A Functional and Degenerate Pair of EF Hands Contains the Very High Affinity Calcium-binding Site of Calbindin-D$_{28K}$*

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Calbindin-D$_{28K}$, a member of the troponin C superfamily of calcium-binding proteins, had six putative EF hand domains containing one very high affinity and two to three lower affinity calcium-binding sites. The location and binding activity of the calcium-binding sites were examined with a recombinant calbindin-D$_{28K}$ protein. This protein (Calb I-II) only contained EF hand domains 1 and 2 of calbindin-D$_{28K}$. Binding of calcium and calcium analogs, the lanthanides, by the recombinant protein was determined in fluorescence emission experiments. Calb I-II bound 1 mol of terbium/mol of protein. Terbium was displaced from Calb I-II by other lanthanides and calcium. Fluorescence from terbium was not quenched by holmium. These results and Hill plots of calcium binding activity, determined from intrinsic protein fluorescence measurements, indicated the presence of a single high affinity calcium-binding site on Calb I-II. The properties of the binding site indicated that the very high affinity site of calbindin-D$_{28K}$ was located in EF hand domains 1 and 2 of the protein. In addition, these findings indicated the NH$_2$-terminal pair of EF hands in calbindin-D$_{28K}$ did not depend on interactions with other domains in the protein for high affinity calcium binding activity. The results suggested at least one calcium-binding domain of calbindin-D$_{28K}$ can exist as an independent EF hand pair.

Calbindin-D$_{28K}$ is induced in avian intestinal tissue by the hormonally active form of vitamin D. There is a temporal correlation between its induction following vitamin D administration and calcium transport (1). The intracellular protein binds calcium (2) and may modulate effects occurring in response to changes in intracellular calcium concentrations (3–5). Its expression is related to the efficiency of transcellular calcium transport. Presence of the protein in numerous additional avian and mammalian tissues suggests an important physiological function (6).

The protein is a member of the troponin C superfamily that may have arisen by multiple gene duplication events (7, 8). Amino acid sequences of calbindin-D$_{28K}$ from different species have been determined by mass spectrometry (9), Edman degradation (10–14), analysis of CDNA, and genomic clones (15–17). The protein has 261–262 amino acids, depending upon species, and contains six putative EF hand domains (1–6). Two of the EF hands (2 and 6) are degenerate, in that their amino acid sequences contain less than three oxygen-containing amino acid side chains at putative positions for ligands with calcium (8). Consistent with this observation, the protein only binds 3–4 mol of calcium/mol of protein (17). One very high affinity ($K_d \sim 10^{-9}$) and two to three lower affinity ($K_d \sim 10^{-6}$) calcium-binding sites are found on the protein. Energy transfer experiments suggest that the very high affinity calcium-binding site is located at EF hand domain 1 and the lower affinity calcium-binding sites in EF hand domains 3–5 (17). In addition, these experiments indicate the very high affinity calcium-binding site is located a relatively long distance (>12Å) from all other calcium-binding sites on the protein.

The EF hand locations of calbindin-D$_{28K}$ calcium-binding sites and the EF hand pairings for the calcium-binding domains are unknown. The presence of six putative EF hands and a long distance between a single site and other sites suggests unique possibilities for this troponin C superfamily protein. All troponin C superfamily proteins have calcium-binding domains with an EF hand motif. The description of this motif is based on x-ray crystallographic studies of parvalbumin (18, 19) and other proteins. It consists of a helix-loop-helix structure with calcium bound to amino acid residues in the loop amino acid sequence. Two or three helix-loop-helix structures can be paired in calcium-binding domains. Calmodulin and troponin C each have two EF hand pairs separated by a long a-helix giving the proteins a dumbbell structure (20, 21). Parvalbumin, on the other hand, has three EF hands in close contact and a globular structure (18). Bovine calbindin-D$_{28K}$ consists of a single pair of EF hands (22).

