons. The protein was estimated to bind 19Ca\(^{2+}\) ions per molecule with a \(K_d\) for Ca\(^{2+}\) of 3.4 mM. Ca\(^{2+}\) was bound to the protein even in the presence of high concentrations of Mg\(^{2+}\) or K\(^{+}\). The results suggested that the protein bound Ca\(^{2+}\) with high ion selectivity, high capacity, and low affinity.

Key words: Ca\(^{2+}\)-binding protein; recombinant protein; plant vacuole; Raphanus sativus

Materials and Methods

Expression of RVCaB in E. coli and protein purification. To produce a recombinant protein in E. coli, cDNA of the radish RVCaB was ligated into the EcoRI/NotI site of a pET23(b) expression vector (Novagen). In this experiment, DNA for RVCaB without a His-tag or the like was used to rule out any effect of the tag on the Ca\(^{2+}\)-binding properties of the recombinant protein. The recombinant plasmid was introduced into E. coli BL21(DE3) (Novagen), and production of the recombinant protein was brought about by the addition of isopropyl thio-\(\beta\)-D-galacto-
Radish Vacuolar Ca$^{2+}$-Binding Protein

Expression and purification of recombinant RVCaB

A large amount of RVCaB was produced in *E. coli* in the presence of IPTG, which could be detected in the gel stained with “Stains-all” (Fig. 1(A)). The recombinant RVCaB, which was stained blue with “Stains-all”, was recovered in the soluble fraction of the cell lysate but not in the membrane or the inclusion body (Fig. 1(A)). The purified RVCaB gave a single protein band of 43 kDa on SDS-PAGE and was stained blue with “Stains-all” (Fig. 1(B)). We concluded that the purified preparation was suitable for examination of the Ca$^{2+}$-binding properties of RVCaB. The recombinant protein could be purified from the *E. coli* cell extract at a yield of a few milligrams per liter of culture medium.

**Ca$^{2+}$ binding to purified RVCaB.** The Ca$^{2+}$-binding properties of RVCaB were measured by equilibrium dialysis. Proteins (20 µl; 40 µg of protein) of the purified recombinant RVCaB were put into wells of small dialysis buttons (Hampton Research) and then dialyzed for 16 h at 25 °C against 40 ml of a solution of 25 mM MES-KOH, pH 6.0, containing 150 mM KCl and 0.15 MBq of $^{45}$Ca$^{2+}$ (37 GBq/mmol, Amersham Biosciences) as CaCl$_2$ at various concentrations. In some experiments, MgCl$_2$ or KCl was added to the Ca$^{2+}$-binding buffer to various concentrations. After dialysis, the RVCaB solution in the well of each dialysis button was collected with a needle and syringe. Portions (10 µl) of the collected solutions were spotted on a nitrocellulose membrane (13 mm in diameter) and then the membranes were dried in the air. The total radioactivity associated with the filter membrane was measured with a liquid scintillation counter. Unbound Ca$^{2+}$ was measured from the radioactivity of the external solution.

**$^{45}$Ca$^{2+}$ overlay assay, PAGE, and staining.** The $^{45}$Ca$^{2+}$-overlay assay, SDS-PAGE, and staining of the gel with “Stains-all” were done as described previously. The $^{45}$Ca$^{2+}$-overlay assay was done by the method of Maruyama et al. with PVDF membranes. SDS-PAGE in 12% gel was done by the standard method. “Stains-all” was purchased from Sigma. After electrophoresis, the gel was fixed with 25% (v/v) isopropyl alcohol containing 30 mM Tris, and then was stained in the dark for 24 h with a mixture of 0.0025% (w/v) “Stains-all”, 25% (v/v) isopropyl alcohol, 7.5% (v/v) formamide, and 30 mM Tris, pH 8.8. “Stains-all” has used for the identification of CaBPs, such as calreticulin ad calsequestrin.

