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# The Use of ESI-MS to Probe the Binding of Divalent Cations to Calmodulin

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Proteins have evolved with distinct sites for binding particular metal ions. This allows metalloproteins to perform a myriad of specialized tasks with conformations tailor-made by the combination of its primary sequence and the effect on this of the ligated metal ion. Here we investigate the selectivity of the calcium trigger protein calmodulin for divalent metal ions. This ubiquitous and highly abundant protein exists in equilibrium between its apo and its holo form wherein four calcium ions are bound. Amongst its many functions, calmodulin modulates the calcium concentration present in cells, but this functional property renders it a target for competition from other metal ions. We study the competition posed by four other divalent cations for the calcium binding sites in calmodulin using electrospray ionization mass spectrometry (ESI-MS). We have chosen two other group II cations  $Mg^{2+}$ ,  $Sr^{2+}$ , and two heavy metals  $Cd^{2+}$ ,  $Pb^{2+}$ . The ease with which each of these metals binds to apo and to holo CaM[4Ca] is described. We find that each metal ion has different properties with respect to calmodulin binding and competition with calcium. The order of affinity for apo CaM is  $Ca^{2+} \gg Sr^{2+} \sim Mg^{2+} > Pb^{2+} \sim Cd^{2+}$ . In the presence of calcium the affinity alters to  $Pb^{2+} > Ca^{2+} > Cd^{2+} > Sr^{2+} > Mg^{2+}$ . Once complexes have been formed between the metal ions and protein (CaM:[xM]) we investigate whether the structural change which must accompany calcium ligation to allow target binding takes place for a given CaM:[xM] system. We use a 20 residue target peptide, which forms the CaM binding site within the enzyme neuronal nitric-oxide synthase. Our earlier work (Shirran et al. 2005) [1] has demonstrated the particular selectivity of this system for CaM:4Ca<sup>2+</sup>. We find that along with Ca<sup>2+</sup> only Pb<sup>2+</sup> forms complexes of the form CaM:4M<sup>2+</sup>:nNOS. This work demonstrates the affinity for calcium above all other metals, but also warns about the ability of lead to replace calcium with apparent ease. (J Am Soc Mass Spectrom 2009, 20, 1159–1171) © 2009 American Society for Mass Spectrometry

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Calmodulin is a small ubiquitous calcium binding protein. It has been extensively studied (see for example [2–5] and is known to bind four calcium ions into four EF hand motifs [6]. CaM interacts with an impressive number of proteins, with wide physiological diversity [7], and a corresponding broad biological role in the body. In its apo form, calmodulin has two globular domains connected by a flexible linker. Its NMR solution structure was solved independently by Finn et al. [8] and Kuboniwa et al. [9] in 1995. These structures showed the protein possess two domains, each consisting of two helix-loop-helix EF hand Ca<sup>2+</sup> binding regions [10]. Upon exposure to calcium, calmodulin undergoes a major structural change as revealed by its X-ray crystal structure [11], the two anti-parallel helices in each EF hand of apo CaM become perpendicular. The resultant movement of the Ca<sup>2+</sup> binding loops causing the domains to become

more rigid and less solvent accessible. A central linker region, in the form of a helix is now exposed.

As a group II divalent cation, calcium will bind successfully to negative charges, supplied from aspartic acid and glutamic acid side chains. It can form several coordination geometries; however in the EF hand favors a seven coordination arrangement. Binding of calcium ions to calmodulin has been extensively studied. A variety of methodological approaches have established that four calcium ions bind specifically to EF hands of calmodulin [5]. These four sites possess some homology and are predictable from the sequence; the affinity for these sites is in the range  $1 \times 10^{-6}$ – $1 \times 10^{-4} M^{-1}$ .

Numerous studies have probed the mechanism of calcium binding to calmodulin [12] and there is still debate over the order of calcium ion binding and the cooperativity between site binding which can be summarized into three possible models:

1. four independent and equivalent sites;
2. four independent sites but two affinity classes, i.e., two pairs of independent sites;
3. cooperative binding amongst all the sites.

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The first model is in agreement with experimental data obtained by direct binding studies and is somewhat simplistic. The second model is based on early NMR and other spectroscopy studies, titrating in calcium, and monitoring spectral changes. The third model is now the most widely supported and suggests a coupling between the four sites and the two lobes of calmodulin, with either positive or negative cooperativity.

