EVIDENCE FOR CALCIUM-MEDIATED CHANGES IN DREAM STRUCTURE*

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DREAM, an EF-hand protein, associates with and modulates the activity of presenilins and Kv4 potassium channels in neural and cardiac tissues and represses prodynorphin and c-fos gene expression by binding to DNA response elements in these genes. Information concerning the metal-binding properties of DREAM and the consequences of metal binding on protein structure are important in understanding how this protein functions in cells. We now show that DREAM binds 1 mol of calcium/mol of protein with relatively high affinity and another 3 mol of calcium with lower affinity. DREAM binds 1 mol of magnesium/mol of protein. DREAM, pre-loaded with 1 mol of calcium, binds 1 mol of magnesium, thus demonstrating that the magnesium-binding site is distinct from the high affinity calcium-binding site. Analysis of metal binding to mutant DREAM protein constructs localizes the high affinity calcium-binding site and the magnesium-binding site to EF-hands 3 or 4. Binding of calcium but not magnesium changes the conformation, stability, and α-helical content of DREAM. Calcium, but not magnesium, reduces the affinity of apodynorphin and c-fos genes. We conclude that DREAM binds calcium and magnesium and that calcium, but not magnesium, modulates DREAM structure and function.

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The abbreviations used are: PS, presenilin; DTT, dithiothreitol; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; mPC-LC, membrane preconcentration-capillary liquid chromatography; μESI-MS, microelectrospray ionization mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; sFL, short full-length.

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The Metal-binding Properties of DREAM

The Metal-binding Properties of DREAM and how such binding affects DREAM structure and function might be important to our understanding of the pathogenesis of some forms of Alzheimer’s disease and in understanding of the modulation of potassium channel function in neural and cardiac tissues. We show for the first time that both calcium and magnesium are bound by DREAM at different sites and that the binding of calcium, but not magnesium, to the protein is associated with distinct structural changes in the protein that affect the affinity of the protein for DNA.

EXPERIMENTAL PROCEDURES

General—Protein amino acid composition, protein amino-terminal and carboxy-terminal sequencing, and DNA sequencing were carried out as described (26–28). Oligonucleotide synthesis (29) was performed using an Applied Biosystems DNA/oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). UV spectra of proteins and nucleic acids were recorded using a model DU-70 or DU-640 Beckman spectrophotometer (Beckman Instruments, Fullerton, CA). Protein concentrations were determined by amino acid analysis, by the Bradford method (30), or by measuring UV absorbance using the molar absorptivity of DREAM at 281 nm of 29.6 ± 0.3 mm−1 cm−1 obtained by quantitative determination of nitrogen by the indophenol blue method (31). Spectra were corrected for turbidity. SDS and non-denaturing PAGE was carried out using a PhastGel apparatus (Amersham Biosciences) and precast gels.

Bioisynthesis of DREAM and DREAM Mutant Proteins—We synthesized the following DREAM proteins by methods detailed below for the full-length DREAM protein. The DREAM protein constructs prepared are as follows: full-length DREAM (amino acid residues 1–256, see Fig. 1, A and B); short full-length or sFL DREAM (amino acid residues 95–256, lacking the first 94 residues of the full-length protein); Δ1 DREAM (amino acid residues 114–256, lacking EF-hand 1); ΔΔ DREAM (amino acid residues, 95–196, lacking EF-hand 4); Δ1,2 DREAM (amino acid residues, 95–256, lacking EF-hand 1 and EF-hand 2).
Metal-binding Properties of DREAM

Full-length DREAM and mutant DREAM proteins were biosynthesized in *Escherichia coli* BL21 cells, transformed with pGEX-6P-1 plasmid (Amersham Biosciences) containing the DREAM complementary DNA sequence (GenBank™ accession number AJ131730) using procedures described previously (32–34). The primers shown in Scheme I were used to reverse-transcribe and amplify DREAM mRNA from human brain total RNA (CLONTECH, Palo Alto, CA) using the Titan One Tube Reverse Transcriptase-PCR System (Roche Molecular Biochemicals). A NaCl gradient (0–1 M in Mono-Q start buffer) was developed over 1 h. Fractions containing DREAM were pooled and dialyzed against buffer (50 mM Tris, 1.5 mM EDTA, pH 7.5). For the Δ3,4 DREAM construct and the 1–101 DREAM construct on-column PreScission Protease treatment was carried out, and pure proteins obtained in this manner were used for analysis.

