# $Ca^{2+}$-Binding Stoichiometry of Calbindin $D_{28k}$ As Assessed by Spectroscopic Analyses of Synthetic Peptide Fragments†

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*Received November 27, 1995; Revised Manuscript Received January 26, 1996*⊗

ABSTRACT: Calbindin $D_{28k}$ is an intracellular $Ca^{2+}$-binding protein noted for its abundance and specific distribution in mammalian brain and sensory neurons. This protein contains six putative $Ca^{2+}$-binding sites, referred to as EF-hands. Due to the presence of the large number of putative sites, previous studies have been unsuccessful in definitively establishing the stoichiometry of $Ca^{2+}$ binding. We describe a synthetic approach to identify the number of $Ca^{2+}$-binding sites in which 6 33-residue peptides, designated EF1−EF6, corresponding to the 6 EF-hand sequences of calbindin $D_{28k}$, were made. The response of each peptide to $Ca^{2+}$ addition was assessed by $^1H$ NMR spectroscopy, circular dichroism (CD) spectroscopy, and agarose gel electrophoresis. The $Ca^{2+}$ binding by CD experiments was performed at two peptide concentrations, 20 and 200 $\mu$M, and the NMR studies at peptide concentrations ranging from 20 to 100 $\mu$M. The CD and $^1H$ NMR data show that five of the six peptides bind $Ca^{2+}$ as isolated peptides, namely, EF1, EF3, EF4, EF5, and EF6. The EF6 peptide appears to bind $Ca^{2+}$ with lower affinity than the other four functional sites. In contrast, EF2 does not appear to bind $Ca^{2+}$ under any of the spectroscopic conditions tested. The data suggest that at least five of the six putative sites in the native protein bind $Ca^{2+}$, although their relative affinities cannot be deduced from studies of the isolated peptides.

Calbindin $D_{28k}$ belongs to a large class of eukaryotic cytosolic proteins which bind $Ca^{2+}$ to a specific helix−loop−helix structure referred to as the EF-hand motif (Kretsinger & Nockolds, 1973). More than 500 individual EF-hands in a large number of proteins have been identified (Marsden et al., 1990; Nakayama et al., 1992), and their affinities for $Ca^{2+}$ range from $10^4$ to $10^{10}$ $M^{-1}$ (Falke et al., 1994; Linse & Forsén, 1995). These ubiquitous proteins belong to the calmodulin superfamily and play central roles in a wide variety of $Ca^{2+}$-mediated cellular processes including cell division and growth, ion transport, secretion, motility, and muscle contraction. The superfamily contains both $Ca^{2+}$ buffers, like parvalbumin and calbindin $D_{9k}$, whose structure is largely insensitive to $Ca^{2+}$ binding, and $Ca^{2+}$ sensors, like calmodulin and troponin C, which have a regulatory role and undergo conformational changes upon $Ca^{2+}$ binding that affect their interactions and regulation of target proteins [for a recent discussion, see Chazin (1995)]. A consensus sequence has been deduced for the highly conserved EF-hand motif (Figure 1; Kretsinger, 1987), which can bind one calcium ion; however, this ability has in some cases been lost during evolution, resulting in an "empty" site. The $Ca^{2+}$ coordinating oxygen atoms form a pentagonal bipyramid and are, in general, provided by one water molecule, one backbone amide carbonyl oxygen, and five oxygen atoms in amino acid side chains (McPhalen et al., 1992; Strynadka & James, 1989). However, there are variant EF-hands with more backbone carbonyls and fewer side-chain oxygen atoms in the coordination (Szebenyi & Moffat, 1986).

Most proteins in the calmodulin superfamily contain 2−4 EF-hands, while calretinin (6 EF-hands; Cheung et al., 1993), calbindin $D_{28k}$ (6 EF-hands; Fullmer & Wasserman, 1987), and the LpS1 protein from sea urchins (8 EF-hands; Xiang et al., 1988) are examples of higher order members. There are very few proteins with an odd number of EF-hands, as the functional domain generally consists of a pair of EF-hands that form an antiparallel 4-helix bundle. The $Ca^{2+}$-binding loops are positioned at the same end of the bundle and are connected by a short antiparallel $\beta$-sheet. Within the domain, the two $Ca^{2+}$ ions are generally bound with positive cooperativity. Due to intensive investigations in the field, the mechanisms of $Ca^{2+}$ activation are starting to be understood for a few systems with 2−4 sites. Studies on larger EF-hand proteins that contain more than 4 sites may contribute new insights into the understanding of, in particular, the interactions between sites and their cooperativity in $Ca^{2+}$ binding.

Calbindin $D_{28k}$ is present in a subset of neurons within the central nervous system and in epithelial tissue involved in $Ca^{2+}$ transport (Andressen et al., 1993; Christakos et al., 1989), and the protein displays a high degree of conservation. In the intestine and kidney, but not in the brain, the expression of calbindin $D_{28k}$ is induced by vitamin D. Recent studies of knock-out mice lacking the calbindin $D_{28k}$ gene indicate that the protein has a role in $Ca^{2+}$ homeostasis within nerve cells (M. Meyer, Max Planck Institute, Martinsried, personal communication). The protein is likely to act as a buffer to keep cytoplasmic free $Ca^{2+}$ below toxic levels

† This project was supported by the Research Council of Rutgers University, the Wenner−Gren Foundations, and the Swedish Natural Science Research Council (Grant K 10178-305).

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

0006-2960/96/0435-3662$12.00/0 

within brain and sensory neurons and during transcellular $Ca^{2+}$ transport. It appears to provide a protective role against cell-death and seizures, in which large amounts of $Ca^{2+}$ are released into certain cells (Dowd et al., 1992; Rami et al., 1992; Kohr, 1991). It may facilitate signal transmission in hair cells (Roberts, 1993) where the calbindin $D_{28k}$ concentration can be above 1 mM (Oberholtzer et al., 1988), and it is thought to play a role in $Mg^{2+}$ homeostasis (Hemmingsen et al., 1994). Interestingly, calbindin $D_{28k}$ displays a high sequence homology to calretinin, another protein noted for its specific neuronal distribution (Cheung et al., 1993).