Further dispute exists over the secondary, or auxiliary, calcium ion binding sites on calmodulin [13–15]. Milos et al. [14] provide evidence of four capital and six auxiliary cation binding sites. The auxiliary sites are devolved further to two subclasses with different binding affinities [13]. These auxiliary sites are also reported to bind other cations [14], as do the EF hands. In this work, we use native electrospray ionization mass spectrometry (ESI-MS) to establish the affinity of divalent cations for both the EF hand calcium sites and the postulated auxiliary sites. We also consider whether the mechanism for binding other metals deviates from that described above for calcium, and how they compete for the EF hands in the presences of calcium.

The ionic environment inside a cell is extremely complex; in mammalian cells up to eight different metals can be present in a variety of concentrations and oxidation states, and it is imperative for metal binding sites within proteins to exhibit considerable selectivity. The EF hands of CaM have evolved to bind  $\text{Ca}^{2+}$ , but several studies have examined the ability for other metals to compete for the sites in this ubiquitous protein [15–17]. Of particular interest is the effect induced by a metal which is foreign to the biological environment for example lead, mercury or cadmium, since these divalent heavy metals certainly remain sequestered in our bodies.

Chao et al., in a preliminary study, link the activation of CaM by various metal cations to the ionic radii of the ion [16]. Using several methods including size exclusion gel electrophoresis and fluorescence spectroscopy both di- and trivalent cations with similar radii to  $\text{Ca}^{2+}$  ( $\text{La}^{3+}$ ,  $\text{Tb}^{3+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Sm}^{3+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$ ) are shown to induce a similar structural change. Some of these metals compete for the calcium binding sites whilst others have effects beyond those produced by calcium alone. Mills and Johnson propose that CaM has four main cation binding sites which must be occupied for functional interaction with a target [18]. However they also suggest that CaM contains a further as yet undefined number of cation binding sites, which produce an allosteric conformational change in CaM, allowing the protein to bind the small molecule feldipin more tightly than when the additional (auxiliary) binding sites are not occupied. Using microcalorimetry Milos et al. [15] further define these interactions, proposing that calcium and strontium have affinity for the primary sites, the EF hands, whilst zinc, manganese, copper and mercury bind specifically to the auxiliary sites. A third group, lanthanum, terbium, lead and cadmium can bind both to the capital and the auxiliary sites although with very different affinities. It has been

suggested that calmodulin may be a mediator of some of the toxic effects of these heavy metals [16]. The ability of heavy metals to displace or disrupt  $\text{Ca}^{2+}$  in CaM can be linked to their toxicity and the inability of the body to remove heavy metal ions is well reported. Analysis of the binding of several alternative divalent cations to calmodulin and their subsequent ability to retain the tertiary holo structure is investigated here using native electrospray ionization mass spectrometry (ESI-MS).

The use of ESI-MS to observe and analyze protein complexes has expanded rapidly in the last ten years, and either uses direct infusion methods to discover intrinsic properties of the complex or indirect methods [19] to probe solution properties [20, 21]. There are definite caveats which must be applied to the analysis of data obtained via direct infusion mass spectrometry. These are principally due to the effects of the ESI process on retaining non-covalence into the gas-phase, and as such direct infusion results are most appropriately considered to (at best) provide relative quantitation on a protein ligand system. Nonetheless, when compared with other techniques such as NMR and X-ray crystallography, ESI-MS provides rapid analysis, can be performed on a very small amount of sample, above all it possesses the ability to resolve the numerous components that can be present in a protein ligand solution. It has a particular suitability for the analysis of metal binding proteins, as shown by Loo [22], Fenselau [23, 24], and others. The mass of the metal ion and (with high-resolution MS) the isotope patterns therein can unequivocally identify which metal ions are binding, and even which oxidation state of the metal is interacting with the protein [25].

## Materials and Methods

### *Protein and Peptide Preparation*

Bovine calmodulin was prepared from *E. coli* cells expressing the protein as described previously [26] and stored as a freeze dried solid. Calmodulin was dissolved in ultrapure water at 20 mg/mL. Approximately 100  $\mu\text{L}$  was dialyzed before analysis against ammonium acetate (10 mM pH6.8) using Slide-a-lyzer dialysis cassettes from Pierce (Pierce Biotechnology, Rockford, IL). To chelate calcium from CaM, 0.4M EGTA was added to the fractions in the presence of 10 mM ammonium acetate. The sample was then passed along a 10DG column (Biorad Laboratories, Hercules, CA) and protein containing fractions pooled and concentrated on a spin cartridge concentrator. The concentration of protein in solution was calculated using Beer's law. Insufficient protein was available to calculate an extinction coefficient, and a value of 2906  $\text{L mol}^{-1} \text{cm}^{-1}$  at 280 nm was used [27]. UV absorbance was measured on an Eppendorf Biophotometer (Fisher Scientific, Loughborough, UK) using disposable Eppendorf UVettes (Fisher Scientific). Concentrations calculated by use of the extinction coefficient and Beers law are accurate to  $\pm 5\%$ .