Matrix-assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) and On-Line Membrane Preconcentration-Capillary High Pressure Liquid Chromatography-MS/MS Analysis (mPC-LC-MS/MS)—DREAM protein sequence was verified by tryptic digestion of the intact protein, followed by mapping of the resulting tryptic peptides by MALDI-MS. MALDI-MS data was obtained on a Voyager DE-STR focusing mass spectrometer (PE Biosystems, Framingham, MA) using the reflector mode over mass range 400–4500 Da with 20-kV accelerating voltage. Sequence information was obtained from the identified peptides using on-line mPC-LC-MS/MS performed with a Micromass Q-Tof II mass spectrometer (Micromass, Danvers, MA) equipped with a modified Micromass nano-ESI interface (37). Membrane PC-LC-MS spectra were recorded over mass range 300–1900 every 2 s. Mass resolution was 8000–10,000. The mass axis was calibrated over the range of 172.88 to 80000, at a scan speed of 10,000 every 2 s. Collision energy values were automatically chosen from MS survey scans over mass range 375–1500. Collision energy values were automatically chosen as a function of precursor ion charge state and mz value. Argon was used as the collision gas, at collision energies varying from 15 to 50 eV.

Matrix-assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS)—μESI-MS analyses were carried out on a Finnigan MAT 900 double focusing mass spectrometer (MAT, Bremen, Germany) of E (electrospray ionization)-B (magnet) geometry. Analyses of DREAM and the metal ion titration experiments were performed in positive ion mode, using a modified μESI source introduction interface described previously (38). Measurements were carried out in the presence of SF 6. The mass spectra were scanned from mass to charge (m/z) 1000–6000, at a scan speed of 10 s per decade and instrument resolution of 2000. Ions were detected at the position and time resolved ion counter (PATRIC), using a channel plate voltage of 850 V, and raw data were transformed using Finnigan MAT software.

Working solutions of 10 or 24 μM of DREAM were prepared in ~5 μM EDTA, pH 7.8. Metal ion titration experiments were carried out by incubating DREAM with appropriate concentrations of metal salts for 30 min. Samples were analyzed directly or put through a cation ion...
Intrinsic Protein Fluorescence and CD Measurements—Fluorescence measurements of apo-DREAM (5–10 μM) were performed in 60 mM MOPS, 2 mM EGTA, 0.5 mM DTT, pH 7.0, buffer before and after the addition of CaCl₂ or MgCl₂. The entire corrected fluorescence emission spectrum from 300 to 450 nm was measured using a SPEX 1680 spectrofluorimeter (SPEX, Edison, NJ). Final values were corrected for dilution and normalized.

CD spectra of DREAM (10–20 μM) in 60 mM MOPS, 2 mM EGTA, 0.5 mM DTT, pH 7.0, were collected on a J-715 spectropolarimeter (JASCO, Japan). Spectral and temperature-dependent measurements were performed at a bandwidth of 2 nm using a U-type quartz cell of path length 0.233 mm (for far-UV measurements, 188–250 nm) and with a rectangular 1-cm cell (for near-UV measurements, 245–320 nm) in a temperature-controlled cell holder. CD spectra were recorded at 10 °C using 5 accumulations, each at scan speed of 20 nm/min and response time of 2 s. The continuous temperature dependence of ellipticity at 222 nm was measured using a scan rate of 50 °C/h and a response time of 8 s. Solvent evaporation was prevented by placing a drop of oil on the sample in the cell. CD spectra were smoothed using a Jasco noise reduction routine. CD data are presented in units of molar ellipticity per residue. Secondary structure determinations were carried out as described (39–46).

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were carried out using DREAM apoprotein and oligonucleotides containing response elements for DREAM: prodynorphin oligonucleotide, 5′ GAAGCCGGATCGAAGGAGGCCCCTG 3′; mutant prodynorphin oligonucleotide, 5′ GAAGCCGTAACCGTAAAGGCCTTG 3′; c-fos oligonucleotide, 5′ CTGCGACCGAGCAATGAAATCCAGAC 3′; mutant c-fos oligonucleotide, 5′ CTGCGAGCGACAAGAATCCAGAC 3′.

Double-stranded DNAs were prepared by annealing complementary DNA strands to the above noted oligonucleotides. Interaction of DREAM protein with DNA was carried out in 50 mM Tris, pH 7.5, 0.5 mM DTT, 0.15 μg/ml poly(dI-dC)poly(dI-dC), 50 μM EDTA, 10% glycerol with appropriate amounts of Ca²⁺ ion or Mg²⁺ ion. In some instances, buffer containing 10 mM Hepes, pH 7.8, 10% glycerol, 0.1 mM EDTA, 8 mM MgCl₂, 1 mM DTT, 0.15 μg/ml poly(dI-dC)poly(dI-dC) was used. Supershift analysis was carried out by adding a specific anti-DREAM antibody raised in rabbits against biosynthetic full-length DREAM (Cocalico Biologicals, Reamstown, PA).