Several structural features of the troponin C superfamily proteins are important determinants of their calcium affinities. Calcium ligands with 3–5 highly conserved oxygen-containing residues in the EF hand loop amino acid sequences. EF hand loops with less than 3 oxygen-containing residues in the conserved positions have very low affinities for calcium. EF hand loop amino acid sequences and calcium affinity have been examined extensively by protein modeling studies and with synthetic peptides (23). Synthetic peptides of loop amino acid sequences have lower affinities for calcium than the same loops in intact proteins. Although the loop amino acid sequence is an important factor in calcium affinity, the presence of helices and EF hand pairing are also essential components for high calcium affinity (24, 25). The removal of a few carboxyl-terminal amino acid residues from parvalbumin, with three EF hands, causes a loss in calcium affinity of five orders of magnitude (26). Proteolysis studies of calmodulin indicate a loss of calcium affinity with removal of one EF hand within an EF hand pair containing calcium-binding domain (27). Further elucidation of the helical function in determining calcium affinity may be aided by the analysis of additional troponin C superfamily proteins and calcium-binding domains.

Full-length rat calbindin-D$_{28K}$ expressed in Escherichia coli...
has calcium and terbium (a calcium analog) binding properties similar to those reported for calbindin-D_{28K} isolated from chicken intestine (17). In this report, we describe the expression and analysis of a truncated calbindin-D_{28K} containing putative EF hand domains 1 and 2 only (Calb I-II). Calb I-II contains amino acids 1 through 93 of calbindin-D_{28K} and an NH_{2}-terminal hexa-histidine purification tag. The correct expression of Calb II is verified by NH_{2}-terminal sequencing and mass spectrometric analysis of the purified protein. Calcium and lanthanide binding experiments indicate the presence of a calcium-binding site with properties similar to those of the high affinity calcium-binding site found in the full-length protein. That is, Calb I-II contains the very high affinity calcium-binding site of calbindin-D_{28K}. The absence of putative EF hand domains 3-6 only has a small apparent effect on the calcium affinity of the high affinity calcium-binding site. Implications regarding calbindin-D_{28K} structure are given in the discussion.

EXPERIMENTAL PROCEDURES

Materials—Lanthanum chloride heptahydrate, terbium (III) chloride hexahydrate, and holmium (III) chloride hexahydrate were greater than 99.999% pure and obtained from Aldrich. The sesquisodium salt of PIPES, sodium chloride, calcium chloride dihydrate, sodium phosphate, and EDTA were of the highest commercial grade available.

Expression of Rat Calbindin-D_{28K} EF Hands I and II, 6His [Calb (I-II)prCaBPL+II, 6xHis] was cotransfected with calcium absorption domain cloning vector pDS66EtBSI1,6xHis (28,29) and the resulting plasmid coding rat calbindin-D_{28K} amino acids 1-93 was cloned into the NcoI and HindIII sites of pDS66/RBSII, 6His (28,29). The resulting plasmid pCaBPI+II, 6His encodes a protein with the amino acids 1-93 of calbindin-D_{28K} preceded by a hexa-histidine tail, with the amino acid sequence M-R-G-S-H-H-H-H-G-S-M (M being the first amino acid of calbindin-D_{28K}). This plasmid was expressed by transfection into E. coli M-15 that contains the lac-repressor expression plasmid pDM1.1 (29).

Protein Purification—E. coli cultures were grown in LB medium to a density of 0.6-0.8 OD_{600} units. Isopropyl-1-thio-p-o-galactopyranoside was added to a final concentration of 1 mM, and the cultures were grown was done with a Phast gel system using gradient gels (8-25%) and ultraviolet wavelengths had absorbances up to 0.05. Minimal changes

Calcium and Lanthanide Binding: Measurements of Fluorescence Emission—All measurements of fluorescence were made on a Perkin-Elmer Cetus MFP-44A fluorescence spectrophotometer. The cell path length was 1.0 cm for all experiments except excitations with 488-nm light (cell path = 0.25 cm). Slit widths were 8 nm for excitation and emission wavelengths in energy transfer, direct and intrinsic protein fluorescence experiments. The geometry of fluorescence detection was 90°, and sample temperatures were maintained at 25 °C with a circulating water bath. Binding experiments were done in PIPES buffer (50 mM, pH 7.0). Some experiments were done in phosphate buffer (0.1 M, pH 7.4) containing 0.15 M NaCl as well as PIPES to examine buffer effects.