Calbindin $D_{28k}$ binds $Ca^{2+}$ with high affinity; $K_a = 10^6-10^7$ $M^{-1}$ in 150 mM KCl (Bredderman & Wasserman, 1974) and $10^8$ $M^{-1}$ at low ionic strength (2 mM Tris/HCl,[1] pH 7.5; Leathers et al., 1990). Its amino acid sequence (Fullmer & Wasserman, 1987) contains six putative EF-hands (numbered 1−6 starting from the N-terminus). The large number of sites has made it very difficult to determine the exact stoichiometry of $Ca^{2+}$ binding, and the suggested number of high-affinity sites are 3 (Gross et al., 1987), 3−4 (Cheung et al., 1993), 4 (Bredderman & Wasserman, 1974), and 5−6 (Leathers et al., 1990). There are no data available on the three-dimensional structure of calbindin $D_{28k}$; however, an NMR structure of a construct comprising the two N-terminal EF-hands is under way (D. Kallick, University of Minnesota, personal communication). Inspection of the amino acid sequence reveals that four of the sites, EF-hands 1, 3, 4, and 5, agree well with the EF-hand consensus (Figure 1), and these have been suggested to provide sites with high affinity for $Ca^{2+}$ (Fullmer & Wasserman, 1987). EF-hands 2 and 6, on the other hand, contain certain anomalies to the EF-hand consensus sequence. Prediction of their $Ca^{2+}$-binding properties is not a straightforward task as some sequences may adopt a variant EF-hand conformation, similar or different from the pseudo-EF-hand in calbindin $D_{9k}$ (Szebenyi & Moffat, 1986). Calbindin $D_{28k}$ mutants have been made (Gross et al., 1988; Kumar et al., 1994) that contain deletions encompassing portions of one or both of the noncanonical EF-hands. The mutants were reported to bind $Ca^{2+}$ although, unfortunately, no binding constants were determined.

In the present work, we describe a synthetic approach toward studying the $Ca^{2+}$-binding properties of calbindin $D_{28k}$. Our goal was to establish which sequences bind $Ca^{2+}$ by synthesizing the 6 putative EF-hands as 33-residue peptides and study their binding characteristics individually. The peptides are designated EF1 through EF6, with EF1 representing the N-terminal site. Their response to $Ca^{2+}$ addition has been characterized using $^1H$ NMR, circular dichroism (CD) spectroscopy, and electrophoresis. To our knowledge, this represents the first attempt at using a synthetic approach to determine which sites bind $Ca^{2+}$ in a multisite protein.

## MATERIALS AND METHODS

*Materials.* All reagents were purchased from commercial suppliers and used without further purification. The following Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids were used in the syntheses: Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OBut)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OBut)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(But)-OH, Fmoc-Thr(But)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(OBut)-OH, Fmoc-Val-OH, Fmoc-Ala-OPfp, Fmoc-Asn(Trt)-OPfp, Fmoc-Asp(OBut)-OPfp, Fmoc-Gln(Trt)-OPfp, Fmoc-Glu(OBut)-OPfp, Fmoc-Gly-OPfp, Fmoc-His(Boc)-OPfp, Fmoc-Ile-OPfp, Fmoc-Leu-OPfp, Fmoc-Lys(Boc)-OPfp, Fmoc-Met-OPfp, Fmoc-Phe-OPfp, Fmoc-Pro-OPfp, Fmoc-Ser(But)-ODhbt, Fmoc-Thr(But)-ODhbt, Fmoc-Trp-OPfp, Fmoc-Tyr(But)-OPfp, Fmoc-Val-OPfp, and were, together with *N*-hydroxybenzotriazole (HOBt) and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), obtained from Advanced ChemTech. 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid−MBHA (PAL) resin was purchased from Milligen. Reversed-phase HPLC C4 columns, preparative (22 × 250 mm, 10 m) and analytical (4.6 × 250 mm, 5 m), were obtained from Vydac. DEAE-Sephacel cation exchange resin and Sephadex G-25 (fine) were acquired from Pharmacia. Chelex-100 resin was purchased from Bio-Rad.

*Peptide Synthesis and Purification.* The peptides were synthesized by solid-phase methodology employing a Milligen 9050 synthesizer using software version 1.5. In order to improve yields, the following amino acids were, in general, coupled twice: Phe, Ala, Trp, His, Arg, $\beta$-branched amino acids, amino acids directly following $\beta$-branched amino acids, amino acids following two consecutive Leu, and the C-terminal amino acid. Standard synthetic protocols were employed with the following modifications: 45-min coupling steps were achieved by activation of the free carboxylic acids with HBTU. The double-coupling step (30 min) used the commercially available pentafluorophenyl (OPfp) ester derivatives or 3,4-dihydro-4-oxo-1,2,3-benzotriazine (ODhbt) ester in the case of Ser and Thr, in combination with HOBt, except for Arg which was coupled as the free carboxylic acid using HBTU. The N-terminal residue of the completed peptide was acetylated with 0.5 M pyridine and 0.5 M acetic anhydride in DMF for 15 min at 21 °C. The cleavage of the peptide from the resin was achieved with TFA/thioanisole/1,2-ethanedithiol/anisole (9.0:0.5:0.3:0.2) for 2 h. After evaporation of the solvent with a stream of $N_2$, the crude peptide was precipitated with ice-cold diethyl ether, collected by filtration, dissolved in water, lyophilized, and purified as described below.

The peptides were purified by reversed-phase HPLC using a preparative C4 column on a Rainin HPXL instrument, equipped with a Dynamax UV-D dual-wavelength detector. Prior to purification by HPLC, each peptide was passed through a DEAE-Sephacel cation exchange column (12 × 180 mm), eluting with a linear gradient of 0−0.5 M NaCl in 10 mM Tris/HCl containing 1 mM EDTA, pH 7.5 (500 mL each). The fractions (7.5 mL) were monitored at 280 nm, pooled, lyophilized, and desalted by passage through a Sephadex G-25 size exclusion column (35 × 175 mm), using water as eluent. The 4 mL fractions were monitored at 214 and 280 nm, pooled, and lyophilized. EF3 was purified by a DEAE-Sephacel cation exchange column a second time using a linear gradient of 0−0.25 M NaCl in 10 mM Tris/HCl containing 1 mM EDTA, pH 7.5 (500 mL each), and desalted as above. The following HPLC gradients were used in the purification of the peptides (flow rate, 10 mL/min;

[1] Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Tris, tris(hydroxymethyl)-aminomethane.

