Calbindin D$_{28k}$ Exhibits Properties Characteristic of a Ca$^{2+}$ Sensor*

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Calbindin D$_{28k}$ is a member of the calmodulin superfamily of Ca$^{2+}$-binding proteins and contains six EF-hands. The protein is generally believed to function as a Ca$^{2+}$ buffer, but the studies presented in this work indicate that it may also act as a Ca$^{2+}$ sensor. The results show that Mg$^{2+}$ binds to the same sites as Ca$^{2+}$ with an association constant of $\sim 1.4$ x $10^{9}$ M$^{-1}$ in 0.15 M KCl. The four high affinity sites in calbindin D$_{28k}$ bind Ca$^{2+}$ in a non-sequential, parallel manner. In the presence of physiological concentrations of Mg$^{2+}$, the Ca$^{2+}$ affinity is reduced by a factor of 2, and the cooperativity, which otherwise is modest, increases. Based on the binding constants determined in the presence of physiological salt concentrations, we estimate that at the Ca$^{2+}$ concentration in a resting cell calbindin D$_{28k}$ is saturated to 40-75% with Mg$^{2+}$ but to less than 9% with Ca$^{2+}$. In contrast, the protein is expected to be nearly fully saturated with Ca$^{2+}$ at the Ca$^{2+}$ level of an activated cell. A substantial conformational change is observed upon Ca$^{2+}$ binding, but only minor structural changes take place upon Mg$^{2+}$ binding. This suggests that calbindin D$_{28k}$ undergoes Ca$^{2+}$-induced structural changes upon Ca$^{2+}$ activation of a cell. Thus, calbindin D$_{28k}$ displays several properties that would be expected for a protein involved in Ca$^{2+}$-induced signal transmission and hence may function not only as a Ca$^{2+}$ buffer but also as a Ca$^{2+}$ sensor. Digestion patterns resulting from limited proteolysis of the protein suggest that the loop of EF-hand 2, a variant site that does not bind Ca$^{2+}$, becomes exposed upon Ca$^{2+}$ binding.

Calbindin D$_{28k}$ is a Ca$^{2+}$-binding protein expressed in brain as well as in kidney, bone, pancreas, and other tissues (1). In some tissues it is exceptionally abundant. For example, calbindin D$_{28k}$ constitutes between 0.1 and 1.5% of the total soluble protein in brain, and protein levels in auditory neurons are estimated to reach concentrations of up to 2 mM (2). Calbindin D$_{28k}$ contains 261 amino acid residues forming six EF-hands, which are organized in a single globular domain (Fig. 1) (3, 4). The protein is a member of the calmodulin superfamily (5).

Some members of this superfamily are Ca$^{2+}$ sensors that undergo Ca$^{2+}$-induced conformational changes resulting in the exposure of a hydrophobic surface. The hydrophobic patch typically serves as a binding surface for target molecules, which become activated or attenuated upon complex formation (6). The targets include many membrane transport proteins and enzymes (7). In this way, intracellular Ca$^{2+}$ influx triggers the regulation of cellular processes, such as muscle contraction and the phosphoinositide cascade.

Another class of Ca$^{2+}$-binding proteins are the Ca$^{2+}$ buffers or signal modulators. Parvalbumin and calbindin D$_{28k}$ are examples of Ca$^{2+}$-buffer proteins. Unlike Ca$^{2+}$ sensors, the Ca$^{2+}$-buffer proteins do not expose hydrophobic surfaces upon Ca$^{2+}$ binding. In fact, the exposure of hydrophobic surfaces would be unfavorable for these proteins because it may limit their stability and Ca$^{2+}$ affinity. Ca$^{2+}$-buffer proteins are thought to be involved in deactivation of signal transducers and/or quenching of Ca$^{2+}$ signals, thus protecting the cell against toxic effects of Ca$^{2+}$, such as the formation of insoluble calcium phosphates (for a review see Ref. 8). The Ca$^{2+}$ affinity is generally higher for buffer proteins than for sensor proteins, and the rates of Ca$^{2+}$ binding and release are lower in the buffer group. Calbindin D$_{28k}$ is thought to prevent sustained elevations of Ca$^{2+}$ by acting as an intracellular Ca$^{2+}$ buffer. There is evidence that calbindin D$_{28k}$ has a Ca$^{2+}$-buffering function in neurons. For example, increased intracellular levels of calbindin D$_{28k}$ causes blunted intracellular Ca$^{2+}$ elevations (9), and Ca$^{2+}$ transients are increased in calbindin D$_{28k}$ null mutant mice (10). A number of examples where Ca$^{2+}$ buffering by calbindin D$_{28k}$ has an effect on the electrophysiological behavior of cells have also been reported (11–13). The notion that calbindin D$_{28k}$ acts as a Ca$^{2+}$-buffering system in the cytoplasm has led to the hypothesis that calbindin D$_{28k}$ may protect neurons against large fluctuations in free intracellular Ca$^{2+}$ and, hence, prevent cell death (14–17). However, experiments using calbindin D$_{28k}$-null mutant mice subjected to cerebral ischemia did not support a cytoprotective effect of the protein (18). Although most reports deal with the Ca$^{2+}$-buffering function of calbindin D$_{28k}$, several lines of evidence suggest that the protein also acts as a Ca$^{2+}$ sensor. Spectroscopic investigations (19) and in vitro studies using antibodies (20) have shown that calbindin D$_{28k}$ undergoes a conformational shift upon Ca$^{2+}$ binding. Additionally, a number of putative Ca$^{2+}$-dependent interactions with target proteins or brain membrane fractions have been reported (20–23), and erythrocyte membrane Ca$^{2+}$-ATPase and 3’,5’-cyclic nucleotide phosphodiesterase have been shown to be stimulated in a dose-dependent, saturable manner with calbindin D$_{28k}$ (24). Moreover, the finding that a fraction of calbindin D$_{28k}$ (9–55%) (20, 25–27) is specifically associated with particulate structures in the cell indicates that the protein
In our previous study (28), we showed that although a conformational change occurs upon Ca$^{2+}$ binding, both the Ca$^{2+}$-free and Ca$^{2+}$-loaded forms of calbindin D$_{28k}$ have exposed hydrophobic surfaces. Thus, the protein behaves neither like a classical Ca$^{2+}$ sensor nor like a Ca$^{2+}$ buffer. The aim of the present study was to further characterize the Ca$^{2+}$-induced conformational change and to determine whether calbindin D$_{28k}$ is likely to respond structurally to changes in the intracellular concentration of Ca$^{2+}$ in the presence of physiological levels of Mg$^{2+}$ and salt.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Human recombinant calbindin D$_{28k}$ was expressed in *Escherichia coli* and purified to homogeneity as described (29). Bovine brain calbindin D$_{28k}$ was purified from brain homogenate as described (28). F123 is a fragment containing EF-hands 1–3 (residues 1–132) of human calbindin D$_{28k}$ whereas F456 contains EF-hands 4–6 (residues 133–261). F123 and F456 were produced by cloning techniques and expressed in *E. coli* (4).