Terbium was excited by energy transfer from tryptophan irradiation with 405-nm light. Excitation time was 1 s. Some fluorometric experiments were done as a titration of terbium-Calb I-II complexes (1 to 1 molar ratio of terbium and Calb I-II) with calcium, holmium, and lanthanum. For each experiment, the concentration of Calb I-II or terbium-Calb I-II complexes was between 2.0 and 3.8 μM. This solution (2.0-2.5 ml) was titrated by the addition of 1.0-5.0-pl aliquots of lanthanides (669.6 pmol) to 2000 x g for 15 min (two times) and 3000 x g for 10 min (one time). Following each spin, the supernatant was removed and transferred to a new tube. The spins were at 4 °C, and the final preparations were free of suspended particles. Protein concentrations were determined by ultraviolet absorbance (280 nm) and the method of Smith et al. (31). The extinction coefficient (ε_{280} for Calb I-II at 280 nm) was determined for solvent (31). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was done with a Phast gel system using gradient gels (8-25%) and molecular weight markers from Pharmacia LKB Biotechnology Inc. Proteins were detected by Coomasie Blue staining. Approximately 1.0 mg of protein was applied to each gel lane.

Amino Acid Sequencing and Mass Spectrometry—Edman degradation of Calb I-II was done on an Applied Biosystems 470A gas phase sequencer. The protein was applied with polybrene and phenylthiohydantoin-derivatives were identified by an on-line HPLC with a small-bore C_{18} reverse-phase column (Siirner-S, Applied Biosystems). The chromatography was developed with 66 mM sodium acetate, pH 3.9, 1 mM trimethylamine in tetrahydrofuran and acetonitrile.

Mass measurements of Calb I-II were taken with a reflector-type, time-of-flight microprobe mass spectrometer (LAMMA 1000). This instrument is equipped with a nitrogen laser (wavelength = 337 nm), whose pulse of light is focused by a microscope quartz lens system onto the sample at 45° (32). The protein sample was dissolved in 0.1% aqueous acetic acid, and a concentration of 280 nm light was decreased by a 500-nm emission cutoff filter (Corion LL-500). Light transmission was measured at 322 nanometers (200 pl of Calb I-II (12.0 μM) in a microcuvette (cell path = 0.25 cm) were used for direct excitation experiments. Terbium was added in 1.0-2.0-μl aliquots, and the total amount added did not exceed 20 μl to a 400-μl solution. Corrections were made for sample dilution as necessary. Scattered light was decreased by a 500-μm emission cutoff filter (Corion LL-500). Light transmission was 8% at 488 nm and 88-90% at 530-560 nm for this filter.

Terbium binding to Calb I-II was determined by measurements of intrinsic protein fluorescence. First, solutions of Calb I-II (2.0 μM, 2.5 μl) were titrated with EDTA (final concentration 220 μM) to remove residual bound calcium. Then, the Calb I-II/EDTA solution was titrated with calcium and measurements taken of the intrinsic protein fluorescence. The excitation wavelength was 280 nanometers, and fluorescence emission was measured at 322 nanometers. EDTA and calcium were added in amounts not exceeding 30 pl in 2.5 ml of Calb I-II solution. Final calcium concentrations were greater than 1.0 μM. Free calcium concentrations were calculated by the method of Storer and Cornish-Bowden (33). Hill plots were done as described in the methods, and emission wavelengths up to 0.05 pl of EDTA occurred in this value due to the small volumes of added solutions, and

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2 The abbreviations used are: PIPES, pipazine-N,N,N,N-bis(2-ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis.
Characterization of a Calcium-binding Site on Calbindin-D_{28K}