Equilibrium-binding Assay with Radiolabeled Calcium and DREAM—The Kᵢ values for calcium in DREAM were determined by adding increasing amounts of ⁴⁴Ca to 100 pmol of purified DREAM in 1 ml of 0.1 M Tris, pH 7.5, and incubating the reaction mixture for 90 min at 37 °C. 100 μl of anti-DREAM monoclonal antibody (1:50 dilution) were added to the mixture, and the reaction was allowed to proceed overnight at 4 °C. 100 μl of a 10% suspension of IgSorb protein A in 0.1 M Tris, pH 7.5 (Enzyme Center, Bedford, MA), was added to each tube, and the mixture was vortexed. After 2 min the tubes were centrifuged at 1,000 × g, and radioactivity in the pellet was determined by scintillation spectrometry. Nonspecific binding of ⁴⁴Ca to DREAM was determined by measurement of binding in the presence of 0.1 M CaCl₂. The Kᵢ values for the protein were determined using established methods for fitting equilibrium binding data (47).

Nondenaturing Gel Electrophoresis of DREAM—Microliter additions of full-length DREAM (970 μM) in 0.5 mM DTT were combined with EDTA (sodium salt, 0.5 M, pH 8.0 stock) and 100 mM Tris, pH 7.6, with or without either or both calcium acetate and magnesium acetate to give ~1:1 or ~8:1 metal/protein ratios. The final concentrations are as follows: 200 μM DREAM, 60 mM Tris, 80 μM EDTA. Bovine serum albumin was run in one lane of gel.

### RESULTS

**Metal-binding Properties of DREAM**

**TABLE II**

| Construct     | Observed mass | Predicted molecular mass |
|---------------|---------------|--------------------------|
| FL-DREAM      | 29,643        | 29,641                   |
| sFL-DREAM     | 19,198        | 19,198                   |
| Δ1 DREAM      | 17,071        | 17,072                   |
| Δ4 DREAM      | 12,096        | 12,096                   |
| Δ1,2 DREAM    | 12,543        | 12,544                   |
| Δ3,4 DREAM    | 9,360         | 9,360                    |
| Δ1,2,3,4 DREAM| 11,722        | 11,723                   |

**FIG. 2**. Positive ion μESI-MS of DREAM after calcium metal ion titration. Transformed mass spectra of 24 μM DREAM in the presence of 25 μM EDTA. A, apo-DREAM, with no Ca²⁺ ions added; B, DREAM plus 125 μM calcium ions; C, DREAM plus 125 μM calcium ions after cation exchange.

**Protein Identification**—Amino acid composition and amino-terminal and carboxyl-terminal sequencing afforded data consistent with the predicted sequences of all constructs (Fig. 1, A and B). Analysis of full-length DREAM by μESI-MS afforded a multiply charged ion series, which on transformation revealed a relative molecular mass of 29,643 Da (expected 29,641 Da, Fig. 2A and Table II). This experimental value is well within the 0.01% accuracy for such measurements. Tryptic peptides
derived from DREAM were analyzed directly by MALDI-MS. A tryptic peptide map was obtained covering \( \sim 75\% \) of the expected protein sequence. The tryptic peptide mixture was analyzed by mPC-LC-MS/MS, and a total of eight peptides were subjected to collision-induced dissociation, and the resulting product ion spectra were submitted to the Sequest program (48). All eight peptides were identified as being derived from human DREAM protein. One of these peptides (MH\(^+\) = 985.5) had the sequence GPLGSMQPAK, identifying it as the amino terminus of the protein. The first five amino acids are derived from the PreScission Protease cleavage site and the linker region. Thus, the biosynthetic protein was unambiguously characterized as human DREAM. Subsequently, all mutant DREAM proteins were subjected to ESIMS.