| PEPTIDE | AMINO ACID SEQUENCE | CHARGE pH 8 | CHARGE pH 6 |
|---|---|---|---|
| **EF1** | Ac-A A Q F F E I W H H Y D S D G N G Y M D G K E L Q N F I Q E L Q Q-$CONH_2$ | -5 | -3 |
| **EF2** | Ac-T P E M K A F V D Q Y G K A T D G K I G I V E L A Q V L P T E E N-$CONH_2$ | -3 | -3 |
| **EF3** | Ac-S E D F M Q T W R K Y D S D H S G F I D S E E L K S F L K D L L Q-$CONH_2$ | -4 | -3 |
| **EF4** | Ac-T E Y T E I M L R M F D A N N D G K L E L T E L A R L L P V Q E N-$CONH_2$ | -4 | -4 |
| **EF5** | Ac-A K E F N K A F E M Y D Q D G N G Y I D E N E L D A L L K D L S E-$CONH_2$ | -6 | -6 |
| **EF6** | Ac-N N L A T Y K K S I M A L S D G G K L Y R A E L A L I L S A E E N-$CONH_2$ | 0 | 0 |
| **Consensus**[a] | E J - - J J - - J D - D - D G - I S - - E J - - J J - - J - - | | |
| | N N L T |
| | S V G |
| | N |
| | D |
| | E |

a) J in the consensus sequence (Kretsinger 1987) stands for a hydrophobic residue.

FIGURE 1: Amino acid sequences of synthesized peptides and the EF-hand consensus sequence. Consensus residue positions are indicated by shading. Cysteine residues have been replaced by serine.

solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in $CH_3CN$/water, 9:1): EF1, 37−47% B/20 min; EF2, 35−45% B/20 min; EF3, 38−48% B/20 min; EF4, 39−49% B/20 min; EF5, 37−47% B/20 min; EF6, 38−53% B/30 min. EF6 was mixed with 1000 wt % guanidinium thiocyanate prior to injection.

*Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF).* Mass spectra were generated in the linear mode on a time-of-flight PerSeptive Biosystems/Vestec Products LaserTec ResearcH Model instrument (Framingham, MA). The mass spectrometer was equipped with a 1.2 m flight tube and a 337 nm nitrogen laser with a pulse duration of 3 ns. The acceleration voltage was +20 kV. The laser power used was the minimum necessary to obtain the threshold of ionization. The signal was summed over 256 laser pulses to obtain an average time-of-flight spectrum for each sample. All data acquisition and reduction was controlled by a custom Grams/386 (Galactic Industries Corp., Salem, NH). Matrices used in the analyses were either α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.) or 2,4,6-trihydroxyacetophenone (Fluka Chemical Corp.) in a solution of 10 mg of matrix/mL of acetonitrile. Approximately 1.5 mL of a 1:2 mixture of peptide (ca. 3 mM in 0.1% aqueous TFA) to matrix solution was deposited on the tip of the sample target pin and allowed to air-dry before being analyzed in the mass spectrometer.

*Agarose Gel Electrophoresis.* Agarose gel electrophoresis was run on a 2 × 110 × 205 mm 1% agarose plate in 75 mM sodium barbitone buffer, pH 8.6, with either 2 mM EDTA or 2 mM $CaCl_2$. The buffer was prepared by dissolving 2.065 g of diethylbarbituric acid (BDH) and 13.05 g of sodium diethylbarbitone (BDH) per liter of doubly distilled water. The peptide concentrations were ca. 200 μM except in the case of EF2 which was more concentrated due to poor staining.

*CD Spectroscopy.* Circular dichroism spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C (thermostated) in a 0.1 mm or 1 mm quartz cuvette in 2 mM Tris/HCl, pH 7.5. The buffer was prepared in doubly distilled water and was stored with a dialysis bag with Chelex-100 in the container to keep the free $Ca^{2+}$ concentration at 0.1−0.5 μM. Both cuvettes were sealed to prevent evaporation during the course of recording the spectra. The peptide concentration was determined by amino acid analysis after acid hydrolysis. Using the 1 mm cuvette, five different spectra were recorded for each EF-hand peptide at a concentration of ca. 20 μM under the following conditions: (1) no added $Ca^{2+}$; (2) 10 mM $Ca^{2+}$; (3) 50% TFE; (4) 50% TFE + 10 mM $Ca^{2+}$; and (5) 8 M urea with no $Ca^{2+}$ added. Additional CD spectra were recorded at higher peptide concentrations as some peptides might have lower affinities for $Ca^{2+}$ and/or lower dimerization constants. Using the 0.1 mm cuvette, spectra were recorded for each peptide at a concentration of ca. 200 μM in 2 mM Tris/HCl, pH 7.5, with (1) no added $Ca^{2+}$ and (2) 10 mM $Ca^{2+}$. Base lines were recorded separately at each solution condition and subtracted from the observed spectra before they were imported in ASCII format into KaleidaGraph for plotting. $Ca^{2+}$ titrations were monitored by the change in ellipticity at 222 nm for EF-hands 1, 3, 4, and 5 at 20 μM peptide concentration in 2 mM Tris/HCl, pH 7.5. The sample volume was 400 μL, and $Ca^{2+}$ was added in small portions (1−5 μL) from 1 to 100 mM stock solutions prepared in the same buffer.

*$^1$H NMR Spectroscopy.* One-dimensional $^1$H NMR spectra were recorded at 500.11 MHz on a GE-Omega 500 spectrometer. The spectra were recorded at 27 °C, pH 7.0, in 90% $H_2O$ + 10% $D_2O$. The peptide concentrations were in the 20−100 μM range, and spectra were recorded for each peptide in the absence and presence of $Ca^{2+}$. $Ca^{2+}$ was added stepwise to obtain information on the $Ca^{2+}$ exchange rates.

## RESULTS

*Peptide Synthesis and Purification.* The sequences of the six peptides are summarized in Figure 1 together with the consensus EF-hand sequence. The purity of the peptide analogs after HPLC purification exceeds 95%, as determined by analytical HPLC. Their identity was established by laser desorption mass spectroscopy and the correct composition confirmed by amino acid analysis after acid hydrolysis.