**Chemicals**—CaCl$_2$ and MgCl$_2$ of Pro Analysi quality were from Sigma and Merck, respectively. BAPTA$^1$ was from Molecular Probes, Eugene, OR. All chemicals were of the highest grade commercially available. Buffers were made Ca$^{2+}$-free by incubation with a dialysis tube filled with Chelex 100 (Bio-Rad) for 2 weeks.

**Preparation of Apoprotein**—All apo solutions were made from Ca$^{2+}$-depleted protein, which was produced as follows. Purified protein was dissolved in 1 ml of doubly distilled water, containing an excess of EGTA (10–20 eq) at pH 8. It was then applied to a 3.4 × 20-cm Sephadex G-25 superfine gel filtration column. To abolish EGTA binding, the protein was applied to the column after 15 ml of saturated NaCl had been allowed to penetrate the top of the column. The NaCl solution had been depleted from residual Ca$^{2+}$ by dialysis against Chelex 100 resin. During the gel filtration, the protein was passed through the NaCl zone and eluted, now free from EGTA, with doubly distilled Ca$^{2+}$-free water. The final product contained between 0.2 and 0.6 molar eq of Ca$^{2+}$, as determined from Ca$^{2+}$-titrations in the presence of the chelator quin 2 or by high resolution inductively coupled plasma mass spectrometry (analysis performed by SGAB, Luleå, Sweden).$^1$ H NMR spectroscopy was used to verify that the apo samples were free from EGTA.

**Near UV CD Spectroscopy**—Near UV CD spectra (250–300 nm) were obtained using a Jasco J-720 spectropolarimeter at 25°C (thermostatted) and quartz cuvettes with a path length of 10 mm. A spectrum was first recorded for the apoprotein (25 μM apocalbindin D$_{28k}$ in 0.15 mM KCl, 2 mM Tris, 0.125 mM EGTA, pH 7.3). A second spectrum was recorded after adding 10 mM MgCl$_2$. Finally, 0.5 mM CaCl$_2$ was added, and a spectrum for the Ca$^{2+}$-loaded form was recorded.

**ANS Fluorescence**—Fluorescence spectra were obtained using a PerkinElmer Life Sciences Luminescence Spectrometer LS 50 B connected to a Julabo F52 water bath. All data were collected at 25.0 ± 0.1°C in a quartz cuvette (10 mm path-length). For the 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence experiments, ANS was added to a final concentration of 120 to 10 μM protein in 10 mM DTT, 0.15 M KCl, 2 mM Tris, pH 7.3, with 0, 1, or 5 mM MgCl$_2$. Fluorescence spectra were recorded between 400 and 600 nm (bandwidth 5 nm) with excitation at 385 nm (bandwidth 3 nm) and a scan rate of 50 nm per min. Two spectra were obtained for each sample and averaged.

**Determination of Macroscopic Ca$^{2+}$ Binding Constants**—The affinity and cooperativity of Ca$^{2+}$ binding to F123, F456, or intact calbindin D$_{28k}$ were determined by titration with CaCl$_2$ in the presence of a chromophoric chelator, quin 2, as described previously (30, 31). All experiments were performed in 2 mM Tris/acetate buffer at pH 7.5, either with no added salt, with 0.15 mM KCl, or with 0.15 mM KCl in the presence of 2 mM MgCl$_2$. The macroscopic Ca$^{2+}$ binding constants were obtained by least-squares fitting of the absorbance at 263 nm versus total Ca$^{2+}$ concentration by minimizing the error square sum (e.s.s) as shown in Equation 1,

$$\text{e.s.s} = \sum_{i=0}^{n} (A_{\text{obs}} - A_{\text{calc}})^2 \quad \text{(Eq. 1)}$$

where the sum runs over the n + 1 data points in the titration. The calculated absorbance at each titration point, $i$, was obtained as shown in Equation 2,

$$A_{\text{calc},i} = \left( A_{\text{min}} - A_{\text{max}} \right) \frac{Y_i}{Y_i + K_j} + A_{\text{max}} \quad \text{(Eq. 2)}$$

where $A_{\text{min}}$ and $A_{\text{max}}$ are the respective absorbances for the Ca$^{2+}$-free and Ca$^{2+}$-loaded forms of the protein/chelator solution that had been lyophilized in a hydrolysis mixture with no added salt, and $K_j$ is the dissociation constant of the chelator-Ca$^{2+}$ complex. The free Ca$^{2+}$ concentration, $Y_i$, was solved using the Newton-Raphson method from Equation 3,

$$Y_i = C_{\text{Ca}} \frac{C_Q \cdot Y_i}{Y_i + K_j} \frac{F \cdot C_P \cdot \sum_{k=1}^{N} (k \cdot Y_i - \prod_{j=1}^{k} K_j)}{1 + \sum_{k=1}^{N} (k \cdot Y_i - \prod_{j=1}^{k} K_j)} \quad \text{(Eq. 3)}$$

where $K_j$ through $K_N$ are the $N$ macroscopic binding constants and $C_Q$, $C_P$, and $C_{\text{Ca}}$ are the total chelator, protein, and Ca$^{2+}$ concentrations, respectively, at point $i$ corrected for the dilutions due to the CaCl$_2$ additions. The initial chelator concentration, $C_{Q_0}$, was determined by withdrawing an aliquot of the solution and record the absorbance at 239.5 nm in the presence of excess Ca$^{2+}$ (using $e = 4.2 \times 10^4$ liter mol$^{-1}$ cm$^{-1}$). The initial Ca$^{2+}$ concentration, $C_{\text{Ca}_0}$, was determined by high resolution inductively coupled plasma mass spectrometry (at SGAB, Luleå, Sweden). The initial protein concentration, $C_P$, was determined by amino acid analysis after acid hydrolysis on a withdrawn aliquot of the protein/chelator solution that had been lyophilized in a hydrolysis tube (Biomedical Centre, Uppsala, Sweden). The adjustable parameters in the fit were $A_{\text{min}}, A_{\text{max}},$ and the $N$ macroscopic binding constants. The parameter $F$ was either fixed at 1.0 or could be used as an adjustable parameter to analyze if the assumed stoichiometry (number of macro-

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$^1$The abbreviations used are: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N'','N''-tetraacetic acid; HSSQC, heteronuclear single quantum coherence; ANS, 8-anilinonaphthalene-1-sulfonic acid; DTT, 4-dithiothreitol; DSS, 2,2-dimethyl-2-silapentane sulfonate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
Calbindin D$_{28k}$, a Ca$^{2+}$ Sensor?