TABLE I

| Cycle | Amino acid | Yield | Predicted amino acid |
|-------|------------|-------|---------------------|
| 1     | M          | 1522.7| M                   |
| 2     | R          | 205.1 | R                   |
| 3     | G          | 952.4 | G                   |
| 4     | S          | 473.4 | S                   |
| 5     | H          | 297.5 | H                   |
| 6     | H          | 243.6 | H                   |
| 7     | H          | 298.1 | H                   |
| 8     | H          | 313.9 | H                   |
| 9     | H          | 324.5 | H                   |
| 10    | H          | 343.1 | H                   |
| 11    | G          | 443.5 | G                   |
| 12    | S          | 183.4 | S                   |
| 13    | M          | 683.5 | M                   |
| 14    | A          | 533.2 | A                   |
| 15    | E          | 341.3 | E                   |

*One-letter code.

RESULTS

Calb I-II was produced with the expression vector, prCaBPI+II, 6xHis, in E. coli. The resultant protein was soluble and had a yield of approximately 20 mg of Calb I-II/liter of culture. Nonetheless, denaturation improved purification efficiency and protein purity. Extraction and nickel chelate resin purification of the protein were done under denaturing conditions. Removal of the denaturing agent by dialysis resulted in purification of the protein were done under denaturing conditions. Removal of the denaturing agent by dialysis resulted in the ready renaturation of Calb I-II. Analysis of the final product by SDS-PAGE showed a homogenous preparation (data not shown). The NH\textsubscript{2}-terminal amino acid sequence of Calb I-II is given in Table I. The NH\textsubscript{2}-terminal sequence, determined by Edman degradation, was identical to the amino acid sequence encoded by the expression vector, prCaBPI+II, 6xHis. A single NH\textsubscript{2} terminus was found with no indication of additional NH\textsubscript{2} termini. The matrix-assisted laser desorption mass spectrum of Calb I-II is shown in Fig. 1. This spectrum was dominated by the molecular ion signal, M\textsuperscript{+}. Signals corresponding to the doubly charged molecular ion M\textsuperscript{2+} and the dimer 2M\textsuperscript{+} were also present. The presence of dimers and doubly charged ions was consistent with other matrix-assisted laser desorption mass spectra (35). The spectrum was characterized by a very low noise background and showed no signs of protein fragmentation. Average molecular mass of Calb I-II calculated from the geometrical centroid of the molecular ion peak was 11,986 Da. This compared well with the average mass calculated from the cDNA-derived protein sequence of 11,994 Da (mass measurement accuracy better than 0.1%). The mass measurement indicated the presence of a hexa-histidine containing short fusion peptide and correct expression of the full-length protein. Additional confirmation was provided by plasma desorption and electrospray mass spectrometric analysis of Calb I-II (data not shown). A complete mapping of the protein by a combination of proteolytic cleavages, chromatographic separation, and mass spectrometric analysis were presented in a separate publication. The calcium and lanthanide binding properties of Calb I-II were determined in several binding experiments. Terbium binding to the recombinant protein was measured by energy transfer from tryptophan. The results of these experiments are shown in Fig. 2. Calb I-II was titrated with terbium, and the fluorescence emission intensity was monitored at 545 nm. Fluorescence was seen immediately following the addition of terbium, and there was an increase in fluorescence until a molar ratio of approximately 1:1, terbium to Calb I-II. The further addition of terbium did not result in an additional increase in fluorescence emission intensity. Experiments, using an excitation wavelength of 280, 290, or 295 nanometers, yielded similar results. These results indicated the presence of a terbium-binding site in close proximity to the single tryptophan found in Calb I-II. Moreover, the results suggest that Calb I-II was folded appropriately for terbium binding. Calculations of energy transfer distances between tryptophan and terbium found the terbium bound in Calb I-II was within 12 Å of tryptophan. The presence of a terbium-binding site located in close proximity to tryptophan, as determined by energy transfer experiments, was reported previously for the intact rat and chicken calbindin-D\textsubscript{28K} proteins (17). Terbium-Calb I-II complexes were titrated with the lanthanides, holmium, and lanthanum. The results of these titrations are shown in Fig. 3. Titrations of terbium-Calb I-II complexes with either of the two lanthanides yielded similar results; that is, the addition of either lanthanide caused a significant decrease in fluorescence resulting from energy transfer. Decreases in fluorescence were linear over a molar range from 0 to 1.5. The amount of fluorescence decrease was approximately equivalent with equal molar ratios of terbium to lanthanide. The mass-to-charge ratio is given on the x axis. Relative intensity is given on the y axis. The spectrum is an accumulation of 10 single laser shots.