Additional errors in concentration can arise from the small volumes of sample handled. Routinely, 1  $\mu\text{L}$  stock protein was diluted by 7  $\mu\text{L}$  of 10 mM ammonium acetate. These small samples were manipulated by Gilson micropipettes (Fisher Scientific, Loughborough, UK) which at worst case (0.5  $\mu\text{L}$  from a 2  $\mu\text{L}$  pipette) have an error of  $\pm 5\%$ .

All glassware and plastic ware were acid-washed with 2M HCl and rinsed with ultrapure water to minimize calcium contamination.

Ammonium acetate, calcium acetate, strontium acetate, magnesium acetate, cadmium acetate, and lead acetate were purchased from Sigma Aldrich, UK. Solutions of protein and metal salt were incubated at room temperature for 40 min before each experiment to allow the binding to equilibrate. No significant differences were seen in the stoichiometries of bound metal ions for a given test solution over the timescales of any experiment (typically 1–2 h of data collection).

The nNOS peptide with the sequence Ac-KRRRAIGFKKLA EAVKFS AKLM-NH<sub>2</sub> which forms the calmodulin binding region of neuronal Nitric Oxide Synthase (nNOS) [28] was synthesized and purified as described previously [1].

### Mass Spectrometry

Measurements were taken with a QTOF mass spectrometer (Micromass/Waters, Manchester, UK) equipped with a Z-spray source. Gold/palladium plated borosilicate glass nanoelectrospray needles (Proxeon Biosystems A/S, Odense, Denmark) were used to spray the sample into the mass spectrometer. Source conditions were kept the same for all experiments and consisted of: capillary (needle) voltage 800–1200 V, cone voltage 50 V, source temperature 80 °C. When measuring the full  $m/z$  range of the sample the quad was used in rf-only mode as a wide bandpass filter. Ions pass through the hexapole collision cell, which is pressurized to 10 psi with argon gas and pass into the TOF where they are detected on a microchannel plate detector. For collision induced dissociation experiments used here to probe the strength of metal ion binding, the energy of ions entering the collision cell is increased.

Data were collected using MassLynx software version 4.0 (Waters, Manchester, UK). A Savitzky-Golay smoothing algorithm was applied to raw data spectra. Data were deconvoluted to represent 'true mass' using the transform package supplied by MassLynx version 4.0. The instrument was calibrated daily before experiments using horse heart myoglobin (Sigma Aldrich).

## Results and Discussion

### Calmodulin with Calcium

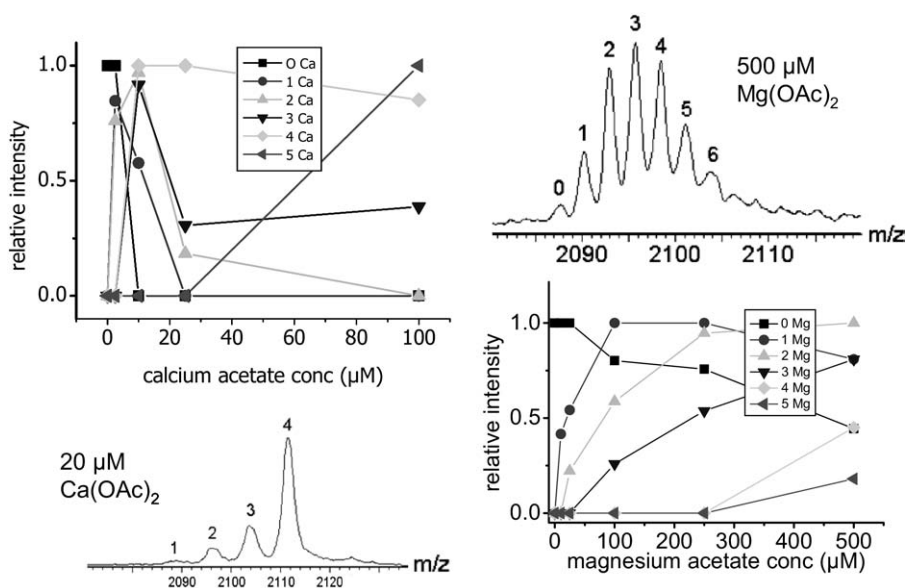
Initial studies were carried out on calcium ion incorporation to provide a standard for comparison with the other metal ion effects. Calmodulin was exposed to

increasing concentrations of calcium ions (Figure 1S in supplementary information, which can be found in the electronic version of this article). As published previously [1, 29] the charge state decreases as the number of bound calcium ions bound increases, and titration of  $\text{Ca}(\text{OAc})_2^-$ , provides evidence for both cooperative binding and the higher affinity of 1 pair of EF hands for calcium.