Scopnic binding constants used) is correct (F will end up close to 1.0) or not (F will deviate from 1.0). For presentation, the data and fitted curves were normalized according to Equation 4,

$$A_{\text{normalized}} = (A_A - A_{	ext{min}})/(A_{\text{max}} - A_{	ext{min}})$$

(Eq. 4)

The apparent Ca$^{2+}$-binding constants in the presence of Mg$^{2+}$ were derived using the same method as above but with 5,5'-Br$_2$-BAPTA instead of quin 2. The chelator 5,5'-H$_9$262/BAPTA has a high level of selectivity against Mg$^{2+}$ ($\log K_{\text{Mg}} = -5.65; \log K_{\text{Mg}} < 1$ at 0.15 m KCl). Hence, differences in titration curves obtained in the interval 0–10 mM Mg$^{2+}$ are due to Mg$^{2+}$ effects on the protein. The resulting binding constants are apparent Ca$^{2+}$ binding constants for the protein in the presence of Mg$^{2+}$. These constants result from the Ca$^{2+}$ affinity, the Mg$^{2+}$ affinity, and competition or coupling between the two events.

**Determination of Ca$^{2+}$ and Mg$^{2+}$ Binding Constants by Fluorescence Spectroscopy—Mg$^{2+}$ or Ca$^{2+}$ binding constants were determined by monitoring the intrinsic (tryptophan) fluorescence during Ca$^{2+}$ or Mg$^{2+}$ titration in 0.15 m KCl, 2 mM Tris, pH 7.3. Fluorescence spectra were obtained using a PerkinElmer Life Sciences Luminescence Spectrometer LS 50 B connected to a Julabo F25 water bath at 25.0°C. Quartz cuvettes with a path length of 10 mm were used. The excitation wavelength was 280 nm, and the emission scanned was between 300 and 450 nm. The protein concentration was 25 μM. Ca$^{2+}$-free protein was titrated with increasing amounts of metal ion (Ca$^{2+}$ or Mg$^{2+}$) beyond saturation. The intensity at each point was corrected for dilution. Ca$^{2+}$ and Mg$^{2+}$ titrations were also performed in the presence of 120 μM ANS (excitation at 385 nm, emission scanned between 400 and 600 nm) with 10 μM calbindin D$_{28k}$ in 10 mM DTT, 0.15 mM KCl, 2 mM Tris, pH 7.1. In some experiments, MgCl$_2$ was added to a final concentration of 1 or 5 mM. The excitation wavelength was 385 nm (bandwidth 5 nm), and the emission was scanned between 400 and 600 nm. The experimental data were fitted according to Equation 5,

$$I = I_0 + (I_b - I_0) / (1 + Y/K_a)$$

(Eq. 5)

where Y is the free Ca$^{2+}$ or Mg$^{2+}$ concentration; $K_a$ is the apparent binding constant, and $I_b$ and $I_0$ are the intensities for the free and bound state, respectively.

**Limited Proteolysis of Calbindin D$_{28k}$—Calbindin D$_{28k}$ was dissolved in 50 mM Tris, 150 mM KCl, containing either 1 mM CaCl$_2$, 1 mM EDTA, or 2.5 mM MgCl$_2$ + 0.5 mM EGTA (EGTA has a very high selectivity for Ca$^{2+}$ over Mg$^{2+}$) at a protein concentration of 0.5 mg/ml. The pH was adjusted to 7.5. Sequencing grade modified trypsin (Promega) was dissolved in the supplied buffer, yielding a stock solution with a concentration of 0.5 mg/ml. Proteolysis was initiated by mixing 100 μl of calbindin D$_{28k}$ (0.5 mg/ml) with 1 μl of the trypsin stock at room temperature. Aliquots of 5 μl were withdrawn at various time points, and the digestion was blocked by the addition of 1 μl of soybean trypsin inhibitor (1 mg/ml) (Roche Molecular Biochemicals). The digested fragments were then separated by SDS-PAGE (15%). Following electrophoresis, the protein bands were either stained by Coomassie Blue and excised for mass spectrometry analysis (see below) or blotted onto a poly(vinylidene difluoride) membrane (Immobilon, Millipore) and subjected to N-terminal amino acid sequencing (automated Edman degradation using an Applied Biosystems 477 A sequencer with on-line detection of phenylthiohydantoin-derivatives with an Applied Biosystems 120A high pressure liquid chromatography).

**Sample Preparation for Mass Spectrometry—Coomassie-stained protein fragments were excised from the gel and washed with water, followed by 40% acetonitrile in 25 mM NH$_4$HCO$_3$, pH 7.8, until the gel piece was transparent. The gel piece was dried in a SpeedVac vacuum centrifuge. Reduction using 10 mM DTT at 48°C for 30 min was followed by alkylation in 55 mM iodoacetamide for 30 min in darkness at room temperature. The gel piece was washed and dried again before digestion with sequencing-grade trypsin (Promega) in 25 mM NH$_4$HCO$_3$, overnight at 37°C. The digestion was terminated, and the peptides were eluted by adding 10 μl of 2% trifluoroacetic acid. Peptides were purified from buffer using C-18 reversed phase tips (Ziptips, Millipore).

**Mass Spectrometry—Mass spectrometric studies were performed using a Bruker Scout 384 Reflex III matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometer. The instrument was operated in the positive ion mode with delayed extraction and an acceleration voltage of 25 kV. Peptide samples were analyzed using the reflector detector with 2,5-dihydroxybenzoic acid as matrix, whereas larger fragments and intact protein were measured in the linear mode using ferulic acid as matrix. Improved signal-to-noise ratios were obtained by the accumulation of 50–150 single shot spectra. Autolysis fragments of trypsin were used for internal calibration, whereas an external calibration standard was used for the analysis of intact protein fragments.