**Metal Ion Binding Studies**—We have demonstrated previously that \( \mu \)ESI-MS can be used to detect protein-metal ion interactions (33, 34, 49–51). Furthermore, using this approach, it is possible to determine exact stoichiometries of metal ion binding to the protein, because uptake of specific metal ions does not significantly alter protein ionization efficiencies (49–51). To investigate the metal-binding properties of DREAM, we analyzed both apo-DREAM and metal ion-titrated DREAM solutions in positive ion \( \mu \)ESI-MS. In all cases, we carried out systematic titration studies adding to \( \sim 25 \mu \text{M} \) DREAM, containing 25 \( \mu \text{M} \) EDTA, metal ions to concentrations of 10, 20, 50, 100, 125, 150, 175, 200, 300, and 500 \( \mu \text{M} \). As noted above, mass spectral analysis of apo-DREAM revealed a mass of 29,643 Da (Fig. 2A). This corresponds to monomeric DREAM; there was no detectable evidence for the presence of dimeric or other multimeric forms of the protein. However, as increasing amounts of calcium are added to the protein solution, a significant change in the \( \mu \)ESI spectrum is noted. For example, addition of calcium to 125 \( \mu \text{M} \) results in the uptake of 4 eq of Ca\(^{2+}\) ions/mol of DREAM (Fig. 2B). Uptake of 1 Ca\(^{2+}\) ion (peak ion abundance 100%) and 2 Ca\(^{2+}\) ions (peak ion abundance \( \sim 80\% \)) are the predominant ion species. However, there is also clear evidence for uptake of an additional 3 Ca\(^{2+}\) ions (\( \sim 60\% \) peak ion abundance) and 4 Ca\(^{2+}\) ions (\( \sim 30\% \) peak ion abundance) (Fig. 2B), indicating that all four EF-hands bind Ca\(^{2+}\) ions. When the amount of calcium added to apo-DREAM reaches or exceeds 175 \( \mu \text{M} \), additional binding of small amounts of calcium occur, up to 6 mol eq/mol of protein. This observation was highly reproducible, but increasing the \( \mu \)ESI source voltages that affect skimmer collision-induced dissociation results in facile loss of these additional Ca\(^{2+}\) ions, indicating that they were nonspecifically bound metal ion-protein adducts (51). Binding of the first four calciums occurs within the EF-hands of the protein, as others have shown (1) that mutant DREAM, in which the acidic residues within EF-hand binding loops are mutated to alanine, fails to dissociate from a DNA response.

**FIG. 3. Positive ion \( \mu \)ESI-MS of DREAM after magnesium or magnesium/calcium metal ion titration.** Transformed mass spectra of 24 \( \mu \text{M} \) DREAM in the presence of 25 \( \mu \text{M} \) EDTA. A, apoDREAM with no metal ions added; B, DREAM plus 125 \( \mu \text{M} \) magnesium; C, DREAM plus 125 \( \mu \text{M} \) magnesium after cation exchange; D, DREAM plus 125 \( \mu \text{M} \) calcium and 125 \( \mu \text{M} \) magnesium.
element in a calcium-dependent manner. It is also interesting to note that when the DREAM protein solution containing 125 μM calcium (Fig. 2B) is passed through a cation exchange micro-cartridge, stripping of 2 mol eq of Ca\textsuperscript{2+} ions is observed (Fig. 2C). This indicates that there are two distinct classes of binding sites present in the DREAM protein. The predominant forms of metal ion-bound protein correspond to the uptake of 1 and 2 mol of Ca\textsuperscript{2+} ion/mol eq of DREAM. We also conducted equilibrium binding assays with radiolabeled Ca\textsuperscript{2+} and DREAM and show that there are two classes of binding sites for calcium in DREAM, the first one with higher affinity (K\textsubscript{D} = 6 \pm 1 \times 10^{-7} M), and a second class with a much lower affinity.

We next added magnesium to apo-DREAM (Fig. 3A). As can be seen in Fig. 3B, the apoprotein binds 1 mol eq of Mg\textsuperscript{2+} ion/mol of protein. Increasing the amount of magnesium did not result in additional specific uptake of magnesium by the protein. Interestingly, when the protein containing the single magnesium atom (as seen in Fig. 3B) was passed through a cation exchange micro-cartridge, the metal ion was readily stripped away to give apo-DREAM as shown in Fig. 3C. This indicates that the relative metal ion binding affinity of Mg\textsuperscript{2+} ion is similar to that of the third and fourth binding sites for Ca\textsuperscript{2+} ions as shown in Fig. 2C. Calcium metal ions bound to these sites are readily stripped away on passage of calcium-loaded DREAM through a cation exchange micro-cartridge. To investigate further the binding of magnesium and calcium to DREAM, both metals were added to apo-DREAM. Although there was still detectable apo-DREAM, two other distinct ions corresponding to uptake of 1 Ca\textsuperscript{2+} ion, and 1 Ca\textsuperscript{2+} ion plus 1 Mg\textsuperscript{2+} ion were observed (Fig. 3D). It appears that the relatively higher affinity Ca\textsuperscript{2+} ion-binding site first takes up the Ca\textsuperscript{2+} ion, followed by uptake of Mg\textsuperscript{2+} ion at another EF-hand-binding site. We next examined the effects of increasing amounts of magnesium on calcium already bound to the “high affinity” Ca\textsuperscript{2+} ion-binding site. DREAM titrated with 75 μM calcium showed predominantly the high affinity Ca\textsuperscript{2+} ion-binding site filled with metal ion (−1:1 apo-DREAM, 1 calcium-DREAM ion abundance signals). Subsequently, 75, 150, and 300 μM magnesium was titrated into the 1 calcium-DREAM sample. Although the Mg\textsuperscript{2+} ion-binding EF-hand was also filled, no reduction in the binding of calcium to DREAM was observed (data not shown). We also assessed the binding properties of DREAM in the presence of a series of other metal ions including Tb\textsuperscript{3+}, Zn\textsuperscript{2+}, Mn\textsuperscript{2+}, and Cu\textsuperscript{2+}. No binding of these metals to DREAM was observed.

