Determination of Calcium-Binding Sites in Rat Brain Calbindin D_{28K} by Electrospray Ionization Mass Spectrometry†

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ABSTRACT: Calbindin D_{28K}, a member of the troponin-C superfamily of calcium-binding proteins, contains six putative EF-hand domains. Calcium-binding studies of the protein by different groups of investigators have yielded discordant results with respect to the stoichiometry of calcium-binding. It has been suggested that the protein binds anywhere from 3−6 mol of calcium/mol of protein. We used negative ion electrospray ionization mass spectrometry in order to definitively determine the exact calcium-binding stoichiometry of calbindin D_{28K} and two mutant forms of the protein, one lacking EF-hand 2 (Δ2) and the other lacking EF-hands 2 and 6 (Δ2,6). The full-length protein bound 4 mol of calcium/mol of protein, while both of the deletion mutants bound 3 mol of calcium. Since terbium has been used extensively as a probe for the determination of the calcium-binding stoichiometries of calcium-binding proteins, we also examined the binding of terbium to the three proteins under the same conditions. Full-length calbindin D_{28K} bound 4 mol of terbium/mol of protein, while calbindin Δ2 and Δ2,6 each bound 3 mol. These results clearly show that calbindin D_{28K} binds 4 mol of calcium/mol of protein and that terbium-binding stoichiometry is similar to that of calcium.

Calbindins are a group of vitamin D-dependent calcium-binding proteins that belong to the troponin-C family of proteins (Barker et al., 1977). These proteins are found in several calcium-transporting tissues such as the intestine, kidney, and placenta as well as other tissues such as pancreas, bone, parathyroid gland, and the brain (Wasserman & Corradino, 1971; Bredderman & Wasserman, 1974; Feher, 1983; Gross & Kumar, 1990), although the expression of these proteins is not vitamin D-dependent in the brain of the adult animal. Calbindins bind calcium in "EF-hand" domains which are composed of a helix-calcium-binding loop-helix motif (Babu et al., 1985; Kretsinger, 1976). In cases such as troponin-C, myosin light chain, and calmodulin, the binding of calcium results in a conformational change within the protein which leads to the transmission of a biologically important signal, such as the alteration of enzyme or muscle activity (Kretsinger, 1976). Calbindin D_{28K} and calbindin D_{9K} also undergo a conformational change on binding calcium, although the exact consequences of such a change have not been precisely elucidated. Experimental evidence suggests that the calbindins D may alter calcium transport by influencing plasma membrane calcium pump activity (Walters, 1989; Reisner et al., 1992).

Recombinant rat brain calbindin D_{28K} has a molecular mass of 29 866 Da as measured by mass spectrometry. Analysis of the primary structure of the protein suggests that it contains six putative EF-hand calcium-binding site domains. Several attempts have been made to determine the correct calcium-binding stoichiometry of calbindin D_{28K} using many different techniques. Traditional methods used to determine metal-binding properties of proteins are prone to error caused by sample preparation or accurate molar ratio matches and often lead to conflicting literature reports. Equilibrium dialysis studies suggest that the protein contains three or four calcium-binding sites (Bredderman & Wasserman, 1974; Cheung et al., 1993). Terbium fluorescent studies of chick (Gross et al., 1987) and recombinant rat brain calbindin D_{28K} (Veenstra et al., 1995) also showed between three and four terbium binding sites within the protein. Furthermore, terbium fluorescent studies of deletion mutants of rat brain calbindin D_{28K} suggest that EF-hand domains 2 and 6 are not required for metal-binding to the protein. Calcium titration of the fluorescent calcium chelator, Quin-2, in the presence of calbindin D_{28K} suggested that the protein binds between 5 and 6 mol of calcium/mol of protein (Leathers et al., 1990). Recently, synthetic peptides representing the six EF-hands of calbindin D_{28K} were prepared and the response of the isolated peptides to the addition of calcium was assessed by proton nuclear magnetic resonance spectroscopy and circular dichroism spectroscopy (Akerfeldt et al., 1996). This approach suggested that five of the six EF-hand domains, namely EF-hand domains 1, 3, 4, 5, and 6, bound calcium.

Electrospray ionization mass spectrometry (ESI-MS[1]) has been used extensively in the analysis of protein solutions (Smith et al., 1991). More recently, it has proved useful in

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[1] Abbreviations used: ESI-MS, electrospray ionization mass spectrometry; *m*/*z*, mass:charge ratio; calbindin Δ2, mutant calbindin D_{28K} protein lacking EF-hand domain 2; calbindin Δ2,6, mutant calbindin D_{28k} protein lacking EF-hand domains 2 and 6; Ca^{2+}, calcium ion; Tb^{3+}, terbium ion; EDTA, ethylenediaminetetraacetic acid; CsI, cesium iodide.

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Domain 1 [1]MAESHLQSSLITASQ **FFEIWLHFDADGSGYLEGKELQNLIQEL LQARKKA**[50]
Domain 2 [51]**GLELSPE MKTFVDQYGQRDDGKIGIVELAHVLPTE** ENFLLLF[92]
Domain 3 [93]RCQQLKSCEE **FMKTWRKYDTDHSGFIETEELKNFLKDL** LEKANKT[137]
Domain 4 [138]VDDTKLAEY **TDLMLKLFDSNNDGKLELTEMARLLPVQ** ENFLLKF[181]
Domain 5 [182]QGIKMCGKE **FNKAFELYDQDGNGYIDENELDALLKDL CEKNKQE**[225]
Domain 6 [226]**LDINNI STYKKNIMALSDGGKLYRTDLALILSAG** DN[261]

FIGURE 1: Amino acid sequence of full-length calbindin D_{28K}. The putative EF-hand domains are underlined. The amino acid residues removed in the deletion mutagenesis of full-length calbindin D_{28K} to create calbindin Δ2 and calbindin Δ2,6 are in bold.