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either lanthanum or holmium. Holmium, unlike lanthanum, can quench fluorescence from terbium, i.e., the presence of a lanthanide-binding site in close proximity with a terbium-binding site would result in quenching of terbium emission by titration with holmium. The absence of a significant difference between the lanthanum and holmium titration curves indicated both holmium and lanthanum competed directly with terbium located at a single site on Calb I-II. Furthermore, the absence of a second lanthanide-binding site on Calb I-II was indicated by a 50% displacement of terbium from terbium-Calb I-II (1:1 molar complex) with an equal molar amount of lanthanum or holmium.

Terbium-binding to Calb I-II was also measured by the direct excitation of terbium with 488-nm light and monitoring fluorescence emission at 545 nm (Fig. 4). Fluorescence emission intensity increased, with the titration of terbium, in a linear manner to a molar ratio of 1:1.3 for terbium to Calb I-II. Further addition of terbium resulted in a gradual increase in fluorescence for a wide range of terbium to Calb I-II molar ratios. The marginal increases in terbium fluorescence, at molar ratios greater than 1:1.3, resulted from very low affinity binding of terbium to the protein. Notably, the concentration of Calb I-II in these experiments was three to six times higher than that in the energy transfer experiments. This amount of protein was essential due to the low quantum yields of terbium fluorescence with direct excitation.

The results of energy transfer, lanthanide competition, and direct excitation of bound terbium experiments suggested the presence of a single high affinity calcium-binding site in Calb I-II. Terbium-Calb I-II complexes (1:1 molar ratio) were titrated with calcium. The results of these experiments are shown in Fig. 5. Fluorescence intensity of the complex decreased with the addition of calcium to the terbium-Calb I-II complexes. The decrease in fluorescence was linear with terbium: calcium molar ratios from 0 to 40. At higher molar ratios of calcium to terbium-Calb I complexes, there was a further, albeit smaller, reduction in terbium fluorescence. Calcium displaced terbium from its binding site on Calb I-II. These results suggested that the terbium binding site on Calb I-II was a calcium-binding site. The amount of calcium required to displace terbium (molar ratio 40 to 1) was similar to that found for the displacement of terbium from the high affinity-binding site on full-length calbindin-D_{28K} (17). The molar ratio of calcium: terbium required for displacement of terbium was consistent with the greater affinity of terbium for EF hand regions as compared with calcium. Terbium had a higher affinity than calcium for calcium-binding proteins (36).

Calcium binding to Calb I-II was measured in intrinsic protein fluorescence experiments. The addition of EDTA resulted in a large decrease in the intrinsic protein fluorescence of Calb I-II (data not shown). It suggested an interaction between the calcium-binding site on Calb I-II and tryptophan located in the NH_{2}-terminal helix of Calb I-II. After addition of EDTA for removal of residual bound calcium, Calb I-II was titrated with calcium. The results of the intrinsic protein fluorescence experiments are shown in Fig. 6. The titration of Calb I-II with calcium in the presence of EDTA resulted in a rapid linear increase in intrinsic protein fluorescence. The initial increase in fluorescence was followed by a sharp break, plateau, and then gradual decline in fluorescence emission. A Hill plot of the data (Fig. 7) indicates the presence of a single calcium-binding site on Calb I-II. This site had an affinity for calcium of approximately 10^{9} (K_{d} \sim 1 \times 10^{-9}) and a Hill coefficient of 1.