A sample of 50  $\mu\text{M}$  calmodulin in ammonium acetate, to which no extra calcium was added, was analyzed under the same nanospray conditions in positive and negative ionization. Different amounts of calcium are observed bound to calmodulin under the different polarities. Charge states  $-7$  to  $-9$  in negative ionization indicate the protein to be in a holo stoichiometry predominantly of the form CaM:4Ca (Figure 1S in supplementary data), however if the full charge ionization series is analyzed, all five stoichiometries exist. When the same sample is analyzed under positive ionization conditions the spectral distribution is distinctly different to the negative distribution and, calmodulin is predominantly found in the CaM:2Ca stoichiometry. Analysis of the full charged ion series under positive ionization conditions is similar to the negative; the protein is present with five distinct stoichiometries, i.e., with 0, 1, 2, 3, and 4 calcium ions bound. However, the positive ionization spectra show a higher degree of non specific metal adducts, particularly from sodium, which is absent in the negative ionization spectra [30] as a consequence all work reported here utilizes negative electrospray conditions.

As discussed above, the concentration of protein in these experiments has a certain error associated with it, and this is supported by our calcium titration experiments (Figure 1a). The CaM:4Ca species is the dominant form by 10–25  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2^-$ , with respect to a calmodulin concentration of  $\sim 8 \mu\text{M}$ , which is somewhat lower than expected. As discussed above, the most likely reason for the observed errors is in the method taken to calculate concentration and also in handling small amounts of protein.

Work by Moorthy et al. [31] utilizing negative ionization ESI-MS revealed the presence of a "special" fifth calcium ion bound to a calmodulin homologue only when the solution pH was close to 7. This correlates well with our data obtained at pH 6.8 (Figure 1a). A fifth calcium ion is also reported by Loo and coworkers [30] at very high calcium ion concentrations and described there as due to non specific binding. The concentrations of free  $\text{Ca}^{2+}$  here are less than the 1000-fold excess employed by Loo and coworkers, and the fifth calcium ion we observe is unlikely to be entirely non-specific as it was only present at pH 6.8, as seen by Murthy, it disappeared at higher pH values. This suggests the auxiliary sites also have differing affinities for calcium, which in turn vary as a function of calcium concentration. As shown below, in competition binding studies between calcium and magnesium, we observe up to seven calcium ions interacting with calmodulin.



**Figure 1.** Comparison of the effect of adding  $\text{Ca}(\text{OAc})_2$  to apo CaM ( $8 \mu\text{M}$ ) with adding  $\text{Mg}(\text{OAc})_2$  to apo CaM. (a) Shows a graph for increasing concentration of  $\text{Ca}(\text{OAc})_2$  versus conformations of CaM:xCa. The first spectra (0 M) were obtained post EGTA treatment, then at increasing concentrations of  $\text{M}(\text{OAc})_2$  as shown on the x axes. The points represent the intensity of the CaM:xCa species, each normalized to the most intense peak in the spectrum, the same method is used for subsequent metals. The inserts show the data at the  $-8$  charge state, for the calcium case after the addition of  $20 \mu\text{M}$   $\text{Ca}(\text{OAc})_2$  and for the magnesium case, after the addition of  $500 \mu\text{M}$   $\text{Mg}(\text{OAc})_2$ . The number of bound metal ions is indicated. For all data shown, spectra were acquired in negative ionization mode, with the concentration of CaM maintained throughout and buffered to pH 6.8 with ammonium acetate at  $10 \text{ mM}$ .

### Calmodulin with Magnesium

The impact of  $\text{Mg}^{2+}$  on  $\text{Ca}^{2+}$  binding to calmodulin is of considerable physiological relevance and has long been a matter of debate [13, 17]. Since primary protein targets of calcium must be able to respond in a background of 100- to 10,000-fold excess of  $\text{Mg}^{2+}$ , the interdependence of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  is required to be managed by the biochemistry of the cell.

Studies of magnesium and calmodulin show that magnesium does not cause the same conformational change as calcium [17] and ESI-MS has shown that there is no propensity for the CaM:4Mg stoichiometry [32]. Unlike calcium, which has a greater affinity for the C domain of calmodulin,  $\text{Mg}^{2+}$  has been shown to bind preferentially to the N domain [33]. Additionally, several groups report that the binding affinity of calcium to calmodulin decreases with raised magnesium concentrations [17, 33, 34]. This indicates either a direct competition between Mg and Ca in the Ca binding loop [34] or allosteric effector status for  $\text{Mg}^{2+}$  [17].