the analysis of protein−metal ion interactions, and specific examples include Zn^{2+}/Cd^{2+}−metallothioneins (Yu et al., 1993), Fe^{3+}−rubredoxin (Petillot et al., 1993), Zn^{2+}−zinc finger protein (Surovoy et al., 1992), and Ca^{2+}/Mg^{2+}−calmodulin (Hu & Loo, 1995; Lafitte et al., 1995) complexes. ESI-MS is an ideal technique to examine noncovalent complexes involving biopolymers since the soft ionization process of transferring ions from solution to the gas phase results in the production of ions less than 1 eV above their ground state energy (Kebarle & Tang, 1993). Furthermore, the mass accuracy and resolution of such measurements using a sector mass spectrometer provides umambiguous determination of metal-binding stoichiometries with the protein (Loo et al., 1995). In order to definitively determine the calcium-binding stoichiometry of calbindin D_{28K}, we have developed a novel method using low-flow, negative ion ESI-MS. We use this approach to examine the calcium- and terbium-binding stoichiometries of native rat brain calbindin D_{28K}, as well as two deletion mutants lacking EF-hand domain 2 (Δ2) or domains 2 and 6 (Δ2,6).

MATERIALS AND METHODS

General. Ultraviolet spectra were obtained on a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). Oligonucleotides were synthesized using phophoramidate chemistry (Matteucci & Caruthers, 1991) on an Applied Biosystems DNA Synthesizer (Applied Biosystems, Foster City, CA). DNA sequencing was carried out using dideoxy sequencing methods (Sanger et al., 1974) on an Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid composition analysis and protein sequencing were carried out using published methods and gas phase sequencing techniques (Bidlingermeyer et al., 1984; Hewick et al., 1981). High-performance liquid chromatography was performed using a Beckman Systems Gold HPLC (Beckman Instruments, Fullerton, CA). Sodium dodecyl sulfate−polyacrylamide gel electrophoreses (SDS−PAGE) were carried out using precast minigels and a Pharmacia Phast-Gel Electrophoresis System (Pharmacia Instruments, Piscataway, New Jersey).

Expression and Purification of Native and Mutant Calbindins. Deletion mutagenesis, expression, and purification of rat brain calbindin D_{28K} proteins were as described previously (Kumar et al., 1994; Veenstra et al., 1995). Briefly, DNA sequences of the calbindin D_{28K} full-length, Δ2, and Δ2,6 plasmids were amplified by polymerase chain

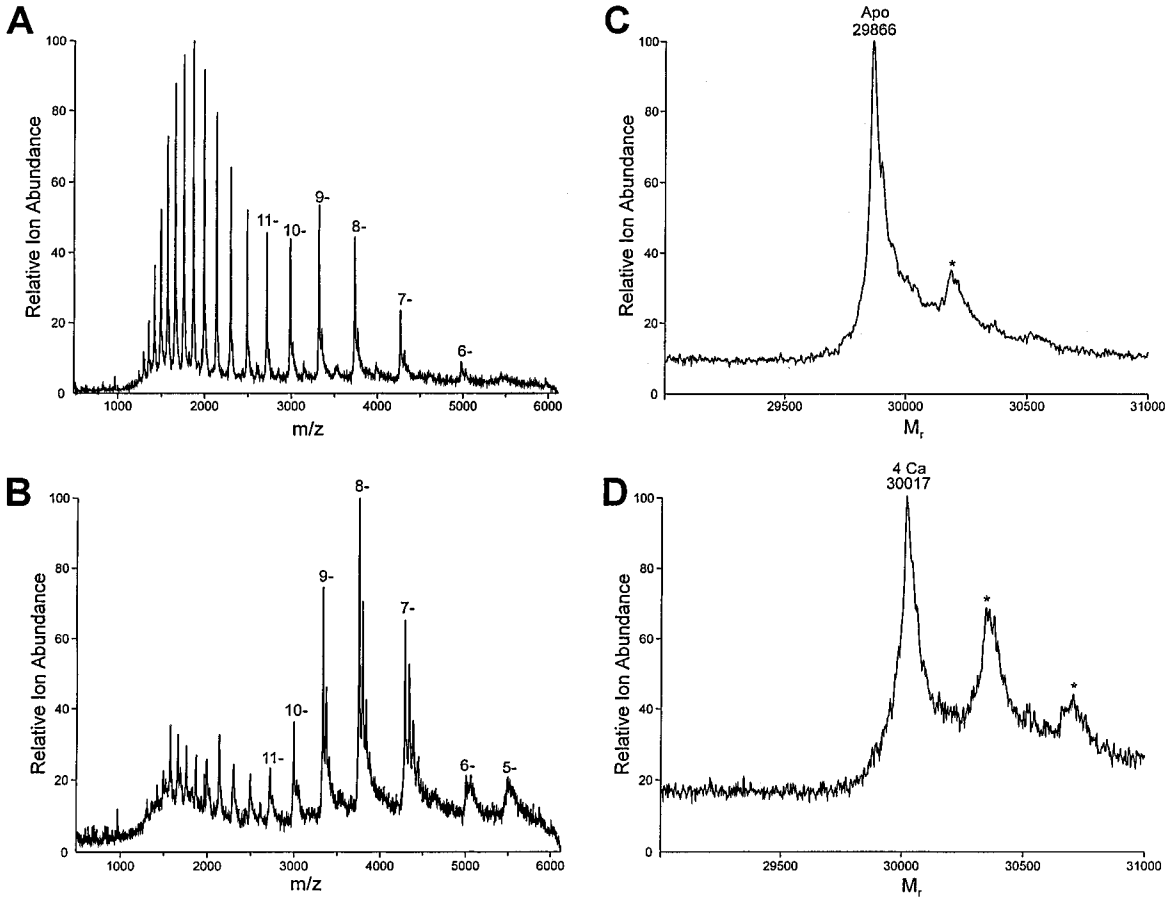

FIGURE 2: Multiply charged negative-ion ESI mass spectra of calbindin D_{28K}: (A) without added calcium acetate and (B) with a final concentration of 1 mM calcium acetate added to the sample. Deconvoluted spectra of calbindin D_{28K}: (C) without added calcium acetate and (D) in the presence of 1 mM calcium acetate. The protein concentration was 60 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0. The charge states used to calculate the deconvoluted spectra of the protein are labeled. The peaks marked by asterisks (*) correspond to Na/EDTA-adducts bound nonspecifically to the protein.