To determine which EF-hands might play a role in Ca\textsuperscript{2+} ion binding and Mg\textsuperscript{2+} ion binding, we constructed a series of DREAM mutants lacking the amino terminus and various EF-hands. Deletion of the NH\textsubscript{2}-terminal region of DREAM (the first 94 amino acids) has little effect on the Ca\textsuperscript{2+} ion or Mg\textsuperscript{2+} ion-binding properties of the protein (Fig. 4, A–C). The apo-sFL DREAM has a M\textsubscript{r} of 19,198 (Fig. 4A) and addition of 150 μM calcium shows the addition of 1–4 Ca\textsuperscript{2+} ions (Fig. 4B). The addition of 150 μM magnesium shows binding of predominantly 1 mol of magnesium ion/mol of protein (Fig. 4C). Additional magnesium (300 μM) does not show any more significant uptake of metal ions (data not shown). All these data are consistent with the notion that the amino-terminal portion of the protein plays no significant role in metal binding.

Binding of metals generally occurs in pairs of EF-hands (6, 54). To ascertain which of the EF-hand pairs was important for metal binding, we deleted either EF-hands 1 and 2 or EF-hands 3 and 4 from the protein. Δ3,4 DREAM lacking EF-hands 3 and 4 and retaining EF-hands 1 and 2 has a mass of 9,360 Da as the apoprotein (Fig. 5A). The addition of 150 μM calcium results in only a small uptake of metal ion (−30% of apoΔ3,4 DREAM, data not shown). Even with the addition of 300 μM calcium, only −30% of the protein contains 1 calcium/mol of protein (Fig. 5B). As seen in Fig. 5C, the addition of 200 μM magnesium results in even less metal ion uptake. Addition of 300 μM magnesium only results in −15% of the magnesium-bound protein compared with apoΔ3,4 DREAM (data not shown).

The data obtained from analysis of Δ3,4 DREAM were in distinct contrast to those seen with the Δ1,2 DREAM. This construct contains EF-hands 3 and 4, and affords a mass of
12,544 Da in the metal-free state (Fig. 5D). Addition of 150 μM calcium results in the ready uptake of predominantly 1 mol of Ca$^{2+}$ ion/mol of protein, with some amount of 2 calcium eq/mol of protein also detected (Fig. 5E). Addition of 300 μM calcium results in uptake of additional Ca$^{2+}$ ions to afford the 2 calcium proteins, with the ratio of apo1,2 DREAM ion abundance of 60:100:90 (data not shown). We also detected some small nonspecific binding of 3–6 Ca$^{2+}$ ions/mol of protein. The addition of 150 μM magnesium shows some significant Mg$^{2+}$ ion binding, representing ~45% of apo1,2 DREAM ion abundance (Fig. 5F). The additional ions responses detected at ~12,584 Da (see * in Fig. 5F) correspond to nonspecific adduction of NH$_4^+$ or Mg$^{2+}$ ions to the 1 magnesium 1,2 DREAM. Upon addition of 300 μM magnesium, the uptake of 1 Mg$^{2+}$ ion by 1,2 DREAM increases to ~60% ion abundance of the apo1,2 DREAM (data not shown). All these data clearly demonstrate that the pair of EF-hands 3 and 4 contains the high affinity Ca$^{2+}$ ion-binding site and the Mg$^{2+}$ ion-binding site.