The purification step of the peptides by ion exchange chromatography on Sephacel resin removed residual scavengers as well as peptide impurities and considerably facilitated subsequent purification by HPLC. A problem with EF6 is that it tends to aggregate and adhere to resin matrixes, especially in the absence of $Ca^{2+}$. We found that the

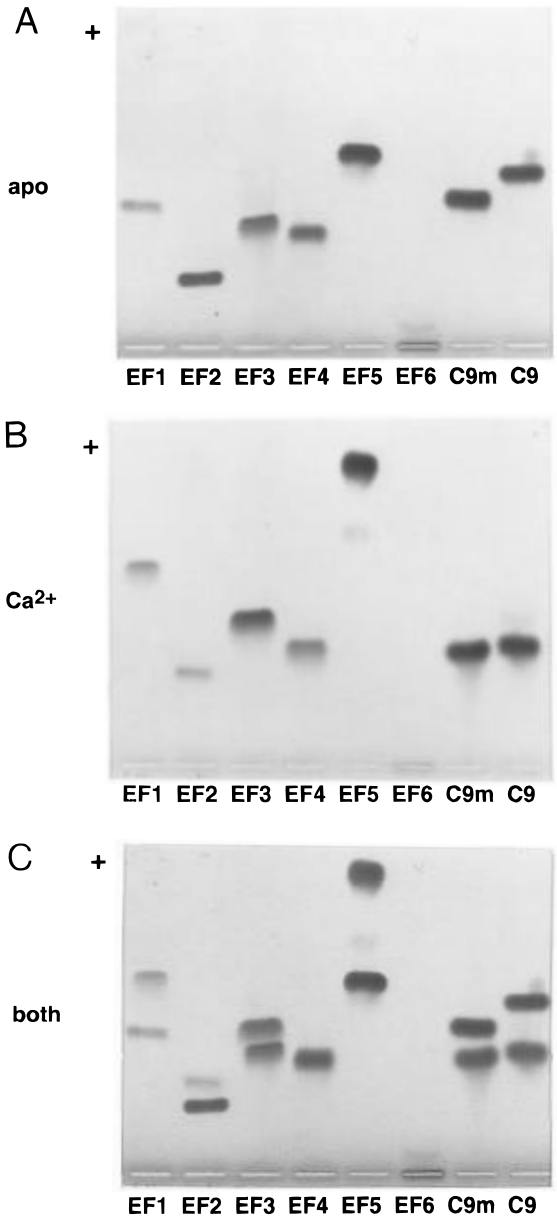


FIGURE 2: Agarose gels in 2 mM EDTA (top, panel A) and 2 mM $Ca^{2+}$ (middle, panel B). The lower panel (panel C) shows both gels superimposed. The peptide concentrations were ca. 200 $\mu$M except in the case of EF2 which, due to poor staining, was considerably higher.

recovery of EF6 was higher overall if the ion exchange purification step was omitted. To avoid aggregation and sticking to the HPLC column, EF6 was mixed with a strong denaturant, guanidine thiocyanate, before injection.

*Matrix-Assisted Laser Desorption Mass Spectroscopy.* The mass spectra were all within 0.1% of the expected mass: EF1, found *m*/*z* 4000.5, expected 4000.8 ($M+H^+$); EF2, found *m*/*z* 3634.2, expected 3633.8 ($M+H^+$); EF3, found *m*/*z* 4040.4, expected 4036.9 ($M+H^+$); EF4, found *m*/*z* 3911.8, expected 3908.9 ($M+H^+$); EF5, found *m*/*z* 3866.6, expected 3866.8 ($M+H^+$); EF6, found *m*/*z* 3641.0, expected 3638.9 ($M+H^+$).

*Agarose Gel Electrophoresis.* Agarose gels of EF1−EF6 run in the presence of 2 mM EDTA and 2 mM $Ca^{2+}$ are shown in Figure 2. In agarose gel electrophoresis, proteins and peptides are run under nondenaturing conditions. The travelling distance of a polypeptide in an agarose gel is to a large extent determined by its total charge. A tight noncovalent complex can move as a unit in the gel and therefore migrates according to the total charge of the entire complex. The relative mobilities observed for the six EF-hand peptides in the presence of EDTA and at pH 8.6 are in agreement with their respective total charge as monomers (Figure 2A). EF6, which has zero net charge, cannot move and is therefore seen as a blue contour of the slit. When the gel is run in the presence of $Ca^{2+}$, the mobilities of the peptides and reference proteins may change due to their complexation with $Ca^{2+}$ and/or to peptide oligomerization (Figure 2B,C). The two reference proteins are wild-type calbindin $D_{9k}$ (C9), which binds two calcium ions, and the Glu27→Gln mutant form of the protein that only binds one $Ca^{2+}$ ion (C9m). As the C9 reference is an old sample, there is a weak extra band due to a 1 unit charge increase by deamidation. The net charge of calbindin $D_{9k}$ is −8, and it is monomeric in the absence, as well as in the presence, of $Ca^{2+}$. The retardation of C9m in the gel due to $Ca^{2+}$ binding is expected to be roughly half of that for C9, as it binds half as many calcium ions. This fact was used to calibrate the relative run times for the two gels, which was necessary as we have separate home-built electrophoresis units for EDTA- and $Ca^{2+}$-containing gels. The mobilities of peptides EF1 through EF6 are observed to either increase or remain unchanged in the presence of $Ca^{2+}$. A significant increase in mobility is observed for EF1 and EF5. These peptides have net charges of −6 (EF5) and −5 (EF1) at pH 8.6 that decrease to −4 and −3, respectively, upon $Ca^{2+}$ binding, which is inconsistent with the observed data. Isolated EF hands have previously been shown to form stable homodimers (Shaw et al., 1992b). The observed increase, rather than decrease, in the mobilities of EF5 and EF1 is indeed compatible with the formation of $Ca^{2+}$-bound homodimers carrying net charges of −8 and −6, respectively. Since other factors than total charge also affect the mobilities, we cannot exclude the possible formation of larger complexes. Dimerization, trimerization, etc. can be expected to lead to structural changes, and it is not possible to predict the difference in the mobilities between different multimers based on their total charge only. In the case of EF3 and EF4, each with a charge of −4, the $Ca^{2+}$-bound forms would have a net charge of −2 and therefore decrease in mobility whereas $Ca^{2+}$-bound homodimers would have a total charge of −4. The mobilities of these two peptides are relatively similar in the two gels (Figure 2C) and are, particularly in the case of EF4, consistent with homodimer formation. (EF3 travels slightly faster in the presence of $Ca^{2+}$.) Again, we cannot exclude that EF3 and EF4 form higher order multimers. Surprisingly, EF2 also experiences an increase in mobility in the presence of $Ca^{2+}$.