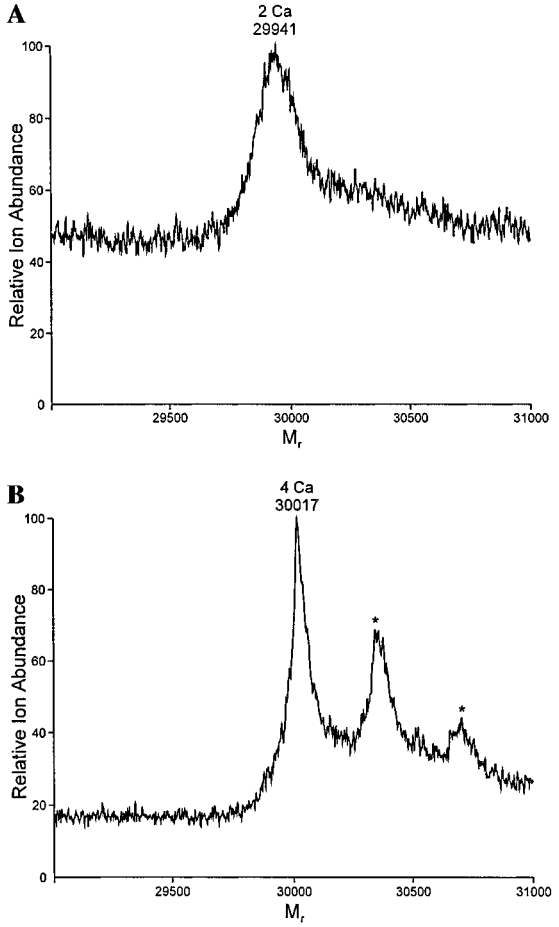

FIGURE 3: Deconvoluted spectra of (A) lower (*m*/*z* 1200−2600) and (B) higher (*m*/*z* 2600−5500) mass:charge species in multiply charged spectrum of calbindin D_{28K} (60 μM) in the presence of 1 mM calcium acetate. The peaks marked by asterisks (*) correspond to Na/EDTA-adducts bound nonspecifically to the protein.

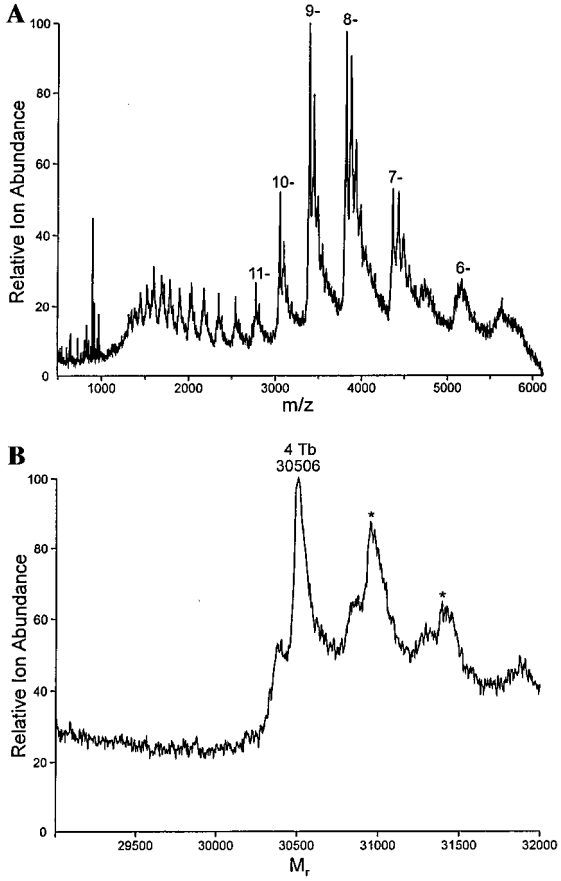

FIGURE 4: (A) Multiply charged negative-ion ESI mass spectrum of calbindin D_{28K} in the presence of 1 mM terbium acetate. (B) Deconvoluted spectrum of calbindin D_{28K} in the presence of 1 mM terbium acetate. The protein concentration was 60 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0. The peaks marked by asterisks (*) correspond to Tb^{3+}/EDTA-adducts bound nonspecifically to the protein.

reaction techniques (Gross et al., 1987; Innis et al., 1990) with Taq polymerase and polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCl, 1.25 mM $MgCl_2$, pH 8.3). The products were subcloned into the pCR vector using the Invitrogen TA Cloning Kit (Invitrogen). Sequences encoding the calbindin D_{28K} proteins were removed from the pCR vector using *Nde*I and *Bam*HI restriction endonucleases and were ligated into gel purified pET3a plasmid vector that had been cut with the same enzymes (Studier et al., 1990). The chimeric plasmids were transformed into competent *Escherchia coli* BL21(DE3) pLysS cells.