**NMR Spectroscopy—NMR spectra were acquired on a 600-MHz Varian Inova spectrometer at 310 K. 15N HSQC experiments utilizing pulsed field gradient and preservation of equivalent path (32, 33) employing water flip-back (34) were performed on calbindin D$_{28k}$ at different Mg$^{2+}$ or Ca$^{2+}$ concentrations. The GARP-1 decoupling sequence (35) was used for 15N decoupling during acquisition. All spectra were acquired with spectral widths of 2000 and 8000 Hz in the F1 and F2 dimensions, respectively, and sampled using 128 and 1024 complex points in the r1 and r2 dimensions, respectively, with 16 transients acquired for each free induction decay. The data were processed and analyzed using the Felix97 Software (Micron Separations, San Diego). The data were multiplied by exponential (F2) and cosine-bell (F1) functions prior to Fourier transformation, and zero-filled to generate matrices of 2048 × 2048 real points. Proton chemical shifts were referenced relative to the water signal, which resonates at 4.64 ppm from the sodium 2,2-dimethyl-2-silapentane sulfonate (DSS) at 310 K. Nitrogen chemical shifts were referenced indirectly relative to DSS, using the nitrogen to proton frequency ratio (36).

The NMR samples contained 0.73 mM apocalbindin D$_{28k}$ (with less than 0.05 eq of Ca$^{2+}$), 0.1 mM DSS, 10 mM deuterated DTT and were prepared by dissolving the lyophilized protein and deuterated DTT in 620 μl of 93% H$_2$O, 7% H$_2$O. The pH was adjusted to 6.8 with 0.1 mM KOH or HCl. Aliquots from a 73 mM CaCl$_2$ stock solution was added to one sample directly in the NMR tube using a Hamilton syringe (5 μl for each point of the titration). The protein concentration was determined by amino acid analysis following acid hydrolysis. The concentration of the CaCl$_2$ stock solution was determined by inductively coupled plasma mass spectrometry. HSQC spectra were also acquired for a 0.8 mM protein sample with 2 or 10 mM MgCl$_2$ as well as with 10 mM MgCl$_2$ and 5 mM CaCl$_2$.

**RESULTS**

**Ca$^{2+}$ Binding Determined from Competition with Chromophoric Chelators—Titration with Ca$^{2+}$ in the presence of a chromophoric chelator was used to determine the macroscopic Ca$^{2+}$ binding constants (30, 37). To minimize experimental errors, care was taken to determine accurately the Ca$^{2+}$ concentrations before and after titration (by high resolution inductively coupled plasma mass spectrometry), as well as the protein concentration before titration (by amino acid analysis after acid hydrolysis). Reliable acid hydrolysis results were obtained only when an aliquot of the sample was freeze-dried in the hydrolysate tube (glass) before shipment to the analysis station. Samples sent as liquid in Eppendorf tubes yielded unreasonably low and inconsistent protein concentrations, probably due to protein adhesion to the plastic.

The stoichiometry of Ca$^{2+}$ binding was analyzed by fitting each titration in a simplified way using Equations 1–3 with $n = 2$. CP$_0$ was set to the value obtained by acid hydrolysis, and the parameter $F$ was used as a variable parameter. After fitting, the stoichiometry was calculated as $2F$. By using this method, the number of high affinity Ca$^{2+}$-binding sites in the intact protein are 4.03 ± 0.11 (average and S.D. of 9 titrations). Thus the data convincingly show that human calbindin D$_{28k}$ binds 4 Ca$^{2+}$ ions with high affinity.

**Table I**

| [KCl] | K$_1$ | K$_2$ | K$_3$ | K$_4$ |
|-------|-------|-------|-------|-------|
| 3.9×10$^6$ | 1.2×10$^6$ | 8.1×10$^7$ | 1.7×10$^7$ |
| 2.0×10$^6$ | 2.7×10$^6$ | 1.5×10$^7$ | 1.0×10$^7$ |
| 1.4×10$^6$ | 2.3×10$^6$ | 1.7×10$^7$ | 9.8×10$^7$ |
| 3.5×10$^6$ | 3.2×10$^6$ | 6.0×10$^7$ | 5.6×10$^7$ |

($F$ (1F23) is a recombinant fragment containing EF-hands 1–3. ($F$ (F456) is a recombinant fragment containing EF-hands 4–6.)}
To obtain the values of the four macroscopic binding constants, each titration was then fitted using Equations 1–3 with \( n = 4 \). CPD was set to the value obtained by acid hydrolysis, and \( F \) was fixed at 1.0. The intact protein displayed macroscopic binding constants of \( 1.5 \times 10^2 \) to \( 3.5 \times 10^4 \) \( M^{-1} \) at low ionic strength and \( 8.9 \times 10^5 \) to \( 7.9 \times 10^6 \) \( M^{-1} \) in 0.15 M KCl (Table I).

The \( \text{Ca}^{2+} \) titration curves display a close to linear decrease of the absorbance as a function of total \( \text{Ca}^{2+} \) concentration. The lack of sigmoidal shape may indicate a low degree of cooperativity of \( \text{Ca}^{2+} \) binding. More quantitative information on the cooperativity is derived from the relationships between the binding constants, each titration was then fitted using Equations 1–3 with \( n = 4 \).

Addition of \( \text{Mg}^{2+} \) to the protein sample causes significant changes in the HSQC spectrum. The HSQC spectrum of the apo state is probably broadened due to exchange between at least two conformational states with a fast-to-intermediate rate on the NMR chemical shift time scale. During the titration, the cross-peaks initially remain broad.
Calbindin D<sub>28k</sub> a Ca<sup>2+</sup> Sensor?

process involving one apo state, four Ca<sub>1</sub> states, six Ca<sub>2</sub> states, four Ca<sub>3</sub> states, and one Ca<sub>4</sub> state. Using the macroscopic calcium binding constants, determined by the chelator method, we have calculated for each point of the HSQC Ca<sup>2+</sup> titration the fraction of the protein in (i) the apo, (ii) any of the Ca<sub>1</sub> states, (iii) any Ca<sub>2</sub> state, (iv) any Ca<sub>3</sub> state, and (v) the Ca<sub>4</sub> state (Fig. 3D). Comparisons of the calculated curves with the NMR data reveal that a majority of the NMR titration curves monitor the appearance of the Ca<sub>4</sub> state. This shows that most protons have a unique chemical shift in the Ca<sub>4</sub> form. A minority of titration curves follow the disappearance of the apo state, whereas none of the monitored peaks seems to correspond to curves expected for any of the Ca<sub>1</sub>, Ca<sub>2</sub> or Ca<sub>3</sub> forms. This suggests that the intermediate states are interconverting rapidly, leading to severe broadening of the resonances. The small number of resonances appearing before addition of the 3rd eq of Ca<sup>2+</sup> may represent protons having the same chemical shift in one or more intermediate states as in the Ca<sub>4</sub> species. The NMR data hence show that all four sites have similar affinities and are filled in a parallel fashion and that the Ca<sub>4</sub> species has structural features distinct from those of the intermediate states.