DISCUSSION

Calb I-II is a truncated form of calbindin-D_{28K} containing the first two EF hand domains from the NH_{2}-terminal portion of the protein and a hexa-histidine tail. It is encoded on an expression vector and can be produced in high yield by E. coli (20 mg/liter of culture). The NH_{2}-terminal sequence of purified Calb I-II, determined by Edman degradation, is in complete agreement with that predicted from the expression vector.
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Fig. 6. Intrinsic protein fluorescence measurements of calbindin-D<sub>28K</sub> domains I-II titrated with CaCl<sub>2</sub>. Calb I-II in 50 mM PIPES, 0.15 mM NaCl buffer, pH 6.70, is titrated with EDTA to a total EDTA concentration of 220 μM. Subsequently, Calb I-II is titrated with CaCl<sub>2</sub>. The free calcium concentration is given on the x axis. Free calcium is calcium not bound to EDTA. Fluorescence emission intensity measured at 322 nm is given on the y axis.

Fig. 7. Hill plot. Y/Y − 1 is calculated based upon the intrinsic protein fluorescence measurements of Calb I-II. The y axis is the log of free calcium concentration and equals the non-EDTA-bound calcium in the reaction mixture. The x axis represents the fraction of total binding sites occupied by a ligand.

The expressed protein has terbium-binding activities as demonstrated by direct and energy transfer excitation experiments of terbium fluorescence. Intrinsic protein fluorescence experiments of Calb I-II indicate calcium binding activity. Moreover, the terbium and calcium binding experiments indicate the presence of a single binding site on the protein. Calcium can displace terbium and the two metals appear to have the same binding site on Calb I-II. Several experimental results are consistent with the presence of a single metal-binding site on Calb I-II. These are: 1) terbium binding to Calb I-II, as measured by energy transfer, is saturated at a molar ratio of terbium to Calb I-II of 1.0. 2) Holmium and lanthanum titration of terbium-Calb I-II complexes (molar ratio, 1:1) yield an approximately 50% reduction in terbium fluorescence at a terbium to holmium or lanthanum molar ratio of 1 to 1. 3) Holmium titration did not result in quenching of terbium fluorescence as would be expected by the presence of a second lanthanide-binding site. 4) A breakpoint is found at a molar ratio of terbium to Calb I-II of approximately 1 to 1 in experiments measuring terbium-binding by the direct excitation of terbium. 5) The Hill coefficient for calcium binding to Calb I-II is 1.0. 6) The presence of a single calcium/terbium-binding site on Calb I-II is consistent with the EF hand loop amino acid sequence. EF hand domain 1 contains four oxygen-containing residues at liganding positions for calcium. Three to five oxygen-containing residues at these positions are associated with high affinity calcium binding to proteins. EF hand region II contains only two oxygen-containing residues in the respective positions.

A second important feature of Calb I-II is the very high affinity of its calcium-binding site (K<sub>d</sub> ~ 10<sup>-8</sup>). This affinity is higher than that reported for bovine calbindin-D<sub>28K</sub>, troponin C (37), and calmodulin (27) and similar to that of parvalbumin (38). These findings indicate the truncated form of calbindin-D<sub>28K</sub> contains a sufficient amount of sequence for the formation of stable high affinity calcium-binding EF hand domains. For other calcium-binding proteins, high affinity calcium binding is absolutely dependent on the interaction between two EF hands. Examples of stable calcium-binding pairs of EF hands include proteolytic fragments of calmodulin (27) and troponin C (38). It can be assumed that in Calb I-II there is close contact between the two EF hands. Notably, in this case, a calcium-binding EF region is paired with a non-calcium-binding EF hand.

Properties of the calcium/terbium-binding site on Calb I-II appear identical to those of the very high affinity calcium/terbium-binding site on calbindin-D<sub>28K</sub>. Both sites: 1) have a high affinity for terbium and maximal fluorescence intensity at a terbium-to-protein molar ratio of 1 to 1; 2) are located in close proximity to a tryptophan residue as indicated by energy transfer from tryptophan to terbium; 3) have a K<sub>d</sub> for calcium of approximately 10<sup>-8</sup>; 4) have relative affinities for calcium and terbium of approximately 30 to 1; and 5) show changes in intrinsic protein fluorescence with calcium binding. The consistency of these findings indicate that Calb I-II contains the very high affinity calcium-binding site found on calbindin-D<sub>28K</sub> (17). Notably, the calcium affinity for Calb I-II (K<sub>d</sub> ~ 10<sup>-8</sup>) is slightly less than the very high affinity calcium-binding site on calbindin-D<sub>28K</sub> (K<sub>d</sub> ~ 10<sup>-6</sup>). Additional studies are needed for further characterization of this possible difference.