**Apo calmodulin and magnesium.** Following electrospray ionization, magnesium is observed to bind to apo calmodulin. In contrast to calcium, this does not result in any observable change in the charge state distribution which is the first indication that magnesium will not invoke any significant structural change on chelating to CaM. Additionally, as is clearly illustrated by a

comparison of Figure 1a and b, magnesium's affinity for CaM is less than that of calcium by  $\sim 2$  orders of magnitude. For the magnesium system with  $250 \mu\text{M}$  metal acetate, less magnesium is bound than that shown for calcium at  $20 \mu\text{M}$ . Increasing the concentration of magnesium acetate to  $500 \mu\text{M}$  does increase the mean number of magnesium ions bound; however there is no special propensity to bind four metal ions (Figure 1b). Overall, this infers that calmodulin has a lower affinity for magnesium than calcium and implies that the magnesium is not specifically binding to the EF hands.

With calcium, the uptake of four calcium ions occurs at the same concentration as the uptake of 3, and once 4 Ca ions have bound, the populations of the other forms decreases rapidly (Figure 1a top). This cooperative behavior is not replicated with magnesium, instead, the ions appear to add with similar affinities, (Figure 1b bottom).

**Calmodulin, calcium, and magnesium.** To probe the competition between calcium and magnesium for the metal ion binding sites on CaM, magnesium was added to calmodulin which had not been treated with EGTA to remove the calcium ions. The raw spectra (Figure 2S) show a slight change in the charge state distribution towards a distribution of peaks that are centered on lower charge states as  $\text{Mg}(\text{OAc})_2$  is added, indicative of



more native like forms. Deconvoluted spectra (Figure 3S) reveal addition of  $Mg^{2+}$  to CaM:4Ca, however no displacement of  $Ca^{2+}$  is observed, suggesting that calcium has a significantly higher affinity for the EF hands than magnesium. Some care must be taken in interpretation of the deconvoluted spectra since CaM:4Ca:2Mg has a similar mass to CaM:5Ca, the difference being unresolvable here.

*Calmodulin, calcium, magnesium, and nNOS peptide.* As we have previously shown by ESI-MS, [1] the linker peptide that forms the calmodulin binding region of nNOS shows complete selectivity in binding only to the CaM:4Ca form of CaM. To investigate the specificity of magnesium binding to CaM, a sample of CaM,  $Ca(OAc)_2$ ,  $Mg(OAc)_2$  and the nNOS peptide was analyzed. This heterogeneous mixture proved difficult to analyze, as is clear from the lower signal to noise present in Figure 4SB. Increasing the concentration of  $Mg(OAc)_2$  suppressed the detection of the protein and its complexes.

The deconvoluted spectrum shown in Figure 4SB indicates a mass increase for signal attributed to CaM:4Ca and CaM:4CaMg to  $\sim 19,200$  Da, corresponding to the addition of the nNOS peptide. This suggests that  $Mg^{2+}$  does not affect CaM:4Ca's ability to form complexes, but since it is only seen in addition to  $4Ca^{2+}$  it can be concluded that in the presence of calcium,  $Mg^{2+}$  binds in auxiliary sites. No evidence was found for a complex between apo CaM incubated with high (up to  $500 \mu M$ ) concentrations of  $Mg(OAc)_2$  and the nNOS peptide.

*Magnesium conclusions.* As found by other studies, magnesium ions have a lower affinity for CaM than calcium. Addition of magnesium ions to apo CaM failed to show any change in charge state distribution which would be indicative of a change in structural conformation and further we see no evidence for the formation of a CaM:4Mg stoichiometry over other CaM:xMg combinations. Vainiotalo and coworkers have similar results, stating no preference for a 1:4 stoichiometry of CaM:Mg [32]. They reported that the addition of Mg to calcium loaded calmodulin in the presence of a peptide that forms the calmodulin binding region in myosin light-chain kinase gives a decrease in intensity of CaM:Ca:peptide and an increase in the intensity of CaM:xMg species. It was extrapolated that  $Mg^{2+}$  displaced  $Ca^{2+}$  if present in high excess, but was unable to maintain the CaM:4Ca conformation needed to bind to the target peptide. Magnesium ions are reported to decrease the affinity of calcium for CaM [17, 33, 35] and the loss of signal of the nNOS peptide bound species as described above is probably due to a decreased affinity of calmodulin for calcium in the presence of magnesium, comparable to the results of Vainiotalo et al. The  $Mg^{2+}$  ion is up to 30% smaller than  $Ca^{2+}$  which may explain why it is unable to bind in a similar fashion to calcium ions. Since it has so much lower measurable affinity for the seven co-ordinate EF hands, perhaps it is simply too

small to remain in them. This is supported by the work of Chao and coworkers who report that several cations with significantly smaller radii, than  $Ca^{2+}$  including,  $Be^{2+}$  and  $Mg^{2+}$  do not promote the structural change found with  $Ca^{2+}$  [16].