Expression and purification of the full-length and mutant proteins were performed as previously described (Kumar et al., 1994). Briefly, minicultures of BL21(DE3) pLysS cells containing the chimeric plasmids were used to inoculate six 1 L cultures of M9ZB medium (1 g of N-Z amine A (ICN Biochemicals, Cleveland, OH), 5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4 in 1 L of H_2O, pH 7.0, sterilized by autoclave). Prior to use, the M9ZB medium was brought to 0.02 M glucose, 1 mM $MgSO_4$, 100 μg/mL ampicillin, and 25 μg/mL chloramphenicol. The cultures were grown at 37 °C with vigorous shaking until an OD_{600} of 0.55 was attained, at which point the expression of protein was initiated by adding isopropyl-thio-β-D-galactoside to a final concentration of 0.4 mM. Incubation was continued for another 4 h. Induction of protein expression was assayed by SDS−PAGE. The bacterial pellets were isolated by centrifugation of the cultures at 5000*g* for 10 min. The pellet was resuspended in 50 mL of TE buffer (50 mM Tris, 2 mM EDTA, pH 8.0). Cells were lysed with a Polytron homogenizer (Beckman Instruments, Fullerton, CA) for 1 min and then passed through a 16 gauge needle. After a second homogenization with the Polytron, the cells were passed through a 21 gauge needle. β-Mercaptoethanol was added to a final concentration of 10 mM followed by the addition of Triton X-100 to a concentration of 2% (v/v). This suspension was sonicated three times at an amplitude setting of 20 μ for 1 min using a Soniprep 150 sonicator (MSE Scientific Instruments, Sussex, England). The homogenate was centrifuged at 4000*g* for 10 min, and the supernatant was saved. The pellet was resuspended in 20 mL of TE buffer containing 10 mM β-mercaptoethanol and 2.0% SDS. The supernatant and pellet were pooled and dialyzed extensively against distilled water and lyophilized.

The lyophilized proteins were reconstituted in a buffer of 10 mM Tris-base, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 7.4 (elution buffer), and were applied to a 2.5 × 25-cm Whatman DE-52, diethylaminoethyl (DEAE)-cellulose anion-exchange column. The columns were eluted with 100 mL of elution buffer without salt, followed by a linear gradient from 0 to 0.5 M NaCl in elution buffer. A gradient PhastGel was run on all protein-containing fractions, and those of the appropriate molecular weight were pooled, concentrated, and exchanged into water. Trifluoroacetic acid

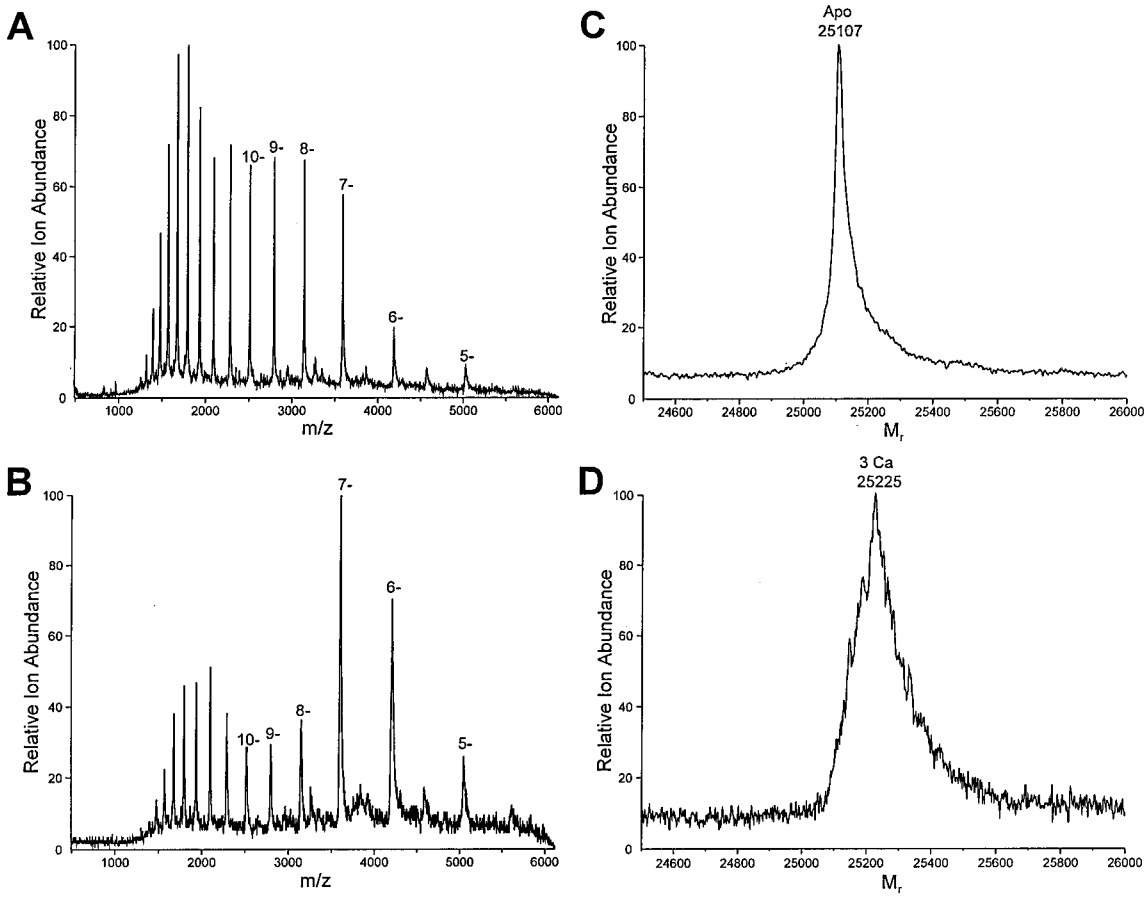

FIGURE 5: (A) Multiply charge negative-ion ESI mass spectra of apocalbindin Δ2 and (B) with a final concentration of 1 mM calcium acetate added to the sample. (C) Deconvoluted spectra of apocalbindin Δ2 and (D) in the presence of 1 mM calcium acetate. The protein concentration was 77 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0.

(TFA) was added to the proteins to a final concentration of 0.1%, and the proteins were applied to a 10 × 250-mm Aquapore C-18 column. Proteins were eluted with a gradient of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient was developed as follows: solvent B was increased in a linear gradient from 20 to 55% over 45 min, then held at 55% B for 20 min, followed by a linear gradient to 100% B over 30 min. The flow rate was 2.0 mL/min, and the eluant was monitored by measuring absorbance at 280 nm. Full-length calbindin D_{28K}, as well as the Δ2 and Δ2,6 deletion mutants, eluted at 55% acetonitrile. Gradient denaturing PhastGels were run on absorbance containing fractions, and those of the appropriate molecular weight were pooled and exchanged into 4 mM NH_4HCO_3, 10 μM EDTA, 15% CH_3OH, pH 8.0. For acquisition of ESI-MS data, protein samples were typically concentrated to a concentration of between 60 and 90 μM and titrated with the highest purity calcium acetate (Sigma, St. Louis, MO) or terbium acetate (Alfa Aesar, Ward Hill, MA). Protein concentration was determined by UV absorbance for full-length calbindin D_{28K} ($E^{1\%}_{280nm}$ = 7.93) (Gross et al., 1987), calbindin Δ2 ($E^{1\%}_{280nm}$ = 8.17), and calbindin Δ2,6 ($E^{1\%}_{280nm}$ = 8.78) (Veenstra et al., 1995).