*CD Spectra at 20 $\mu$M Peptide Concentration.* The far-UV CD spectra recorded for EF1 through EF6 at 20 $\mu$M peptide concentration are shown in Figure 3. In 2 mM Tris, all six peptides appear mostly unfolded in the absence of $Ca^{2+}$. However, spectral changes that occur at ca. 192, 208, and 222 nm are indicative of an increase in α-helix content, and are observed on $Ca^{2+}$ addition to EF1, EF3, EF4, and EF5. With spectra recorded for the apo, $Ca^{2+}$, and the urea-denatured forms, we can estimate that the apo forms of these four peptides maintain approximately 5−10% of the structure present in the $Ca^{2+}$ forms. In the presence of 50% TFE (Figure 4), the shape of the spectra, notably the ratio of the ellipticities at 222 and 208 nm, is different compared to those in Figure 3. In TFE, all six peptides show a significant α-helix content, and $Ca^{2+}$ addition has very little effect.

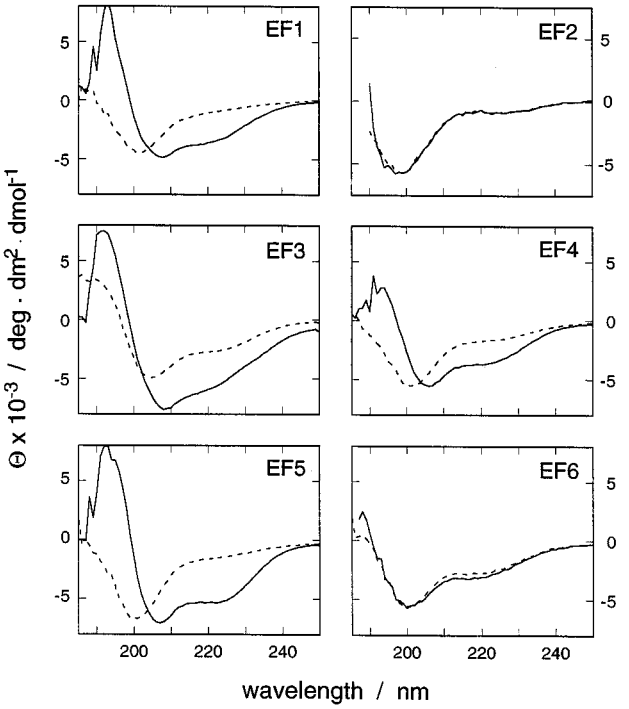


FIGURE 3: CD spectra obtained using a 1 mm cuvette with peptide concentrations around 20 $\mu$M in 2 mM Tris/HCl, pH 7.5. (---) Apo and (—) $Ca^{2+}$.

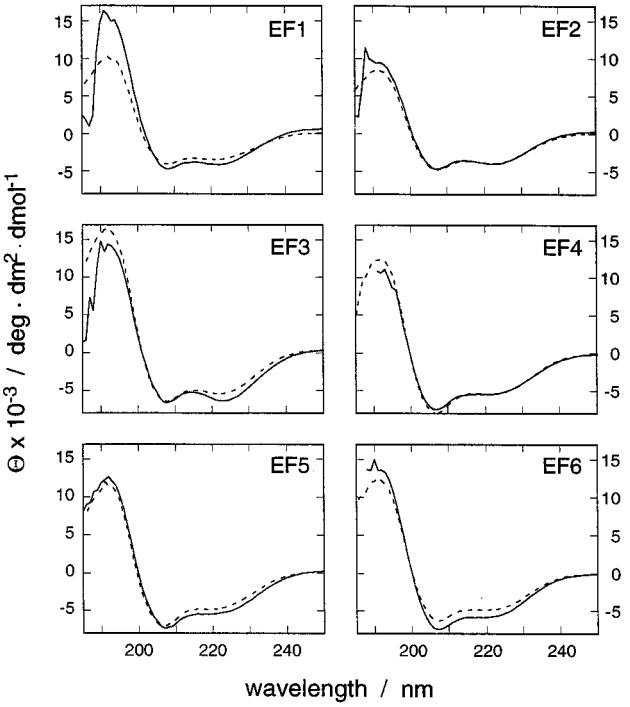


FIGURE 4: CD spectra obtained using a 1 mm cuvette with peptide concentrations around 20 $\mu$M in 2 mM Tris/HCl, pH 7.5, with 50% TFE. (---) Apo and (—) $Ca^{2+}$.

*CD Spectra at 200 $\mu$M Peptide Concentration.* The far-UV CD spectra recorded for EF1 through EF6 at 200 $\mu$M peptide concentration are shown in Figure 5. At this concentration, EF1, EF3, EF4, EF5, and EF6 undergo spectral changes consistent with $Ca^{2+}$ binding. The spectra in the absence and presence of $Ca^{2+}$ differ significantly from each other except in the case of EF3; however, the small changes observed for this peptide are due to the fact that a fair amount of the helix−loop−helix structure is already present in the $Ca^{2+}$-free state. As a control experiment, one

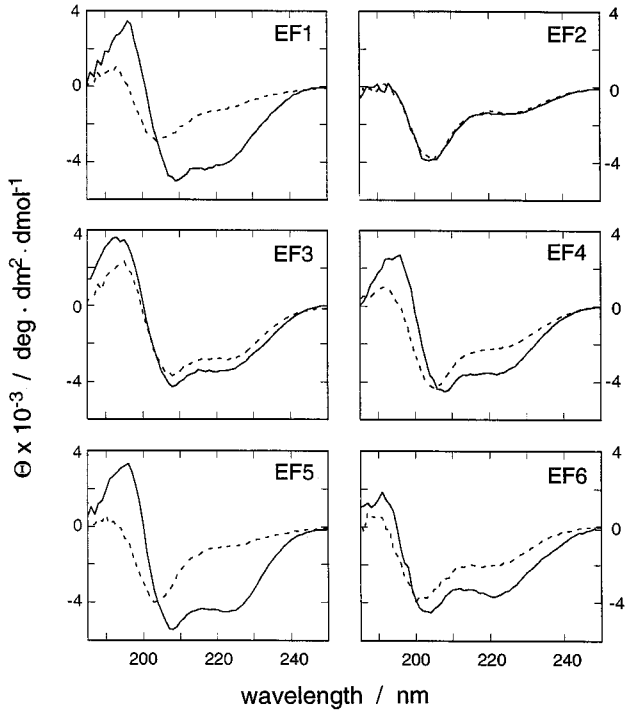


FIGURE 5: CD spectra obtained using a 0.1 mm cuvette with peptide concentrations around 200 $\mu$M in 2 mM Tris/HCl, pH 7.5. (---) Apo and (—) $Ca^{2+}$.