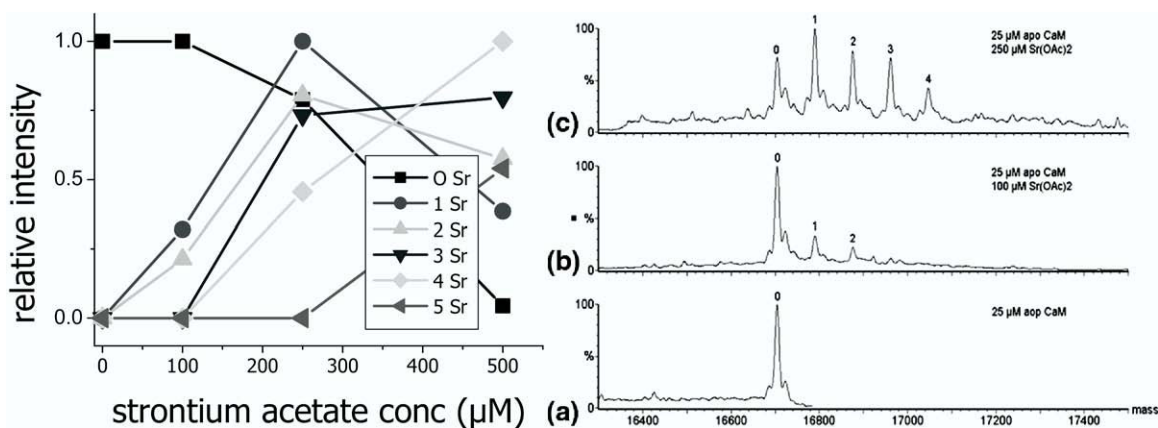
Magnesium has reputedly a higher affinity for the N terminal domain, unlike calcium which has a preferential affinity for the C domain [17]. There is little way of showing which site is bound first via direct infusion mass spectrometry as used here, although a study using a solution based HDX method such as PLIMSTEX [36] perhaps coupled with mutational approaches could reveal the succession of preferred binding sites.

Given that we see no indication of strong magnesium CaM interactions, it is perhaps not surprising that no structure of such a complex is available. NMR data is available for an Mg–Calbindin D9K complex, where the binding of magnesium, which adopts 6-fold coordination, is achieved in an EF hand. This study suggests that  $Mg^{2+}$  can be coordinated by the same ligands as  $Ca^{2+}$ , apart from one interaction, between the  $Ca^{2+}$  and a glutamic acid, which is imparted here by a water molecule [17]. The bidentate  $Ca^{2+}$  ligation by the side-chain carboxylate oxygens of this glutamic acid have been found to be crucial for  $Ca^{2+}$  induced structural rearrangement [37–39], and this provides credible support to our findings that  $Mg^{2+}$  will not induce the conformational change needed to allow CaM to bind to target enzymes.

### *Calmodulin and Strontium*

Like magnesium and calcium, strontium is a member of group II of the periodic table and therefore shares similar properties. However, unlike magnesium and calcium, strontium is not biologically essential in mammals, although it does exist as a trace metal in the human body [40]. Toxic symptoms due to overdosing of strontium have not been reported in humans; nevertheless intravenous administration of high doses of strontium induces hypocalcaemia—deficiency of calcium in the blood—due to increased excretion of Ca [41]. Possibly strontium is acting similarly to calcium in the body and this is the cause of these mild toxic effects.