Because 1,2 DREAM was shown to contain the high affinity Ca$^{2+}$ ion as well as the Mg$^{2+}$ ion-binding sites, we next examined the specific metal-binding properties of the 2 EF-hands. When 1,2 DREAM is incubated with 1:2 calcium/magnesium (75 and 150 μM), the predominant metal ion species is the 1 calcium 1,2 DREAM (Fig. 6A). The apo1,2 DREAM/1 calcium 1,2 DREAM/1 magnesium 1,2 DREAM is ~100:80:40. The 1 calcium/1 magnesium 1,2 DREAM is also present at
Metal-binding Properties of DREAM

Fig. 6. Positive ion μESI-MS of Δ1,2 DREAM; competition between calcium and magnesium ion uptake. Transformed mass spectral data for simultaneous addition of 75 μM calcium and 150 μM magnesium to Δ1,2 DREAM (A). B, simultaneous addition of 150 μM calcium and 150 μM magnesium to Δ1,2 DREAM.

~40% of apoΔ1,2 DREAM (Fig. 6A). However, on increasing the calcium to 150 μM and maintaining the magnesium at 150 μM, the spectrum obtained is now significantly different. The ratio of apoΔ1,2:1 calcium Δ1,2:1 magnesium Δ1,2 is ~80:100:35 (Fig. 6B). Furthermore, the ratio of 1 calcium Δ1,2:1 calcium/1 magnesium Δ1,2:2 Ca2+/1 Mg2+ Δ1,2 is 100:60:60 (Fig. 6B).

The binding of calcium to EF-hand proteins generally occurs when EF-hands are present as pairs (6). To determine whether this occurs with DREAM, we investigated the uptake of both Ca2+ and Mg2+ ions by proteins containing only 3 EF-hands by examining Δ1 and Δ4 DREAM. In the case of Δ4 DREAM, addition of either 150 μM calcium or magnesium resulted in insignificant metal ion uptake. For both titration studies, uptake of 1 calcium or 1 magnesium was <15% of the apoΔ4 DREAM present (Fig. 7, A–C). Even the addition of 300 μM calcium or magnesium did not enhance metal ion uptake (data not shown). Similar data were also obtained in the analysis of Δ1-DREAM (Fig. 7, D–F). The data in Fig. 7, A–F, indicate that there were effects of end EF-hands of EF-hand pairs 1 and 2 and pairs 3 and 4 on global protein structure that preclude efficient metal ion binding.

Metal-induced Conformational Changes in DREAM—Previously, we have shown that μESI-MS can be used to rapidly ascertain whether a protein undergoes gross tertiary or secondary structural change on metal ion uptake (50, 51). We demonstrated that the changes in near-UV CD and/or fluorescence spectra correlate with changes in the μESI-MS charge state distribution of calbindin-D28K on uptake of Ca2+ ions and the charge state distribution of the DNA binding domain of vitamin D receptor on the uptake of 2 mol eq of Zn2+ ions (50, 51). As discussed previously (52) the ESI multiply charged spectrum of a protein is directly related to the number of ionizable side chains at or near the protein surface. As a protein undergoes a conformational change, this number of side chains can increase or decrease and is reflected in a charge state shift in ion distribution in the ESI spectrum. To determine whether metal ions induce conformational changes in the tertiary structure of DREAM, we analyzed the multiply charged spectral data of DREAM in the presence of both calcium and magnesium. The μESI-MS analysis of apo-DREAM reveals an ion series from +19 to +7 (m/z ~1,500–4,250), centered around the +12, +11 charge states (Fig. 8A). However, on titration of 175 μM calcium into the DREAM protein solution, a subtle but reproducible change occurs in the charge state distribution (Fig. 8B). The charge state ions shift to a bimodal distribution with the most abundant ions now being centered around the +16 and +15 charge states. This is indicative of a conformational change occurring for DREAM on uptake of 4 mol eq of calcium (see Fig. 2B for the transformed spectrum of this sample). However, when calcium-loaded DREAM is then subjected to cation exchange resin chromatography to afford DREAM containing predominantly only one calcium, the charge state distribution of DREAM reverts to a pattern identical to apo-DREAM (compare Fig. 8C with Fig. 8A). Also, when the charge state distribution of DREAM containing only 1 mol eq of calcium is analyzed, it closely resembles that seen with apo-DREAM. This indicates that uptake of only 1 mol eq of calcium does not significantly alter the gross tertiary structure of DREAM. It is only when the protein is fully loaded with 4 mol eq of calcium that a change in conformation occurs. Finally, titration of magnesium with DREAM resulting in the uptake of 1 mol eq of the metal ion results in a much smaller (relative to changes induced by calcium) but reproducible change in the charge state distribution pattern of multiply charged ions (Fig. 8D).