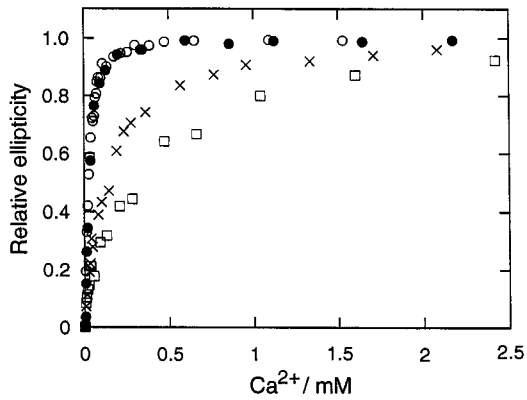


FIGURE 6: $Ca^{2+}$ titrations at 20 $\mu$M peptide as monitored by CD (ellipticity at 222 nm). The data have been normalized. (×) EF1, (○) EF3, (□) EF4, (●) EF5.

spectrum of each peptide was recorded in the presence of 1 mM EDTA, and the results confirm that the purified peptides were free of $Ca^{2+}$.

The largest spectral changes occur for EF1 and EF5. The $Ca^{2+}$-induced spectral changes observed for EF4 and EF6 are moderate and very similar in shape and magnitude. As a control experiment to test that the spectral changes observed were not due to an ionic strength effect as the result of the addition of 10 mM $CaCl_2$, a spectrum of EF6 was run in the absence of $Ca^{2+}$, both with and without 50 mM KCl, and shown to be very similar. No $Ca^{2+}$ binding was observed for EF6 at 20 $\mu$M peptide concentration.

*$Ca^{2+}$ Titrations As Followed by CD.* $Ca^{2+}$ titrations of EF1, EF3, EF4, and EF5 at ca. 20 $\mu$M peptide concentration are shown in Figure 6. It is clear that the full $Ca^{2+}$-induced spectral changes occur before the concentration of $Ca^{2+}$ has reached 0.5 mM in the case of EF3 and EF5, and before 2 mM for EF1 and EF4. The amount of $Ca^{2+}$ used (10 mM) in the CD spectra recorded in Figures 3−5 is thus sufficient to observe the maximum $Ca^{2+}$-induced spectral changes. At

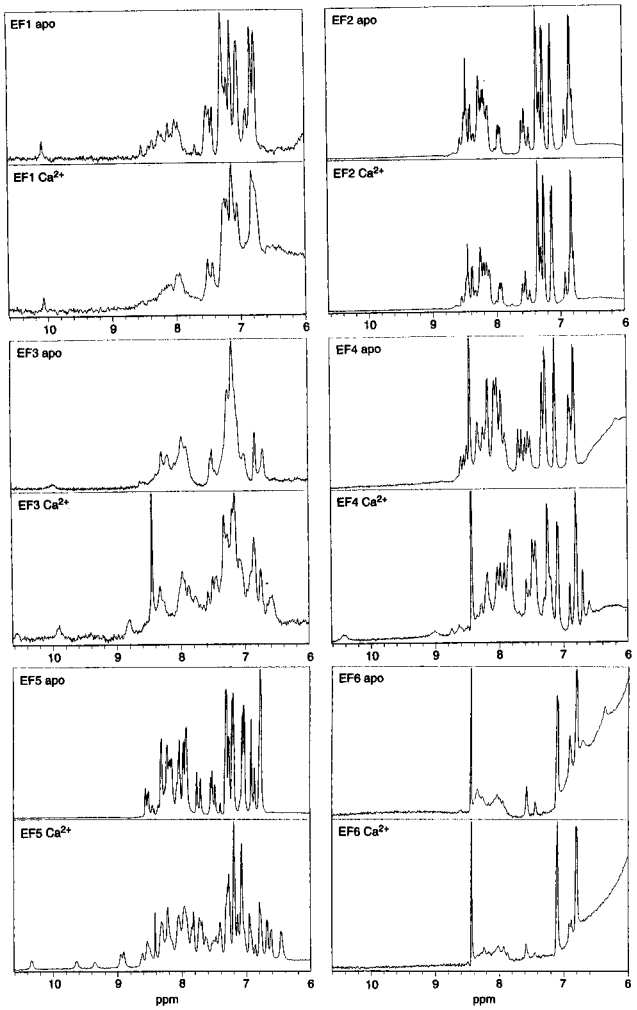


FIGURE 7: $^1$H NMR spectra obtained at 500 MHz in the absence and presence of $Ca^{2+}$ in $H_2O$ at pH 7.0. The peptide concentrations were approximately 20 $\mu$M for EF6, 50 $\mu$M for EF1, EF3, and EF4 and 100 $\mu$M for EF2 and EF5. The spectral region covers aromatic and backbone amide protons.

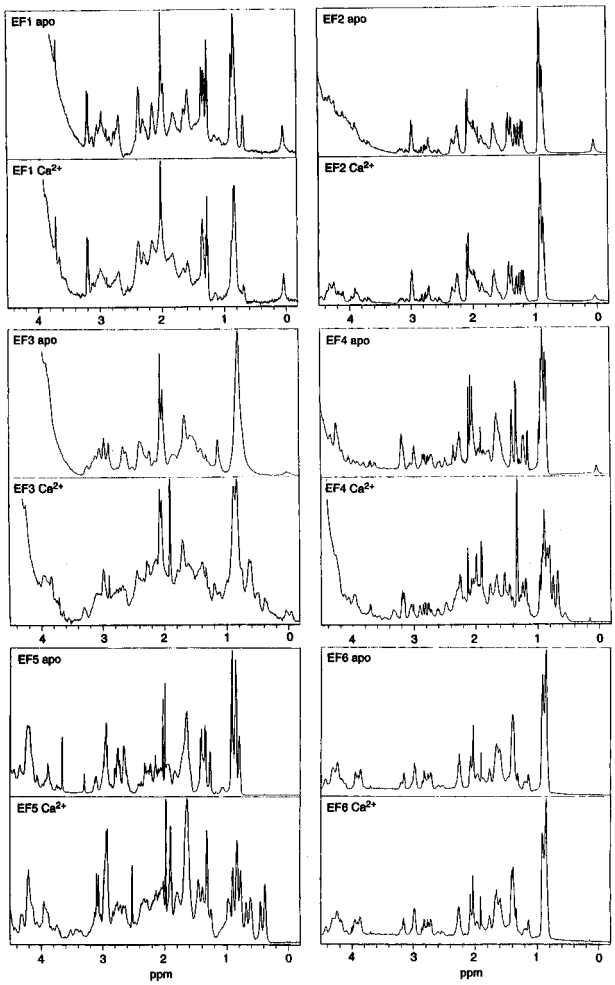


FIGURE 8: Methyl and methylene proton region of the same $^1$H NMR spectra as shown in Figure 7.