*Apo calmodulin and strontium.* Increasing concentrations of  $Sr(OAc)_2$  were added to  $8 \mu M$  apo CaM, and analyzed by nanospray on the Q-ToF. There is no observable shift in the charged ion series to lower charge states to indicate a contraction in the tertiary structure of the protein as seen with calcium ions. However, deconvolution of the data, (Figure 2) shows that the CaM has an affinity for up to four strontium ions at a concentration of  $250 \mu M Sr(OAc)_2$ . This suggests that  $Sr^{2+}$  ions are binding into the EF hands. Increasing the concentration of available  $Sr^{2+}$  still further to  $500 \mu M Sr(OAc)_2$  promotes further binding of  $Sr^{2+}$  to CaM (Figure 2 left hand side) and there is a



**Figure 2.** Graph of increasing concentration of  $\text{Sr}(\text{OAc})_2$  versus species corresponding to  $\text{CaM}:\text{xSr}$ , for a solution of  $8 \mu\text{M}$  CaM. As for calcium and magnesium (Figure 3) the first spectra was obtained post EGTA treatment, the points shown represent the intensity of the  $\text{CaM}:\text{xSr}^{2+}$  species normalized to the most intense peak in the spectrum. For all data the spectra were acquired in negative ionization mode with the concentration of CaM maintained at  $8 \mu\text{M}$ . The right-hand side figure shows deconvoluted mass spectra from  $8 \mu\text{M}$  CaM obtained at different concentrations of  $\text{Sr}(\text{OAc})_2$  bottom 0, middle  $100 \mu\text{M}$ , and top  $250 \mu\text{M}$ . All solutions are buffered to pH 6.8 with ammonium acetate at  $10 \text{mM}$ .

propensity for the  $\text{CaM}:\text{4Sr}$  form, similar to that with  $\text{CaM}:\text{4Ca}$ . Formation of  $\text{CaM}:\text{4Sr}$  takes place at a much higher concentration of  $\text{Sr}(\text{OAc})_2$  ( $250 \mu\text{M}$ ) than the  $\text{CaM}:\text{4Ca}$  form. This suggests that CaM has at least a 5-fold lower affinity for  $\text{Sr}^{2+}$  than  $\text{Ca}^{2+}$ .

Figure 2 shows the relative intensity of each complex with increasing concentration of strontium. The binding plots indicate that there may be also cooperativity in the binding of the first and second strontium and the third and fourth strontium, similar to the cooperativity detailed in the calcium graph (Figure 1a). Binding of the first and second metal ion follows a similar trend, showing a similar degree of binding at  $100 \mu\text{M}$  Sr, both reaching maximum binding at  $200 \mu\text{M}$  before decreasing in intensity, as the  $\text{CaM}:\text{3Sr}$  and  $\text{CaM}:\text{4Sr}$  conformations start to form and dominate. Cooperativity between the third and fourth strontium ions binding is not as defined as that between the first and second, however it is still notable. The fifth strontium binding is similar to that seen with calcium binding; although at much higher concentrations.

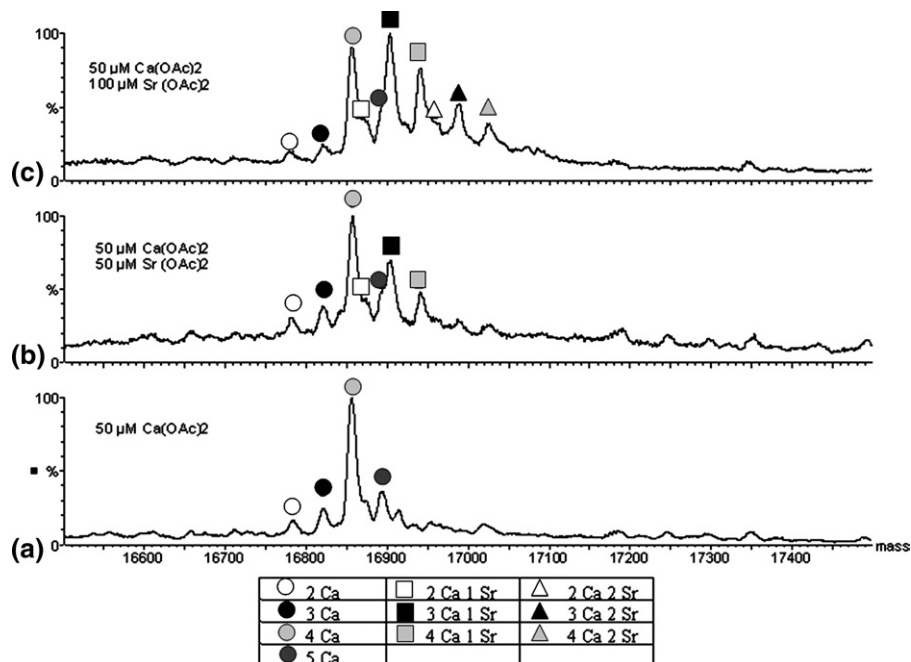
Overall the strontium binding graph is comparable with the calcium binding graph (Figure 1a), indicating that strontium may bind in a similar fashion to calcium in the EF hands.