Results

**Expression and purification of recombinant RVCaB.**

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**$^{45}$Ca$^{2+}$ overlay assay**

The ability of the recombinant RVCaB to bind Ca$^{2+}$ was tested by $^{45}$Ca$^{2+}$ overlay assay on the PVDF membrane. Some RVCaB was retained on the PVDF membrane, but not on the nitrocellulose membrane, as found previously. The purified RVCaB prepared from *E. coli* and that from radish taproots bound $^{45}$Ca$^{2+}$ (Fig. 2), so the recombinant RVCaB could bind Ca$^{2+}$. The signal was more intense with more blotted RVCaB. Bovine immunoglobulin G was put on the same membrane as a negative control. It gave no signal.

**Ca$^{2+}$-binding properties of RVCaB**

Several assay methods can be used to measure the Ca$^{2+}$-binding kinetics of CaBPs: for example, there are equilibrium dialysis, flow dialysis, membrane microassay, and spectrophotometry, used in studies of CaBPs. We could not use these methods, because only a small amount of the purified preparation could be obtained from radish taproots, and little RVCaB was retained on nitrocellulose membranes. Therefore, we used small dialysis buttons (Fig. 3(A)), of the kind usually used for protein crystallization. This method enabled us to do small-scale studies because the well of the dialysis button holds only 20 µl. In this system, $^{45}$Ca$^{2+}$ ions in the dialysis solution enter the well of the dialysis button through a dialysis membrane and bind to RVCaB in the well. After

side (IPTG) at a final concentration of 1 mM to the culture medium at 37°C.

*E. coli* cells expressing RVCaB were treated by sonic oscillation at 50 kHz at 4°C and centrifuged at 5,000 × g for 10 min. The clear supernatant was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, for 16 h. The dialyzed solution was put on a column (gel volume, 15 ml) of QAE-Toyopearl (Tosoh, Tokyo) equilibrated with the same buffer. The column was washed first with 50 ml of 10 Tris-HCl, pH 7.5 and 200 mM KCl and then with 10 ml of 10 mM Tris-HCl, pH 7.5, containing 300 mM KCl. RVCaB was eluted from the column with 10 mM Tris-HCl, pH 7.5, containing 350 mM KCl. The amount of RVCaB in the fraction was estimated from the profile of SDS-PAGE and “Stains-all” staining. The fractions corresponding to the peak were collected and filtered through a nitrocellulose membrane with a pore size of 0.45 µm. In this step, RVCaB passed through the membrane and some other proteins were trapped on the membrane. The pass-through fraction was put on a gel-filtration column (1.0 × 30 cm) of Superdex 200 HR (Amersham Biosciences) equilibrated with 25 mM MES-KOH, pH 6.0, containing 150 mM KCl. The buffer of the purified preparation was replaced with 25 mM MES-KOH, pH 6.0, by using an Ultrafree-MC (Amicon).

In some experiments, RVCaB was purified from radish taproots as described previously.

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$^{45}$Ca$^{2+}$ overlay assay

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thiazolin-2-ylidene)-2-methyl-propenyl] naphtho[1,2-dj thiazolium bromide] was purchased from Sigma. After electrophoresis, the gel was fixed with 25% (v/v) isopropyl alcohol containing 30 mM Tris, and then was stained in the dark for 24 h with a mixture of 0.0025% (w/v) “Stains-all”, 25% (v/v) isopropyl alcohol, 7.5% (v/v) formamide, and 30 mM Tris, pH 8.8. “Stains-all” has used for the identification of CaBPs, such as calreticulin ad calsequestrin.6,14)
Fig. 1. Expression and Purification of Recombinant RVCaB. (A) Recombinant RVCaB expressed in E. coli. Bacterial cells were transformed with cDNA of RVCaB. Lane 1, no IPTG; lane 2, IPTG. The lysate of cells treated with IPTG was sonicated, centrifuged, stained with the supernatant was put through SDS-PAGE and the gel was stained with “Stains-all” (lane 3). The precipitate after centrifugation of the cell lysate was separated into two fractions, the fraction soluble in 6 M urea (lane 4) and the insoluble fraction (lane 5). (B) Purification of recombinant RVCaB. Recombinant RVCaB was purified from an E. coli lysate by QAE-Toyopearl, nitrocellulose membrane filtration, and Superdex 200HR column chromatography. The fractions of RVCaB corresponding to the peak after ion-exchange chromatography (lane 1) and gel filtration (lanes 2 and 3) were treated by SDS-PAGE. Proteins in the gel were stained with Coomassie Blue (lanes 1 and 2) or “Stains-all” (lane 3).