**Mg<sup>2+</sup> Binding as Monitored by Trp Fluorescence**—Titration with MgCl<sub>2</sub> to saturation results in a 10% increase of the fluorescence intensity at 333 nm and gives a small but reproducible blue shift of the intensity maximum from 333.5 to 332.5 nm (not shown). Addition of excess Ca<sup>2+</sup> to the Mg<sup>2+</sup>-saturated sample results in an additional 15% increase in the fluorescence intensity (not shown). This shows that, although Mg<sup>2+</sup> binds to the protein, it does not invoke the same structural response as Ca<sup>2+</sup> binding. The Mg<sup>2+</sup> titration curve in the absence of Ca<sup>2+</sup> yields an apparent Mg<sup>2+</sup> binding constant of 1.4 × 10<sup>3</sup> M<sup>−1</sup> in 0.15 M KCl (average of four independent experiments). The stoichiometry of Mg<sup>2+</sup> binding cannot be deduced from the experiment, because the Mg<sup>2+</sup> concentration at half-saturation is almost 2 orders of magnitude higher than the protein concentration. However, the obtained binding constant implies that under physiological intracellular concentrations of Mg<sup>2+</sup> (0.5–2 mM) and resting levels of Ca<sup>2+</sup>, the Mg<sup>2+</sup>-binding sites in calbindin D<sub>28k</sub> are 40–75% occupied by Mg<sup>2+</sup>.

**Mg<sup>2+</sup> Binding as Monitored by NMR Spectroscopy**—Mg<sup>2+</sup> binding to calbindin D<sub>28k</sub> was also monitored by heteronuclear two-dimensional <sup>15</sup>N-<sup>1</sup>H HSQC NMR experiments. Fig. 4 shows spectra recorded using 0.8 mM Ca<sup>2+</sup>-free protein samples containing 2 and 10 mM Mg<sup>2+</sup>, corresponding to 2.5 and 12.5 eq of Mg<sup>2+</sup>, respectively. Addition of Mg<sup>2+</sup> to the apoprotein causes an increase in the chemical shift dispersion with a number of shifted peaks, although the effects are not as pronounced as during addition of Ca<sup>2+</sup>. Even at 10 mM Mg<sup>2+</sup>, most of the peaks are still broadened and clustered between 8.5 and 7 ppm in the proton dimension and between 121 and 115 ppm in the nitrogen dimension. Addition of 3.2 mM Ca<sup>2+</sup> (4 eq) to the sample with 10 mM Mg<sup>2+</sup> yields an HSQC spectrum that is identical to the one in Fig. 2D (data not shown). This shows that Ca<sup>2+</sup> efficiently displaces Mg<sup>2+</sup>, suggesting that Mg<sup>2+</sup> binds to the same sites as Ca<sup>2+</sup>.

**Mg<sup>2+</sup> Binding to Individual EF-hands as Monitored by CD Spectroscopy**—The far-UV CD spectra were recorded for six synthetic peptides, comprising EF-hands 1–6, at a concentration of 20 μM. The six peptides appear mostly unfolded in the apo form as well as in the presence of 10 or 200 mM MgCl<sub>2</sub> (data not shown). This is in striking contrast to the spectra obtained in the presence of Ca<sup>2+</sup>, which show a high degree of helicity and are consistent with Ca<sup>2+</sup> binding for EF1, EF3, EF4, EF5, and EF6 (39). The results suggest that although Mg<sup>2+</sup> binds to the intact protein, the interaction between Mg<sup>2+</sup> and the indi-

![Fig. 3. Signal intensities of cross-peaks in the two-dimensional <sup>1</sup>H-<sup>15</sup>N HSQC spectra of calbindin D<sub>28k</sub> as a function of the Ca<sup>2+</sup>/calbindin D<sub>28k</sub> ratio. A–C, normalized experimental data grouped according to the shape of the curve. D, fraction of the protein in the apo, Ca<sub>1</sub>, Ca<sub>2</sub>, Ca<sub>3</sub>, and Ca<sub>4</sub> forms during the titration calculated using the four macroscopic binding constants.](image-url)
vidual EF-hands is not strong enough to induce secondary structure in these peptides.

Ca$^{2+}$ Binding in the Presence of Mg$^{2+}$ and a Chromophoric Chelator—Ca$^{2+}$ binding in the presence of Mg$^{2+}$ was determined using a chromophoric Ca$^{2+}$ chelator (5,5'-Br$_2$-BAPTA), which has a high level of discrimination against Mg$^{2+}$. Fig. 5 shows the experimental data for Ca$^{2+}$ titrations of intact calbindin D$_{28k}$ in the presence of 0 or 2 mM Mg$^{2+}$, together with the curves of best fit. Already from the appearance of the curves it is evident that the presence of Mg$^{2+}$ reduces the Ca$^{2+}$ affinity for calbindin D$_{28k}$. The fitting of the experimental data shows that the Ca$^{2+}$ affinity of calbindin D$_{28k}$ is reduced approximately 2-fold to yield $K = 3.5 \times 10^6 - 5.6 \times 10^5$ M$^{-1}$ in the presence of 2 mM MgCl$_2$ (Table I). Only very small ionic strength effects are expected for the addition of 2 mM MgCl$_2$ into 0.15 M KCl. Therefore, the data again suggest that Mg$^{2+}$ competes with Ca$^{2+}$ for the same binding sites.

Changes in the Tertiary Structure as Monitored by Near UV-CD—Ca$^{2+}$ binding induces changes in the near-UV CD spectra of calbindin D$_{28k}$, which indicates a rearrangement of the tertiary structure (not shown, see also Ref. 28), in agreement with the NMR data. In contrast, addition of 10 mM MgCl$_2$ to apocalbindin D$_{28k}$ does not alter the near-UV CD spectrum to any significant degree (not shown).