first sight, EF3 and EF5 appear to bind $Ca^{2+}$ with higher affinity than EF1 and EF4. However, the shapes of these titration curves are dependent not only on the $Ca^{2+}$-binding constants but also on the dimerization or oligomerization constants. For the simplest case, i.e., dimer formation, data analysis involves the fitting of two $Ca^{2+}$-binding constants and one dimerization constant. The most rigorous approach to obtaining a unique solution to these three parameters requires titration data at several different peptide concentrations. Our future plan is to purify enough material for these measurements.

*$^1$H NMR Spectroscopy.* Selected regions of the $^1$H NMR spectra of the six peptides in the absence and presence of $Ca^{2+}$ are shown in Figures 7 and 8, respectively. There is no significant effect of $Ca^{2+}$ addition on EF2. However, distinct changes occur for EF1, EF3, EF4, EF5, and EF6, although the observed effects vary in type and magnitude. For EF1, EF3, EF4, and EF5, $Ca^{2+}$ binding is a slow exchange process on the NMR time scale. This is manifested as a gradual change in the intensity of several resonances, some increasing and some decreasing, as $Ca^{2+}$ is added in portions. For EF6, $Ca^{2+}$ binding is a fast exchange process on the NMR time scale, which is observed as a progressive shift of the resonances as $Ca^{2+}$ is added. The $^1$H NMR signals are considerably broader for EF3 compared to the other five peptides. This is consistent with aggregation as the signals are further broadened at higher peptide concentrations.

## DISCUSSION

Previous studies of fragments the size of a single EF-hand have yielded information that has contributed considerably to the understanding of the respective intact proteins. Most of these studies have targeted the smaller members of the calmodulin superfamily with domains containing only two EF-hands, as in troponin C (Reid et al., 1980, 1981; Shaw et al., 1990, 1992, 1994) and calbindin $D_{9k}$ (Finn et al., 1992; Tsuji & Kaiser, 1991; Linse et al., 1993). None of the single EF-hand fragments fold in the absence of $Ca^{2+}$, but appear as random coils. However, in the presence of $Ca^{2+}$ they adopt a structure very similar to their native structure in the intact protein. Moreover, as they are naturally parts of an EF-hand pair, $Ca^{2+}$-induced folding has been shown to be coupled to homodimerization to bury the very large hydrophobic surface area which would otherwise be unfavorably exposed to solvent (Shaw et al., 1992b; Finn et al., 1992). In the intact protein, this hydrophobic surface interacts with a complementary hydrophobic surface of a specific EF-hand partner to form a hydrophobic core. The peptide corresponding to site III in troponin C, for example, forms homodimers in the presence of $Ca^{2+}$ (Shaw et al., 1990). If this peptide is mixed with its natural partner, site IV, the natural heterodimer is formed exclusively (Shaw et al.,

1992a). Similar experiments with two calbindin $D_{9k}$ fragments show that the natural heterodimer is preferred even if one of the homodimers is stabilized by a disulfide bond linking the monomers (Linse et al., 1993). Fragments encompassing two EF-hands have been derived from larger domains in parvalbumin (Permyakov et al., 1991) and the sarcoplasmic $Ca^{2+}$-binding protein (Durussel et al., 1993).

To our knowledge, the synthesis and study of single EF-hand fragments derived from a larger protein of unknown structure to delineate its functional sites and subdomain architecture are a novel approach. We do not expect that an EF-hand that is excised from its natural environment in the intact protein will retain all of its affinity for $Ca^{2+}$ when studied as an isolated peptide. In the intact protein, it will benefit from stabilizing interactions with other parts of the protein, and side chains in the surroundings may contribute additional $Ca^{2+}$ ligands or stabilize the ligands already present in the site. We do expect that an EF-hand that binds calcium ions as an isolated peptide also binds $Ca^{2+}$ in the intact protein, although results obtained with the peptides might not correctly reflect the relative $Ca^{2+}$ affinities of the sites in the protein. As in the previous studies on fragments excised from the smaller members of the calmodulin family, the EF-peptides of calbindin $D_{28k}$ show very little structure in the absence of $Ca^{2+}$. However, $Ca^{2+}$ addition leads to significant changes in the CD spectra for five of the six peptides, consistent with the formation of the helix−loop−helix motif. In the presence of 50% TFE, all six peptides show a significant α-helix content both in the absence and in the presence of $Ca^{2+}$.

*The Canonical EF-Hands (Numbers 1, 3, 4, and 5).* The amino acid sequences of EF-hands 1, 3, 4, and 5 of calbindin $D_{28k}$ agree well with the EF-hand consensus sequence (Kretsinger, 1987). They have the key hydrophobic residues in the correct position in the helices: a glycine in position 6 of the loop, a glutamate in position 12, and oxygen-bearing side chains in the standard $Ca^{2+}$ ligand positions of the loop (Figure 1). The CD and $^1$H NMR spectral data strongly support that the corresponding fragments, EF1, EF3, EF4, and EF5, bind $Ca^{2+}$ as isolated peptides even at relatively low (20 $\mu$M) concentration, and we find it reasonable to conclude that these EF-hands provide four of the high-affinity sites in the intact protein calbindin $D_{28k}$.

The results of agarose gel electrophoresis suggest that each of these fragments form homodimers or higher order multimers in the presence of $Ca^{2+}$. The $^1$H NMR spectra of EF1, EF3, and EF4, and, in particular, EF5, in the presence of $Ca^{2+}$ contain several features repeatedly observed for native EF-hand pair domains. Resonances are observed at high chemical shifts (around 10−10.5 ppm), typical of the hydrogen-bonded backbone amide protons of the conserved glycine in the sixth position of the loop and of other residues in the $\beta$-sheet connecting the $Ca^{2+}$-binding loops. For EF5, there is also a significant chemical shift dispersion in the methyl region. The CD spectra of the $Ca^{2+}$ forms are identical in the presence or absence of 50% TFE, whereas for the apo peptides a stronger signal is observed at 222 nm in the presence of 50% TFE than in aqueous buffer.