The binding data suggest that EF hand domains 1 and 2 in Calb I-II are similar to EF hand domains 1 and 2 in the wild type protein. Calb I-II EF hand domains can fold independent from the remainder of calbindin-D<sub>28K</sub>, and do not rely on contacts with other domains of the protein for a high calcium binding affinity. Independently folded EF hand pairs are also found in calmodulin and troponin. These proteins have a dumbbell-like shape with a pair of EF hands at each end connected by a long a-helix (20, 21). If the EF hand domains 1 and 2 of calbindin-D<sub>28K</sub> fold independently, a remaining question is how do the residual domains fold. EF hand domains 3-6 could fold into two additional independent pairs of EF hand domains. The protein would have an extended structure with three independent pairs of EF hand domains. Alternatively, EF hand domains 3-6 could form an independent globular domain. An alternative model for calbindin-D<sub>28K</sub> can be based on parvalbumin. Parvalbumin is a globular protein with two functional and one non-calcium-binding EF hand domains packed tightly together. Protein synthesis of the COOH terminus of parvalbumin, to even a limited extent, results in a large loss of calcium-binding activity (26). In this model, calbindin-D<sub>28K</sub> could form two to three EF hand-containing domains. Some evidence comes from experiments on mutations deleting entire EF hand domains. The deletion of domain 2 results in a protein with...
Calcium binding activity (39). Even the combined mutation (2 and 6) that only contains EF hand domains 1, 3, 4, and 5 is found to bind calcium (39). These results support the extended structure model. In the double mutation, the EF hand pair III-IV remains unchanged and could be responsible for the observed calcium binding activity. Alternatively, in the parvalbumin model, all possible pairing of three EF hands are disrupted with the 2 and 6 EF hand deletion mutation, with one exception. The pairing of 3, 4, and 5 could remain intact. However, the complimentary pairing, necessary in this case, of 1, 2, and 6 seems unlikely. Additional studies on the structure of calbindin-D_{28K} are needed to conclusively differentiate between the two models. Several structural components of EF hand domains have a role in the determination of its calcium affinity (23). These include the number and location of side chain oxygen-containing amino acids in the loop, the presence of NH₂-terminal and COOH-terminal helices and helix-helix interactions. A comparison of Calb I-II with the proteolytic fragments of troponin C and calmodulin indicates the presence of similar EF hand loop amino acid sequences with similar residues in highly conserved positions. Yet Calb I-II has a substantially higher affinity for calcium than the proteolytic fragments of calmodulin and troponin C (27, 38). These observations suggest the presence of unique calcium affinity-determining structural features in Calb I-II. Calb I-II may be a good model system for acquiring additional information on the structural elements involved in calcium affinity.

The hexa-histidine sequence of Calb I-II facilitates purification of the protein. Hexa-histidine has a high affinity for nickel and its presence provides for the rapid specific isolation of expressed proteins by nickel chelate chromatography. A one-step isolation procedure with the nickel-chelate column yields large amounts (milligrams) of highly pure Calb I-II (>98%). The high purity and yields of protein are essential for the experiments reported herein. We found lyophilized Calb I-II to be soluble in chaotrope agents (guanidine HCl) but not totally soluble in distilled water. A small amount of protein present as a fine suspension was readily removed by centrifugation at low speed. Analysis of Calb I-II by SDS-PAGE preparations before and after centrifugation yielded a single band. Thus, the water-insoluble material appears to be a denatured form of Calb I-II produced upon lyophilization. Lyophilization can be avoided by keeping the protein under denaturing conditions and dialyzing against water just prior to use.

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