Limited Proteolysis—Limited tryptic digestion of human recombinant calbindin D$_{28k}$ was performed in the presence of EDTA, Ca$^{2+}$, or Mg$^{2+}$. The reaction was quenched at different time points ranging from 1 min to 14 h and was analyzed by SDS-gel electrophoresis (Fig. 6). Both the rate and pattern of tryptic digestion are strongly Ca$^{2+}$-dependent. The Ca$^{2+}$-loaded form of calbindin D$_{28k}$ is clearly more resistant to proteolysis than the Ca$^{2+}$-free form, although many of the potential tryptic cleavage sites are protected in both forms (Fig. 1). Significant digestion of the apo form is seen already in the 1st min, whereas fragments of the Ca$^{2+}$-loaded form appear at a significantly lower rate. The identities of the tryptic fragments were determined by mass spectrometry and N-terminal amino acid sequencing. The deduced cleavage sites are indicated by the arrows in Fig. 7, and the identity of the major fragments are summarized in Table II. Both apo- and Ca$^{2+}$-bound calbindin are cleaved at Lys-59 and Lys-235 in the C-terminal helices of EF-hands 2 and 6. The apoprotein is cleaved also at Arg-169 in the C-terminal helix of EF-hand 4. In contrast, the Ca$^{2+}$-bound protein is not cleaved at this site but mainly at

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**Fig. 4.** Two-dimensional $^1$H-$^1$N HSQC spectra of uniformly $^{15}$N-labeled calbindin D$_{28k}$ recorded at 310 K with 0.8 mM Ca$^{2+}$-free protein samples containing 2 and 10 mM Mg$^{2+}$ (corresponding to 2.5 and 12.5 eq of Mg$^{2+}$, respectively).

**Fig. 5.** Titration of 5,5'-Br$_2$-BAPTA with Ca$^{2+}$ in the presence of calbindin D$_{28k}$ with or without Mg$^{2+}$ in 0.15 M KCl, 2 mM Tris, pH 7.3. The curves of optimal fit to the data points are presented. ○, titration performed without MgCl$_2$. ●, titration performed in the presence of 2 mM MgCl$_2$.
Lys-72 in the loop of EF-hand 2 and at Lys-98 in the linker between EF-hands 2 and 3. In addition, a minor cleavage site in the Ca\textsuperscript{2+} form is found at Lys-245 in the loop of EF-hand 6.

At longer incubation times, a number of small fragments appear. These are mainly due to further cleavage of larger fragments and may therefore represent cleavage sites that are inaccessible in the intact protein. It is noteworthy that in EDTA a small fragment containing residues 181–261 (EF-hands 5 and 6) is fairly resistant to further cleavage as its intensity increases all the way up to the last time point (120 min, band A8 in Fig. 6).

Limited proteolysis of calbindin D\textsubscript{28k} in the presence of 2 mM Mg\textsuperscript{2+} results in nearly the same digestion pattern as for the apoprotein (Fig. 6). Thus, all identified fragments were equivalent in the EDTA and Mg\textsuperscript{2+} samples, except for a very small amount of a peptide (residues 60–261),\textsuperscript{2} which was not found in the EDTA samples. Mg\textsuperscript{2+} seems to offer some protection to proteolytic digestion, although digestion is much faster than in the presence of Ca\textsuperscript{2+}.

Ca\textsuperscript{2+} Binding to F123 and F456 as Monitored by the Chromophoric Chelator Quin 2—Ca\textsuperscript{2+} binding to F123 and F456,
each comprising one-half of calbindin D_{28k}, was measured with the quin 2 method. The stoichiometry of Ca^{2+} binding to each half was analyzed as described for the intact protein, yielding \( n = 2.01 \pm 0.22 \) for F123 and \( 2.14 \pm 0.24 \) for F456. Hence, there are two high affinity sites in each half of calbindin D_{28k}. One site in each half was found to retain the Ca^{2+} affinity of the intact protein (\( K_1 \sim 1 \times 10^{-6} \text{ M}^{-1} \)). The second site in each half binds Ca^{2+} with \(-50\)-fold lower affinity (\( K_2 \sim 2 \times 10^{-4} \text{ M}^{-1} \)).

**Ca^{2+}-induced Conformational Changes as Monitored by ANS Fluorescence**—The fluorescent probe ANS can be used as a sensitive reporter of solvent-exposed hydrophobic patches in proteins. In an attempt to investigate whether F123 and F456 undergo Ca^{2+}-induced conformational changes at the tertiary level, we measured the Ca^{2+} dependence of ANS binding for F123 and F456. In EDTA, both F123 (472 nm) and F456 (485 nm) yield a significant blue shift and intensity increase relative to ANS in buffer (530 nm), indicating binding of ANS. When Ca^{2+} is added, a further blue shift in the fluorescence maximum from 485 to 475 nm is observed for F456 with a 30% increase in intensity (Fig. 8B). In contrast, addition of Ca^{2+} to F123 yields a 43% decrease in intensity, with a shift from 472 to 483 nm. The results clearly indicate that both halves undergo structural changes after binding of Ca^{2+}. The response is different in the two halves with the local environment around the bound ANS becoming less polar in F456 but more polar in F123.

ANS fluorescence spectra were also recorded for an equimolar mixture of F123 and F456 and for the intact protein. These spectra were compared with one another and to the sum of the ANS spectra recorded individually for F123 and F456. No spectral differences were observed between these three cases, nei-
Calbindin D$_{28k}$ a Ca$^{2+}$ Sensor?

TABLE II
Analysis of tryptic peptides of calbindin D$_{28k}$ by Edman degradation and mass spectrometry

Human calbindin D$_{28k}$ was digested with trypsin; fragments were separated by SDS-PAGE (15%), and gels were stained with Coomassie Blue. Samples were collected after various time points. In some experiments, the bands were blotted onto an Immobilon membrane.

| Protein fragment | Amino acid sequence | Method(s) used to identify fragments |
|------------------|---------------------|-------------------------------------|
| Edman degradation$^{a}$ MALDI-TOF$^{b}$ |
| Ca1 2–261 (intact protein) | A7HEEHL | Yes |
| Ca2 2–235 | A7HEEHL | Yes |
| Ca3 60–261 | A7HEEHL | Yes |
| Ca4 73–261 | A7HEEHL | Yes |
| Ca5 99–245 | A7HEEHL | Yes |
| Ca6 99–236 or 235 | A7HEEHL | Yes |
| Ca7 73–? | A7IGIVE | Yes |
| A1 2–261 (intact protein) | A7HEEHL | Yes |
| A2 2–235 | A7HEEHL | Yes |
| A3 60–? | A7TFVDQ | Yes |
| A4 2–169 | A7HEEHL | Yes |
| A5 2–? | A7HEEHL | Yes |
| A6 60–169 | A7TFVDQ | Yes |
| A7 170–261 | A7LPPVQ | Yes |
| A8 181–261 | A8FQQIK | Yes |
| A9 1–59 | A8FQQIK | Yes |
| A10 170–216 | A8FQQIK | Yes |
| Mg1 2–261 (intact protein) | A7HEEHL | Yes |
| Mg2 2–235 | A7HEEHL | Yes |
| Mg3 60–? | A7TFVDQ | Yes |
| Mg4 60–261 | A7TFVDQ | Yes |
| Mg5 2–169 | A7HEEHL | Yes |
| Mg6 60–169 | A7TFVDQ | Yes |
| Mg7 170–261 | A7LPPVQ | Yes |
| Mg8 181–261 | A8FQQIK | Yes |
| Mg9 1–59 | A8FQQIK | Yes |
| Mg10 170–216 | A8FQQIK | Yes |

$^{a}$ The N-terminal sequence of the bands were determined by Edman degradation.