The data on the effects of metal ions on DREAM conformation was further assessed by examining the mobility of DREAM on non-denaturing polyacrylamide gels (Fig. 9). As seen in Fig. 9, lane 1, the calcium-free form of DREAM migrates as a single band. Addition of increasing amounts of calcium causes a progressive decrease in protein mobility (Fig. 9, lanes 2 and 3). In contrast, the addition of magnesium has little effect on protein mobility (lanes 4 and 5). The addition of magnesium to calcium-bound protein has no effect on the decrease in protein mobility brought about by calcium (Fig. 9, lane 6).

Optical Spectroscopy Data—Measurements of intrinsic tryptophan fluorescence of DREAM were conducted in the presence of different amounts of calcium and/or magnesium. The average titration curves with corresponding error bars are shown in Fig. 10. Addition of calcium to apoprotein leads to a decrease in fluorescence intensity in the region of pCa 7–4. The absence of any shoulders on the broad sigmoidal curve does not allow us to distinguish between separate transitions corresponding to particular Ca2+ ion-binding sites. Magnesium titration results in a slight decrease of fluorescence intensity. To determine whether either calcium or magnesium influences the binding of the other metal to apo-DREAM, we carried out sequential titration experiments. DREAM was titrated with calcium to pCa of 6.6; titration was then continued with magnesium. The resultant titration curve coincided within the experimental error with one corresponding to that seen with magnesium alone (Fig. 10). When we performed the calcium titration of DREAM-saturated with magnesium (pMg 3.0), the resulting curve was identical with that seen with calcium alone.

We used far-UV CD spectroscopy to discern differences be-
between the secondary structures of the apo- and calcium- or magnesium-bound protein. Two CD spectra representing the apo- and calcium-saturated state of DREAM are shown in Fig. 11. Table III presents the results of the protein secondary structure calculations obtained by two different methods for fitting the CD data (39–46). Apo-DREAM is 35% α-helical (Table III). However, calcium binding increases α-helical content (by ~6%) at the expense of a decrease in β-turn content (by ~5%). The number of terminal residues (α-distorted) in α-helices is not changed upon Ca²⁺ ion binding, and thus the number of α-helices (11 segments) remains the same. The increase in the number of residues in the central part of the helical segments (α-regular) extends the average helix length by 1 residue (Table III). Addition of magnesium to the apoprotein does not change the protein secondary structure (data not shown). The effect of calcium on fully magnesium-charged DREAM secondary structure is identical to the one seen with calcium addition to the apoprotein. CD spectra in the near-UV region (spectra not shown) exhibit rather weak induced optical activity of DREAM aromatic residues both in the apo-state and in the presence of calcium and/or magnesium.

DREAM thermostability was determined by measuring the

**Fig. 7. Positive ion μESI-MS of DREAM deletion mutants Δ4 and Δ1 after Ca²⁺ or Mg²⁺ metal ion titration.** The transformed mass spectra were obtained on 10 μM DREAM deletion mutants in the presence of ~25 μM EDTA. A, apoΔ4 DREAM with no metal ions added. B, Δ4 DREAM + 150 μM Ca²⁺ ions. C, Δ4 DREAM + 150 μM Mg²⁺ ions. D, apoΔ1 DREAM with no metal ions added. E, Δ1 DREAM + 150 μM Ca²⁺ ions. F, Δ1 DREAM + 150 μM Mg²⁺ ions.
temperature dependence of CD signals at 222 nm at different free Ca\(^{2+}\)/H\(^{1+}\) concentrations (Fig. 12). These data demonstrate an increase in protein stability upon metal ion binding. The mid-point of heat denaturation transition shifts from \(\approx 52^\circ\)C in the apo-state to \(\approx 55^\circ\)C at \(pCa\) 7.0, \(\approx 65^\circ\)C at \(pCa\) 5.0, and to \(\approx 85^\circ\)C in the calcium-saturated state (\(pCa\) 2.5). The heat denaturation of DREAM is irreversible. CD spectra at high temperatures or after cooling do not show any increase in the content of \(-\)structure, suggesting that aggregation is not responsible for the observed irreversibility. Increasing the concentration of DTT does not alter the stability of the protein or affect the irreversibility of heat denaturation. Unlike calcium, magnesium titration minimally stabilizes the protein; at \(pMg\) 5.0, the mid-point of heat denaturation transition shifts to \(\approx 80\)–\(85^\circ\)C. In the presence of excess Mg\(^{2+}\) the shape of the heat denaturation profile changes significantly with an increase of the mid-point of transition to \(\approx 80\)–\(85^\circ\)C. Similar changes for calcium and magnesium at saturating concentrations suggest that the observed effects are nonspecific.