Electrospray Ionization Mass Spectrometry Measurements. Electrospray ionization mass spectrometry (ESI-MS) measurements were performed on a Finnigan MAT 900 mass spectrometer (Bremen, Germany), a double-focusing instrument of EB geometry. ESI measurements were done in the negative mode using a Finnigan MAT electrospray interface. Protein solutions were introduced into the ESI source via 50 μM i.d. fused silica, using a 50 μL syringe, and a Harvard Apparatus Model 22 syringe pump (South Natick, MA). The flow rate of sample introduction was 0.2 μL/min. Sulfur hexafluoride introduced through the auxiliary gas port at 2 L/min was used to prevent formation of corona discharge in the ESI source. An instrument resolution of approximately 2000 was used for the analyses. The instrument was scanned from mass:charge (*m*/*z*) 600 to *m*/*z* 6000 at a rate of 30 s/mass decade. Calibrations of the *m*/*z* axis were done in positive ion mode with liquid secondary ion mass spectrometry using clusters of cesium iodide (CsI) as the mass reference. The calibration was then briefly rechecked in negative ion electrospray using a CsI cluster at *m*/*z* 3504.43. The position and time-resolved ion counter (PATRIC) array detector, one of two detectors supplied in the standard configuration of the instrument, was used for ion detection. Multiple scans were recorded and summed by the instrument data system (Finnigan MAT ICIS software, version 8.01HB). Multiply charged spectra were deconvoluted into the M_r scale using algorithms supplied with the instrument data system.

RESULTS

The amino acid sequence of full-length calbindin D_{28K} is shown in Figure 1. The residues removed in the formation of calbindin Δ2 and calbindin Δ2,6 are also shown. In the preparation of calbindin Δ2, deletion of the second EF-hand domain brings EF-hand domains 1 and 3 in close proximity, separated by a short linker segment. In the preparation of calbindin Δ2,6, EF-hand domains 2 and 6 are removed from the full-length protein. The sequential relationship between

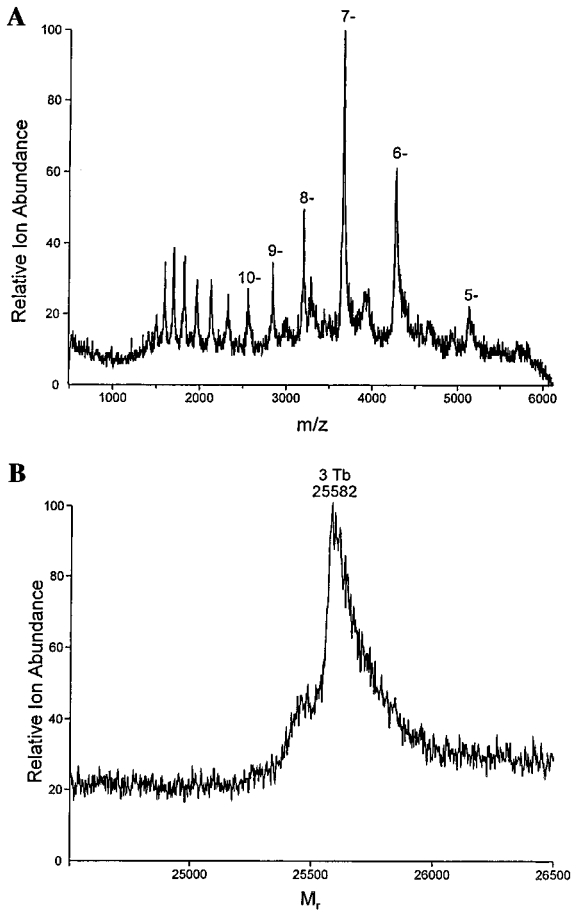

FIGURE 6: (A) Multiply charged negative-ion ESI mass spectrum of calbindin Δ2 in the presence of 1 mM terbium acetate. (B) Deconvoluted spectrum of calbindin Δ2 in the presence of 1 mM terbium acetate. The protein concentration was 77 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0.

EF-hand domains 3, 4, and 5 are not affected in the formation of either mutant protein.

The multiply charged negative ion ESI-MS spectrum of calbindin D_{28K} without any calcium acetate added is shown in Figure 2A. To obtain a spectrum of the protein without any bound calcium ions it was necessary to maintain the protein in a solution containing a low concentration of EDTA ($\approx$10 μM). Two overlapping envelopes of multiply charged ions are observed in the ESI mass spectrum of the protein. When a 10-fold excess of Ca^{2+} is added to the protein, a shift in the bimodal distribution occurs, such that the higher *m*/*z* envelope becomes the dominant species (Figure 2B). The deconvoluted spectra of the high *m*/*z* charge states (*m*/*z* 2600−5500) of the apoprotein gives a single peak at 29 866 Da (Figure 2C), which is in good agreement with the expected molecular mass of the full-length protein (Kumar et al., 1994). Deconvolution of the same region of the multiply charged spectrum of the protein containing a 10-fold excess of Ca^{2+} (Figure 2D) shows a peak at 30 017 Da. The mass difference of 151 daltons is equal to the addition of 4.0 ions of Ca^{2+} to the protein. This assumes the addition of a single Ca^{2+} ion results in a mass increase of 38 Da, which corresponds to the incorporation of the Ca^{2+} ion and the displacement of two protons. The additional peaks marked by asterisks at 30 353 and 30 693 Da arise from disodium EDTA adduction to the protein. The formation of the disodium/EDTA complex creates a neutral species which likely binds to the protein through a hydrophobic interaction. Experiments in our laboratory have shown that calbindin D_{28K} readily interacts with 2-*p*-toluidinonaphthalene-6-sulfonate, a probe for hydrophobic domains within proteins (McClure & Edelman, 1966), and this interaction is enhanced when calcium is added to the protein (unpublished results).