*The Noncanonical EF-Hands (Numbers 2 and 6).* The amino acid sequences of the second and sixth EF-hands agree with the consensus EF-hand in several positions. They both have the predicted helix−loop−helix secondary structure (Fullmer & Wasserman, 1987), with a glycine and a glutamate residue in the sixth and the twelfth position of the loop, respectively, and large hydrophobic residues immediately preceding and succeeding the loop. However, they lack some of the oxygen-containing side chains often found in the first, third, fifth, and ninth positions of the loop. It is therefore commonly anticipated that these two EF-hands are nonfunctional in terms of $Ca^{2+}$ binding (Fullmer & Wasserman, 1987; Kretsinger, 1987; Kawasaki & Kretsinger, 1994). On the other hand, one cannot, without experimental confirmation, rule out the possibility that EF-hands 2 and 6 adopt variant folds, similar or different from the pseudo EF-hand in calbindin $D_{9k}$ (Szebenyi & Moffat, 1986).

None of the spectroscopic data obtained in the present work suggest that EF2 binds $Ca^{2+}$ as an isolated peptide, regardless of peptide or $Ca^{2+}$ concentration. Its CD and $^1$H NMR spectra are unaffected by $Ca^{2+}$ addition (Figures 3, 5, and 7), and it also appears unfolded at high (10 mM) $Ca^{2+}$ concentrations. In the presence of 50% TFE, its CD spectrum has a similar shape and intensity to the other peptides, and it does not change upon addition of $Ca^{2+}$. However, the mobility of EF2 on agarose gels is observed to increase on $Ca^{2+}$ addition. Due to the poor staining of EF2, the concentration used in these gels was considerably higher than those used in the spectroscopic analyses. It is, therefore, plausible that EF2 can bind $Ca^{2+}$ at extremely high peptide concentration.

As an interesting contrast to EF2, several spectral features of EF6 are perturbed by $Ca^{2+}$ addition. At low peptide concentration (20 $\mu$M, Figure 3), the CD spectrum is similar to that of EF2, both in the absence and in the presence of $Ca^{2+}$. No change occurs in the spectrum upon addition of $Ca^{2+}$. However, at higher peptide concentration (200 $\mu$M, Figure 5), the CD spectrum is sensitive to $Ca^{2+}$ addition, and the change is consistent with increased α-helix formation. Its response to $Ca^{2+}$ addition is very similar to that observed for EF4. The $^1$H NMR spectrum obtained with 20 $\mu$M peptide is also affected by $Ca^{2+}$ addition (Figure 7). Several signals in the backbone amide region (between 6.5 and 9.5 ppm) are gradually shifted as $Ca^{2+}$ is titrated into the solution, showing that the interaction with $Ca^{2+}$ is a fast exchange process on the NMR time scale. $Ca^{2+}$ binding does not lead to the occurrence of amide protons at high chemical shifts around 10 ppm as in EF1, EF3, EF4, and EF5, suggesting that the EF6−$Ca^{2+}$ complex does not have a standard EF-hand fold. Taken together, the spectroscopic data show that EF6 binds $Ca^{2+}$ at relatively high peptide and $Ca^{2+}$ concentrations. The failure of this peptide to respond to $Ca^{2+}$ addition on the agarose gel also suggests that the $Ca^{2+}$ is less tightly bound than in EF1, EF3, EF4, and EF5. Assessment of the $Ca^{2+}$ affinity would require relatively large quantities of peptide, as titrations have to be performed at several different peptide concentrations to solve the system of coupled equilibria for peptide dimerization and $Ca^{2+}$ binding. However, it is clear that EF6 fails to bind $Ca^{2+}$ at low peptide concentrations, in contrast to EF1, EF3, EF4, and EF5. Thus, in the form of an isolated peptide, the sixth EF-hand binds $Ca^{2+}$ weakly, which might in part be a consequence of its dimerization (or oligomerization) constant being lower compared to those of the canonical EF-hand peptides. The present findings should stimulate further studies of the $Ca^{2+}$-binding properties of EF-hand 6 in its native environment.

We have shown that five out of the six EF-hands of calbindin D28k bind $Ca^{2+}$ as isolated peptides, namely, the four canonical sequences, EF1, EF3, EF4, and EF5, and the

aberrant sequence, EF6. It therefore seems reasonable to assume that these sequences also bind $Ca^{2+}$ in the intact protein. It is clear that all the EF-hand sequences have lost a substantial amount of their $Ca^{2+}$ affinity in being excised from the protein. Clearly, the interactions between the EF-hands within the intact protein are important. As isolated peptides, it is interesting to note the difference in $Ca^{2+}$ binding between the canonical EF-hands and EF6. Is the lower $Ca^{2+}$ affinity displayed by EF6 of physiological relevance or is it simply due to lack of important interactions to other portions of the protein? Because interactions within the protein may be critical for $Ca^{2+}$ binding, we cannot exclude the possibility that EF2 may bind $Ca^{2+}$ in calbindin $D_{28k}$, in spite of the lack of binding observed for the isolated EF2 peptide.

The approach of studying synthetic $Ca^{2+}$-binding sites shows great promise in the study of multisite proteins. Calbindin $D_{28k}$ is an ideal candidate to test this premise because studies of the intact protein have been marred by the presence of the large number of sites and have been inconclusive. Our initial studies have considerably contributed to our understanding of the properties of the individual sites which should have relevance to the understanding of the properties of intact calbindin $D_{28k}$. This technique should prove useful to the study of other members of this class of protein. The synthetic approach also allows the incorporation of specifically labeled residues at any position of the individual sequences which will aid in their structural elucidation by NMR spectroscopy.

## ACKNOWLEDGMENT

We thank James Hagan and Lida Gifford for aid in the HPLC purification of the peptides; Lida Gifford, Johan Evenäs, and Anders Malmendahl for their critical reading of the manuscript; and Sharon Jackson and Arlene Rockwell for many stimulating discussions. We are indebted to Dr. William F. DeGrado and DuPont Merck Pharmaceutical Co. for the rental of equipment.

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BI9527956