Effect of Metals on DREAM-DNA Interaction—We next investigated the binding properties of DREAM to DNA response elements found in the prodynorphin and c-fos gene promoters. Apo-DREAM binds to the prodynorphin DREAM DNA response elements (Fig. 13, 1st panel, arrows 1 and 2). The addition of 1 and 10 mM calcium diminishes the binding of DREAM to prodynorphin DNA response elements (Fig. 13, 3rd panel, arrows 1 and 2). The addition of 1 and 10 mM calcium diminishes the binding of DREAM to prodynorphin DNA response elements (Fig. 13, 3rd panel, arrows 1 and 2).
and 5th panels, arrows 1 and 2). In contrast, the addition of 10 mM magnesium has no effect on DREAM/DNA binding (Fig. 13, 4th panel, arrows 1 and 2). Similar results were obtained with the c-fos DNA response element (data not shown). Of note, binding of the 1–101 NH₂-terminal fragment of DREAM to the prodynorphin DNA response element was observed.

**DISCUSSION**

The stoichiometry of calcium binding and the metal-binding properties of DREAM have not been determined previously. Various authors (18–20, 24) have speculated that the DREAM binds between 2 and 3 mol of Ca²⁺/mol of protein. A recent report (53), using an ultrafiltration technique, shows that the protein binds 4 mol of calcium/mol of protein. The Mg²⁺-saturated protein. The Mg²⁺ ion-binding site because when DREAM is loaded with 1 mol of calcium/mole of protein, it is still capable of binding 1 mol of magnesium. The affinity of DREAM for Mg²⁺ ions is lower than that for Ca²⁺ ions in the first Ca²⁺ ion-binding site because when DREAM is loaded with 1 mol of calcium/mole of protein, it is still capable of binding 1 mol of magnesium. The affinity of DREAM for Mg²⁺ ions is lower than that for Ca²⁺ ions in the first Ca²⁺ ion-binding site.

We observed only slight changes in the μESI-MS charge distribution of the protein upon the addition of magnesium. This is supported by the absence of changes in intrinsic protein fluorescence observed upon the addition of magnesium. Additionally, there is no change in the mobility of the protein on non-denaturing gels. Hence, it is likely that magnesium associates with the protein but does not significantly alter its struc-
Secondary structure of DREAM in the apo (pCa9.5) and the Ca-saturated state (pCa2.5)

|          | α-Regular | α-Distorted | α-Total | β-Regular | β-Distorted | β-Total |
|----------|-----------|-------------|---------|-----------|-------------|---------|
| pCa2.5  | 23.1 ± 0.2 | 17.4 ± 0.6  | 40.5 ± 0.6 | 6.8 ± 1.1  | 6.5 ± 0.4    | 13.3 ± 1.2 | 18.3 ± 3.4 | 27.6 ± 1.5 |
| pCa9.5  | 18.5 ± 1.1 | 16.2 ± 0.4  | 34.7 ± 1.2 | 6.5 ± 0.8  | 7.1 ± 0.7    | 13.6 ± 1.4 | 22.9 ± 2.6 | 28.4 ± 2.6 |

Table III

We also demonstrated that DREAM does not bind other biologically relevant metal ions such as zinc, copper, and manganese. Interestingly, DREAM does not bind terbium, a fluorescent lanthanide that is often used to study the properties of calcium-binding proteins (49, 56). Other neuronal calcium-binding proteins, such as the neuronal calcium sensor-1, also fail to tightly bind Tb\(^{3+}\) ions (57). The exact reason why certain EF-hand proteins fail to bind Tb\(^{3+}\) ions is not known but indicates subtle differences in the conformation of EF-hand metal-binding sites.

In conclusion, DREAM is a biologically important EF-hand protein that binds both calcium and magnesium. All four of the EF-hands within the protein appear to be capable of binding calcium. EF-hand 3 or 4 binds calcium with much higher affinity than the others. EF-hand 3 or 4 is involved in magnesium binding. The affinity of the magnesium-binding EF-hand for magnesium is considerably lower than the affinity of the high affinity Ca\(^{2+}\) ion-binding site for calcium. The protein undergoes significant structural change upon binding calcium but not upon the binding of magnesium. Calcium binding alters the ability of DREAM to bind to specific DNA response elements in the prodynorphin and c-fos genes.

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