In an attempt to show that the binding of calcium to the protein was specific, the above experiment was repeating using calmodulin as a positive control and β-lactoglobulin as a negative control. Addition of a 16-fold excess of calcium acetate to calmodulin showed that the protein bound four Ca^{2+} ions, in agreement with a previous study (Hu & Loo, 1995). When a 16-fold excess of calcium acetate was added to β-lactoglobulin, there was no evidence of any Ca^{2+} ions bound to the protein, suggesting under our experimental conditions, that any metal ion binding to the protein was specific.

In the multiply charged spectrum of calbindin D_{28K} without any added calcium (Figure 2A), two overlapping envelopes of ion series are observed. Separate deconvolution of these envelopes shows that both the low *m*/*z* envelope (*m*/*z* 1200−2600) and the high *m*/*z* envelope (*m*/*z* 2600−5500) represent the apo-form of the protein. As mentioned above, when a 10-fold excess of Ca^{2+} is added to the protein, the high *m*/*z* envelope becomes the predominant species in the multiply charged spectrum (Figure 2B). Figure 3, panels A and B show the deconvoluted spectra of Ca^{2+}-loaded calbindin D_{28K}. Deconvolution of the low *m*/*z* envelope (*m*/*z* 1200−2600) (Figure 3A) gives a single peak corresponding to the protein bound to two Ca^{2+} ions. Deconvolution of the higher *m*/*z* envelope (*m*/*z* 2600 to 5500) (Figure 3B) gives a peak showing calbindin D_{28K} with four Ca^{2+} ions bound.

The titration of full-length calbindin D_{28K} was repeated using terbium acetate in place of calcium acetate. Terbium has been used extensively as a probe for calcium-binding sites in fluorescence studies. Terbium's larger mass, compared to that of calcium, is particularly advantageous in ESI-MS measurements of calcium-binding sites within proteins. Similar to the calcium experiments, ESI-MS spectra resulting from the titration of the protein with terbium acetate displayed a bimodal distribution of the charge states. The abundance of the higher *m*/*z* envelope was enhanced and eventually dominated the mass spectrum as the terbium concentration was increased to 1 mM (Figure 4A). Deconvolution of these envelopes shows that the low *m*/*z* envelope (*m*/*z* 1200−2600) consists of calbindin D_{28K} with fewer bound Tb^{3+} ions compared to the high *m*/*z* envelope. These data were consistent with those observed in the calcium acetate experiments. Deconvolution of the high *m*/*z* envelope of the multiply charged spectrum of the protein containing an excess of Tb^{3+} (Figure 4B) shows a peak at 30 506 Da. This mass increase of 640 daltons is equal to the addition of 4.1 ions of Tb^{3+} to the protein (the addition of a single Tb^{3+} ion to the protein results in a mass increase of 156 Da, which arises from the incorporation of the Tb^{3+} ion and the displacement of three protons. Additional peaks were observed at 30 952 and 31 392 Da, which correspond to Tb^{3+}/EDTA-bound adducts of the protein. We believe the interaction of the protein with the Tb^{3+}/EDTA complex is hydrophobic in nature and analogous to that observed with the disodium/EDTA complex discussed above.

The multiply charged ESI-MS spectrum of apocalbindin Δ2 is shown in Figure 5A. Again, two overlapping