The total amount of bound Ca$^{2+}$ increased in proportion to the amount of RVCaB (Fig. 4(A)). Under the assay conditions, little Ca$^{2+}$ bound to bovine serum albumin, the negative control (Fig. 4(A)). Figure 4(B) shows the effects of KCl and MgCl$_2$ on the proportion of Ca$^{2+}$ ions that bound to RVCaB. The reaction buffer for this assay contained 1 mM CaCl$_2$. Neither 10 mM K$^+$ nor 1 mM Mg$^{2+}$ affected Ca$^{2+}$ binding, although both ions inhibited binding at 10 mM for MgCl$_2$ and at 100 mM for KCl. The results indicate that RVCaB preferentially binds Ca$^{2+}$.

Discussion

E. coli cells expressing RVCaB grew normally with different Ca$^{2+}$ concentrations (up to 100 mM) in the medium (not shown). It is estimated that RVCaB did not change the Ca$^{2+}$ concentration in E. coli because the affinity of RVCaB for Ca$^{2+}$ was only 3.4 mM. The recombinant RVCaB and native RVCaB had the same properties on SDS-PAGE, in “Stains-all” staining, and in the $^{45}$Ca$^{2+}$-overlay assay. This study showed that RVCaB had not only a low affinity for Ca$^{2+}$ and a high Ca$^{2+}$-binding capacity of 19 ions per molecule. This capacity is consistent with the earlier suggestion that the number of Ca$^{2+}$ ions per molecule was 15, as estimated from the net amount of Ca$^{2+}$ incorporated into radish vacuolar membrane vesicles including RVCaB. The $K_d$ of RVCaB for Ca$^{2+}$ and the number of binding sites, $n$, were different from those of calmodulin and annexin II and similar to that of the recombinant C-domain (Ca$^{2+}$-binding domain) of calreticulin (Table 1). RVCaB is comparable to calreticulin in the ER, which has a low affinity and a high capacity, although the primary structure of RVCaB is different from that of calreticulin.

Both Mg$^{2+}$ and KCl affected the Ca$^{2+}$-binding activity of RVCaB at high concentrations (Fig. 4). Mg$^{2+}$ at 10 mM reduced the Ca$^{2+}$-binding capacity to
Fig. 3. Equilibrium Dialysis of Ca$^{2+}$-Binding of Recombinant RVCaB.
(A) Diagram of the equilibrium dialysis assay of recombinant RVCaB with a dialysis button. Twenty microliters of RVCaB solution was put in the well of a dialysis button 3 mm in diameter, sealed with a dialysis membrane, and then dialyzed at 25°C against 40 ml of 25 mM MES-KOH, pH 6.0, 150 mM KCl, and the indicated concentrations of 45CaCl$_2$. (B) Ratios of numbers of Ca$^{2+}$ bound to RVCaB. (C) Scatchard plot. B and F, bound and the free Ca$^{2+}$, respectively. The number of Ca$^{2+}$ ions bound per molecule of RVCaB can be obtained from the equation, $y = 5.58 - 0.292x$.

Fig. 4. Specificity of Ca$^{2+}$-Binding Activity by Recombinant RVCaB.
(A) Effects of the RVCaB concentration on Ca$^{2+}$-binding properties. Binding of 45Ca$^{2+}$ to recombinant RVCaB was examined at various concentrations of recombinant RVCaB (○) and bovine serum albumin (BSA)(●) at 1 mM CaCl$_2$. (B) Effects of K$^+$ and Mg$^{2+}$ on the Ca$^{2+}$ binding of RVCaB. The Ca$^{2+}$-binding capacity of RVCaB was measured by equilibrium dialysis in the presence in 25 mM MES-KOH, pH 6.0, 25°C, 1 mM CaCl$_2$, and the indicated concentrations of KCl (open boxes) or MgCl$_2$ (shaded boxes).