$^{b}$ The same bands were also in-gel digested with trypsin, and the amino acid sequences of the bands were determined based on the cleavage pattern identified by MALDI-TOF mass spectrometry.

Furthermore, in the absence nor the presence of Ca$^{2+}$. The data suggest that ANS binds to the same exposed hydrophobic patches in the intact protein as in the two halves. Earlier work (4) has shown that each half-fragment has a tendency to form homodimers, hence the contact surface between the two halves in the intact protein would be hidden in the separate fragments and not accessible to ANS binding.

DISCUSSION

The results of the present work clearly demonstrate that the tertiary structure of calbindin D$_{28k}$ is Ca$^{2+}$-dependent. The large Ca$^{2+}$-induced effects observed by two-dimensional (H-$^{15}$N) HSQC NMR spectroscopy (Fig. 2) indicate that the protein undergoes a conformational change. Whereas the spectrum of the apo form shows limited chemical shift dispersion and broad lines, the Ca$^{2+}$-saturated protein displays shift dispersion and narrow lines typical of a well folded helical protein. Far-UV CD data (28) indicate a significant degree of helicity both for the apo and Ca$^{2+}$-loaded states, suggesting that the limited chemical shift dispersion of the apo form represents a loosely organized tertiary structure, similar to a molten globule. As seen in Figs. 2 and 3, there is not a progressive change in the spectrum throughout the Ca$^{2+}$ titration. Rather, fairly small spectral changes are observed until 4 equiv of Ca$^{2+}$ are added, at which point there is a dramatic change with a large number of sharp resonances appearing. For example, four signals appear in a region where the glycine residue in position 6 of the Ca$^{2+}$-bound EF-hand loop is typically found (δ$^{1}$H = 10–10.5 ppm, δ$^{15}$N = 110–114 ppm). These signals most likely represent the corresponding four glycine residues of the regular EF-hands 1 and 3–5, because synthetic peptides corresponding to these four sites also display $^{1}$H chemical shifts of these glycine residues between 10 and 10.5 ppm in the Ca$^{2+}$-bound form (39). Whereas data on the rat protein have been interpreted as one site having much higher affinity than two or three other sites (40), we do not observe sites differing in affinity. Rather, we have strong evidence from the quin 2 and NMR titrations that all four sites bind Ca$^{2+}$ in a parallel fashion with similar affinity and positive cooperativity.

Limited tryptic proteolysis of the apo- and Ca$^{2+}$-proteins (Figs. 6 and 7 and Table II) provides further details on the Ca$^{2+}$-induced structural changes. In accordance with the HSQC results, the protein appears more loosely folded in the apo state, because it is considerably more sensitive to proteolysis than in the Ca$^{2+}$-loaded state. The cleavage patterns are also distinctly different. Whereas the apoprotein is mainly cleaved at Arg-169, the Ca$^{2+}$-form is cleaved at Lys-72 and Lys-98. Arg-169 is located in the middle of the second helix of EF-hand 4, whereas Lys-72 and Lys-98 are found in the loop of EF-hand 2 and in the N-terminal helix of EF hand 3, respectively. This suggests that the conformational changes upon Ca$^{2+}$ binding involve burial or rotation of the second helix in EF-hand 4 and exposure of the loop of EF-hand 2 and of the N-terminal helix of EF hand 3. EF-hand 2 is a variant site that does not appear to bind Ca$^{2+}$ (39), and its Ca$^{2+}$-induced exposure to solvent is intriguing. One could speculate that EF-hand 2 is part of a target-binding surface that becomes exposed upon Ca$^{2+}$ binding.

It is interesting to compare the limited proteolysis of calbindin D$_{28k}$ to the digestion patterns of calretinin. Calretinin is a hexa-EF-hand protein that is homologous to calbindin D$_{28k}$ (58% sequence identity), but in contrast to calbindin, it binds Ca$^{2+}$ to EF-hand 2. In calretinin, regions in EF-hands 2 and 3 are not cleaved in the presence of Ca$^{2+}$, which indicates that this part of the protein is not exposed to solvent in a Ca$^{2+}$-dependent way (41, 42). Instead, cleavage occurs in EF-hand 6 of calretinin, which corresponds to a minor Ca$^{2+}$-induced cleavage point in calbindin D$_{28k}$, EF-hand 6 represents a non-canonical sequence in both proteins. The dissimilarities in digestion pattern may reflect functional and evolutionary differences between the two proteins to adapt to different targets.

The heteronuclear two-dimensional $^{15}$N-$^{1}$H HSQC NMR experiments show that Mg$^{2+}$ efficiently displaces Mg$^{2+}$, suggesting that Mg$^{2+}$ binds to the same sites as Ca$^{2+}$. The results from limited proteolysis, near-UV CD, and NMR spectroscopy show that the structural changes upon Mg$^{2+}$ binding are much smaller than upon Ca$^{2+}$ binding. This indicates that although Mg$^{2+}$ binds to calbindin D$_{28k}$, it coordinates in such a way that it does not drive any substantial conformational change. This is consistent with structural data on other EF-hand proteins, which show that the coordination of Mg$^{2+}$ and Ca$^{2+}$ to the same site generally differs (43). The bidendate Ca$^{2+}$-ligating glutamate at position 12 in the EF-hand loop is important for sensor proteins. In the Mg$^{2+}$-loaded form, the ion is often coordinated by none or only by one of the side chain oxygens of this glutamate side chain.