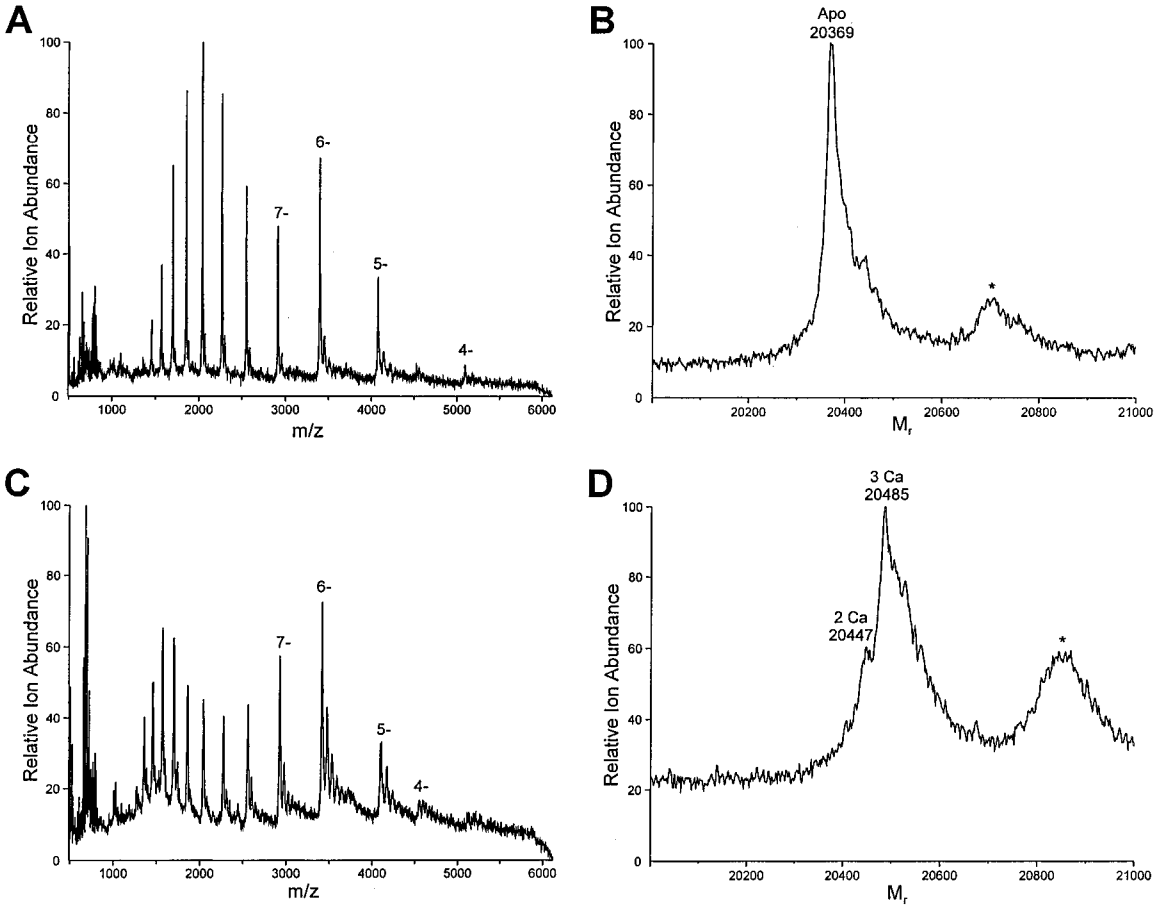

FIGURE 7: (A) Multiply charged negative-ion ESI mass spectra of apocalbindin Δ2,6 and (B) with a final concentration of 1 mM calcium acetate added to the sample. (C) Deconvoluted spectra of apocalbindin Δ2,6 and (D) in the presence of 1 mM calcium acetate. The protein concentration was 90 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0. The peaks marked by asterisks (*) correspond to Na/EDTA-adducts bound nonspecifically to the protein.

envelopes of ion series are observed, with a shift in the bimodal distribution to the higher *m*/*z* envelope when an excess of Ca^{2+} is added to the protein (Figure 5B). Deconvolution of the low *m*/*z* envelope (*m*/*z* 1200−2600) in the presence of excess Ca^{2+} showed that this region represented species of calbindin Δ2 with 0, 1, and 2 bound Ca^{2+} ions. The deconvoluted spectra of the high *m*/*z* charge states (*m*/*z* 2600−5500) of the apoprotein gives a single peak at 25 107 Da (Figure 5C). Deconvolution of the multiply charged spectrum of the protein containing an excess of Ca^{2+} (Figure 5D) shows a peak at 25 225 Da. The mass difference of 117 Da corresponds to the addition of 3.1 ions of Ca^{2+} to the protein. The experiment was repeated using terbium acetate and as shown in Figure 6A a similar pattern in the bimodal distribution of the multiply charged envelopes occurs when terbium is added to the protein. Deconvolution of the multiply charged spectrum of calbindin Δ2 containing an excess of Tb^{3+} gave a peak at 25 582 Da (Figure 6B). This corresponds to the molecular mass of the protein with 3.0 bound Tb^{3+} ions.

The multiply charged ESI-MS spectrum of apocalbindin Δ2,6 is shown in Figure 7A. As was seen for both of the previous proteins, two overlapping envelopes of ion series are observed. However, when Ca^{2+} or Tb^{3+} is added to calbindin Δ2,6, the increase in the intensity of the higher *m*/*z* envelope was not as great as the increase seen when full-length calbindin D_{28K} or calbindin Δ2 are titrated with Ca^{2+} or Tb^{3+} (Figure 7C and 8A). Deconvolution of the low *m*/*z* envelope of the protein in the presence of 1 mM calcium acetate shows that the low *m*/*z* envelope (*m*/*z* 1200−2600) consisted of calbindin Δ2,6 with 0, 1, and 2 bound Ca^{2+} ions. The deconvoluted spectra of the high *m*/*z* charge states of the apoprotein gives a single peak at 20 369 Da (Figure 7B). Deconvolution of the multiply charged spectrum of the protein containing an excess of Ca^{2+} (Figure 7D) shows a peak at 20 485 Da. The mass difference of 116 Da corresponds to the addition of 3.0 atoms of Ca^{2+} to the protein. The experiment was repeated using terbium acetate, and as shown in Figure 8A a similar pattern in the bimodal distribution of the multiply charged envelopes occurs when terbium is added to the protein. Deconvolution of the multiply charged spectrum of calbindin Δ2,6 containing an excess of Tb^{3+} gives two peaks at 20 684 and 20 838 Da (Figure 8B), which corresponds to the molecular mass of the protein bound to 2.0 and 3.0 Tb^{3+} ions, respectively. The peak marked by an asterisk corresponds to a Tb^{3+}/EDTA-adduct bound to the protein.

DISCUSSION

This study provides direct evidence that rat brain calbindin D_{28K} possesses four Ca^{2+}-binding sites. This is the first time that a spectroscopic method has been used to directly examine the calcium-binding stoichiometry to the intact protein. To the best of our knowledge, this is the largest protein whose calcium-binding stoichiometry has been determined using ESI-MS. By using terbium as an isomorphous replacement for calcium, ESI-MS can be extended to measure the number of metal-binding sites on even larger