Table 1. Dissociation Constant ($K_d$) and Ca$^{2+}$-Binding Number (n) of Several CaBPs

<table>
<thead>
<tr>
<th>CaBP (source)</th>
<th>Detection</th>
<th>Assay medium</th>
<th>$K_d$ (mM)</th>
<th>n</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calmodulin (bovine)</td>
<td>Metallochromic indicator</td>
<td>0.1 M KCl in 20 mM MOPS-KOH, pH 6.8</td>
<td>$5.1 \times 10^{-3}$</td>
<td>3.4</td>
<td>19</td>
</tr>
<tr>
<td>Annexin II (bovine)</td>
<td>Aggregating system</td>
<td>0.1 M NaCl in 50 mM Tris, pH 7.5</td>
<td>$10^{-2}$</td>
<td>10–12</td>
<td>20</td>
</tr>
<tr>
<td>Calreticulin (recombinant)</td>
<td>Equilibrium dialysis</td>
<td>0.15 M KCl in 10 mM MOPS, pH 7.0</td>
<td>1.0</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>RVCaB (recombinant)</td>
<td>Equilibrium dialysis</td>
<td>0.15 M KCl in 25 mM MES-KOH, pH 6.0</td>
<td>3.4</td>
<td>19</td>
<td>this study</td>
</tr>
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Ca\textsuperscript{2+} conditions.\textsuperscript{12} In that study, the Mg\textsuperscript{2+} concentration affected neither the transcriptional nor translational level of RVCaB. From these earlier observations and those in this study, we concluded that RVCaB acts mainly as a Ca\textsuperscript{2+}-buffering protein \textit{in vivo}.

There are several possible Ca\textsuperscript{2+}-binding sites in RVCaB. RVCaB consists of 248 amino acid residues and has a calculated mass of 27.1 kDa.\textsuperscript{6} The apparent molecular size on SDS-PAGE is 43 kDa. The low migration rate in SDS-PAGE may be due to the acidity of RVCaB (pI, 3.94). Glutamate residues (82 residues) were 33\% of the amino acid residues in RVCaB. An unusual sequence 22-amino acid residues long, including eight Glu residues, was involved in the repeat sequence. Residues conserved in all four domains are indicated with asterisks. The full sequence of RVCaB is available in sequence data banks under accession number AB035900.

Judging from its low affinity for Ca\textsuperscript{2+}, RVCaB may not be saturated with Ca\textsuperscript{2+} under normal physiological conditions. However, RVCaB can absorb and store Ca\textsuperscript{2+} in vacuoles, because the protein bound several Ca\textsuperscript{2+} ions per molecule even at or near a Ca\textsuperscript{2+} concentration of 1 mM (Fig. 3(B)). This low affinity for Ca\textsuperscript{2+} is essential if vacuoles are to supply Ca\textsuperscript{2+} to the cytosol. RVCaB probably releases bound Ca\textsuperscript{2+} ions through Ca\textsuperscript{2+} channels when the free Ca\textsuperscript{2+} concentration decreases in the vacuoles. If CaBPs in vacuoles had a high capacity and high affinity, vacuoles would not be able to release Ca\textsuperscript{2+} into the cytosol.

RVCaB is weakly associated with the vacuolar membranes, as described previously,\textsuperscript{6} and the membranes contain a number of essential transporters and channels.\textsuperscript{31} Thus, RVCaB may have an additional function as a regulator of these transporters and channels in response to the Ca\textsuperscript{2+} concentrations in the vacuolar lumen.

Acknowledgments

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References

16) Colowick, S. P., and Womack, F. C., Binding of

Fig. 5. Unusual Repeat Sequence in RVCaB.

The numbers show the positions of the residues on both sides of the repeat sequence. Residues conserved in all four domains are indicated with asterisks. The full sequence of RVCaB is available in sequence data banks under accession number AB035900.