Each of the two isolated halves, comprising EF-hands 1–3 and 4–6, respectively, was found to contain two high affinity Ca$^{2+}$-binding sites. In each of the two halves, one site retains the Ca$^{2+}$ affinity as seen for intact calbindin D$_{28k}$, whereas the other site has lost affinity by a factor of 50. Hence, truncation of the protein leads to loss of important interactions between EF-hands at the interface between the two halves. Thus, the Ca$^{2+}$-binding sites of calbindin D$_{28k}$ are clearly not grouped into two largely independent domains, as in calmodulin. The
two halves of calmodulin retain native-like Ca\(^{2+}\) binding behavior even when separated (44). The present Ca\(^{2+}\) binding data, obtained for F123 and F456, support earlier findings that all six EF-hands in calbindin D\(_{28k}\) are part of one globular domain (3, 4).

Calbindin D\(_{28k}\) appears to have evolved through triplication of an ancestral gene coding for two EF-hands (5). EF-hands often associate in pairs as they occur in the sequence, but in calbindin D\(_{28k}\) there are also extensive interactions beyond the pairwise contacts (3, 4). As isolated synthetic peptides, EF-hands 1 and 3–5 in calbindin D\(_{28k}\) are the main Ca\(^{2+}\)-binding sites, whereas EF-hand 2 fails to bind Ca\(^{2+}\) and EF-hand 6 does so only weakly (39). Hence, it seems reasonable to assume that EF-hands 1 and 3 are active in the Ca\(^{2+}\) titrations of the N-terminal half and EF-hands 4 and 5 of the C-terminal half. An attractive model for the intact protein is that EF hands 3 and 4 are paired with one another. A cut in this pair would then have major influences on the Ca\(^{2+}\) affinity for both EF-hands 3 and 4, which could explain the reduced Ca\(^{2+}\) affinity of one site in each half. In this model, the conformational properties and high Ca\(^{2+}\) affinities of EF-hands 1 and 5 depend mostly on interactions within the respective half.

The physiological role of calbindin D\(_{28k}\) as a neuroprotector in conditions related to Ca\(^{2+}\) overload, and as a facilitator of neuronal Ca\(^{2+}\) signaling and renal Ca\(^{2+}\) resorption, has been suggested to be linked to the Ca\(^{2+}\)-buffering capacity of the protein. However, Ca\(^{2+}\)-induced conformational changes, reported in an earlier study (28) and further analyzed in the present work, suggest that some of the physiological findings could be due to Ca\(^{2+}\) sensor activity of calbindin D\(_{28k}\). All cells have transport systems for the extrusion of Ca\(^{2+}\). The intracellular Ca\(^{2+}\) concentration in a resting cell is therefore relatively low, <0.1 \(\mu\)M. The cytosolic Ca\(^{2+}\) concentration can be abruptly raised to 1–10 \(\mu\)M when the cell is activated by influx of Ca\(^{2+}\).

In the activated cell, Ca\(^{2+}\) is bound in a highly selective way to Ca\(^{2+}\)-signaling proteins (Ca\(^{2+}\) sensors) and induce conformational changes, which lead to an altered activity of target proteins. As discussed above, calbindin D\(_{28k}\) clearly undergoes a Ca\(^{2+}\)-induced conformational change, however, to be classified as a Ca\(^{2+}\) sensor, several other conditions also needed to be fulfilled. The intracellular concentration of Mg\(^{2+}\), a potential competitor to Ca\(^{2+}\), is kept at a relatively constant level around 0.5–2 mM. Consequently, a Ca\(^{2+}\) sensor protein must have 

\[K_{d} \approx 10^{3} \text{ M}^{-1}\]  

The condition is met by calbindin D\(_{28k}\) as the protein binds to Mg\(^{2+}\) with an affinity constant around \(1.4 \times 10^{3} \text{ M}^{-1}\) in 0.15 M KCl, where the average Ca\(^{2+}\) binding constant is \(2.5 \times 10^{6} \text{ M}^{-1}\). A Ca\(^{2+}\) sensor must further be able to respond structurally within a biologically relevant range of intracellular Ca\(^{2+}\) concentrations. This is also true for calbindin as its average Ca\(^{2+}\) dissociation constant \(K_{d,\text{av}} = 7 \times 10^{-7} \text{ M}\) falls within the physiological range of intracellular free Ca\(^{2+}\) concentrations. In contrast, a Ca\(^{2+}\)-buffer protein would be expected to have higher affinity \((K_{d} < 10^{-7}\text{ M})\) whereas Ca\(^{2+}\)-binding proteins with a lower affinity \((K_{d} > 10^{-3}\text{ M})\) cannot act as sensors because they are unable to detect the changes in intracellular free Ca\(^{2+}\) concentrations that normally occur in cells.

Fig. 9 shows the Ca\(^{2+}\) saturation curves for calbindin D\(_{28k}\), calmodulin, and parvalbumin calculated from the Ca\(^{2+}\) binding constants in 1–2 mM Mg\(^{2+}\) and 0.15 M KCl, pH 7.3. The Ca\(^{2+}\) concentration intervals of a resting and activated cell are shaded.
be a Ca\(^{2+}\) sensor because it is almost fully saturated with Ca\(^{2+}\) at resting concentrations of Ca\(^{2+}\). By contrast, calbindin D\(_{28k}\) and calmodulin are only \(\leq 9\)% and \(\leq 3\)% saturated with Ca\(^{2+}\), respectively, at resting concentrations of Ca\(^{2+}\) (\(\leq 0.1 \mu M\)). At Ca\(^{2+}\) concentrations similar to those that follow Ca\(^{2+}\) activation of a cell, the saturation levels of calbindin D\(_{28k}\) and calmodulin are changed dramatically, as would be expected for Ca\(^{2+}\) sensors.

If calbindin D\(_{28k}\) is a Ca\(^{2+}\) sensor, by which mechanism could it work? As mentioned above, the Ca\(^{2+}\)-binding sites of calbindin D\(_{28k}\) are clearly not grouped into two largely independent domains, as seen in calmodulin. Therefore, it does not follow the calmodulin paradigm of having two independent domains, which expose hydrophobic surfaces only upon Ca\(^{2+}\) binding, and that together clamp around the recognition sequences of target enzymes (45). Instead, the tryptic digestion patterns suggest that the Ca\(^{2+}\)-induced conformational changes involve the rotation of helices and the reorganization of the interface between EF-hands and the linker loops. The mechanism may resemble the Ca\(^{2+}\)-induced changes observed for recoverin (46), a protein with 4 EF-hands organized in a single globular domain. Alternatively, calbindin D\(_{28k}\) may function by a unique mechanism, possibly shared with other hexa-EF-hand proteins.

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