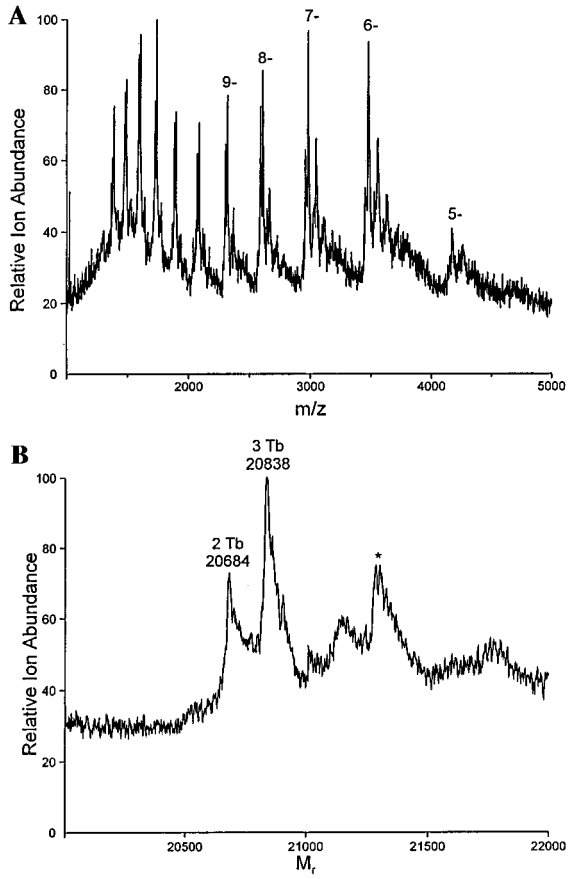

FIGURE 8: (A) Multiply charged negative-ion ESI mass spectrum of calbindin Δ2,6 in the presence of 1 mM terbium acetate. (B) Deconvoluted spectrum of calbindin Δ2,6 in the presence of 1 mM terbium acetate. The protein concentration was 90 μM, and the solvent was 4 mM NH_4HCO_3, 15% CH_3OH, pH 8.0. The peaks marked by asterisks (*) correspond to Tb^{3+}/EDTA-adducts bound nonspecifically to the protein.

proteins due to the greater relative mass of terbium, which increases the mass shift between metal-bound speices in the deconvoluted spectrum.

Both deletion mutants of calbindin D_{28K} bind 3 mol of Ca^{2+} or Tb^{3+}/mol of protein, which is 1 less than that bound in the full-length protein. Since the metal-binding characteristics of the Δ2 and Δ2,6 mutants were similar, it can be concluded that EF-hand 6 is not a Ca^{2+}- or Tb^{3+}-binding domain in calbindin D_{28K}. Therefore, of the remaining EF-hand domains 1−5 in the full-length protein, four of these bind Ca^{2+}. Terbium fluorescence data of the full-length and mutant proteins suggest EF-hands 1, 4, and 5 are Ca^{2+}-binding domains (Veenstra et al., 1995). Comparison of numbers of metals bound by the full-length protein and the Δ2 mutant, would also suggest that EF-hand 2 is a Ca^{2+}-binding domain, however there is little direct evidence from other experiments showing that it functions in metal-binding (Veenstra et al., 1995; Gross et al., 1993). Although the results presented here cannot absolutely determine the exact sites where Ca^{2+}-binds within the protein, all of the available data on metal-binding to calbindin D_{28K} suggests that EF-hands 1, 4, and 5 and one of either EF-hands 2 or 3 are the Ca^{2+}-binding sites within the full-length protein.

It is of interest to note the distribution of the charge states in the apo- and holoproteins. In the multiply charged spectrum of all three proteins, there is a bimodal distribution of low and high *m*/*z* charge states (Figures 2A, 5A, and 7A). In the absence of calcium the lower *m*/*z* envelope is greater in intensity. As calcium, or terbium, is added to each protein there is a shift in the bimodal distribution of the charge states, so that the high *m*/*z* envelope becomes the predominant species at high metal:protein ratios. However, the increase in intensity of the high *m*/*z* envelopes is much greater relative to the lower envelope in the full-length and Δ2 mutant protein compared to the Δ2,6 mutant. Separate deconvolution of the *m*/*z* envelopes show that they represent the protein with differing numbers of bound metal ions, with the lower envelope being species of the protein with fewer ions bound. A similar phenomenon has been observed for rabbit and rat parvalbumin under similar conditions (Hu & Loo, 1995). Loo et al. (1991) have shown similar charge state distributions for ubiquitin under denaturing conditions and postulated that the distribution of peaks at high *m*/*z* represent the protein in its native, globular form while the low *m*/*z* distribution represents the protein in a denatured, extended form. We believe the distribution of the charge states seen in this study may be related to a metal-induced conformational change within the protein. Indeed calbindin D_{28K} has been shown to undergo a calcium-dependent conformational change by circular dichroism and intrinsic fluorescence (Gross et al., 1987). The charge state distribution may also be a product of the stability of the protein. As is seen for the calbindins, the low charge states predominate at higher calcium:protein ratios. It could be reasonably expected that the protein would have a greater stability when all of its calcium-binding sites are occupied; therefore, the lower charge states would dominate the multiply charged spectrum. Although ESI is a mild technique by which to introduce proteins into the gas phase, the energy released into the molecule can still with great enough intensity cause the protein to undergo a change in conformation depending on the energy of the transition state.

Terbium fluorescence has been used extensively to determine the number of calcium-binding sites within proteins, including calbindin D_{28K}. Previous terbium fluorescence studies of the protein have indicated three to four metal-binding sites within the protein (Gross et al., 1987; Veenstra et al., 1995). Using ESI-MS we unequivocally show that calbindin D_{28K} binds 4 mol of terbium/mol of protein. Spectroscopic techniques, including terbium fluorescence, are prone to errors in that they monitor physical changes within the protein upon binding metal ions. Such techniques also require meticulous sample preparation and the exact measurements of protein and ligand concentrations in order to provide reliable results. In terbium fluorescent experiments the emission intensity is the sum of the amount of protein-bound terbium times the quantum yield at each binding site, with the quantum yield being a function of the affinity of terbium at a given binding site (Horrocks & Sudnick, 1981). It is therefore possible that the emission arising from a weakly bound terbium ion may be overshadowed by that originating from higher affinity sites and therefore difficult to interpret as a specific or nonspecific binding site.

This is the first case in which direct spectroscopic evidence has been used to definitively determine the Ca^{2+}-binding stoichiometry of intact calbindin D_{28K}. The conclusions reached in this study show that calbindin D_{28K} binds 4 mol of Ca^{2+}/mol of protein and that two deletion mutants of the protein, calbindin Δ2 and Δ2,6, each bind 3 mol of Ca^{2+}/

mol of protein. The number of Tb^{3+} ions bound was shown to be equivalent to the number of Ca^{2+}-binding sites within each protein, showing that Tb^{3+} is a useful probe for Ca^{2+}-binding sites within these proteins.

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