*Calmodulin, calcium, and strontium.* To assess the competition between calcium and strontium,  $\text{Sr}(\text{OAc})_2$  was titrated into a sample of  $8 \mu\text{M}$  CaM and  $50 \mu\text{M}$   $\text{Ca}(\text{OAc})_2$ . As the strontium acetate concentration increases relative to the CaM concentration the number of strontium ions bound to the calmodulin also increases (Figure 3). However increasing the concentration of strontium does not have a marked effect on the overall charge distribution, suggesting no major changes in tertiary structure.

Addition of  $50 \mu\text{M}$  strontium acetate reveals the formation of  $\text{CaM}:\text{3Ca}:\text{1Sr}$  (Figure 3b). This complex may have formed via  $\text{CaM}:\text{3Ca}$ , or from  $\text{CaM}:\text{4Ca}$  after the displacement of a calcium. It is more likely to be by the latter, as  $\text{CaM}:\text{3Ca}$  still exists in the spectrum. Further addition of strontium acetate, to  $100 \mu\text{M}$  (Figure 3c), produces higher intensity peaks due to  $\text{CaM}:\text{xCa}:\text{ySr}$  complexes. The most abundant complex at this concentration appears to be  $\text{CaM}:\text{4Ca}:\text{2Sr}$ . This means a total of six metals bound and therefore auxiliary metal ion binding sites must be occupied. From the full charged ion series strontium ion binding appears to be greater at low charge states, (higher  $m/z$ ). This may indicate that the strontium is non-specifically binding to the complexes present at lower charge states.

*Calmodulin, calcium, strontium, and nNOS peptide.* The nNOS peptide was added to a sample of protein which has been exposed to calcium. Data obtained with  $50 \mu\text{M}$  peptide:  $50 \mu\text{M}$   $\text{Sr}(\text{OAc})_2$  showed clearly that the peptide binds to the protein. Before binding the peptide yielded a wide ion series with many  $\text{CaM}:\text{Ca}:\text{Sr}$  complexes, this altered to a much narrower distribution peaks due to  $\text{CaM}:\text{4Ca}:\text{nNOS}$ ;,  $\text{CaM}:\text{3Ca}:\text{Sr}:\text{nNOS}$  and  $\text{CaM}:\text{2Ca}:\text{2Sr}:\text{nNOS}$  are observed indicating that with one or two strontium ions bound, the tertiary structure of holo-calmodulin is retained and the hydrophobic linker between the N and C terminus domains is accessible for nNOS binding.

*Strontium conclusion.* From the data collected apparently strontium will bind to calmodulin in a similar fashion to calcium. A preference for the  $\text{CaM}:\text{4Sr}$  form is found at high concentrations (Figure 2 LHS). It however appears to have a lower affinity than calcium and will not displace calcium until present in excess. The prop-



**Figure 3.** The competition between strontium and calcium for binding sites in CaM. Shown are the deconvoluted mass spectra obtained from 8  $\mu\text{M}$  CaM in the presence of  $\text{Sr}(\text{OAc})_2$ . In (a) the protein has been incubated with  $\text{Ca}(\text{OAc})_2$  at 50  $\mu\text{M}$ . In (b) the spectra comes from a solution of 15  $\mu\text{M}$  CaM 50  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$  and 50  $\mu\text{M}$   $\text{Sr}(\text{OAc})_2$ , and in (c) 15  $\mu\text{M}$  CaM 50  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$  and 100  $\mu\text{M}$   $\text{Sr}(\text{OAc})_2$ . The key reveals the species present as identified by their mass. All solutions are buffered to pH 6.8 with ammonium acetate at 10 mM.

erties of  $\text{Sr}^{2+}$  are very similar to  $\text{Ca}^{2+}$ . Unlike  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$  has a VII coordination geometry, and although slightly larger than  $\text{Ca}^{2+}$ , this appears to enable strontium ions to mimic aspects of calcium binding to CaM although with a lower affinity.

The fact that we observe a preference for stoichiometries of  $\text{CaM}:\text{xCa}:\text{ySr}$  where  $x + y = 4$  indicates that strontium may well bind to the EF hands. Strontium does not appear to have a particularly strong affinity for auxiliary metal ion binding sites remote from the EF hands, although binding of more than four cations is seen at high concentrations of strontium. This correlates to the results of Milos et al. [13, 14] who state that calcium and strontium would bind selectively to the principal sites; other metals such as zinc, manganese, copper and mercury would bind specifically to the auxiliary sites and a third class including lanthanum, terbium, lead and cadmium could bind to both types. We also show that one or two strontium ions within a  $\text{CaM}:\text{4M}$  complex (where M is Ca or Sr) still maintains the defined tertiary structure of holo calmodulin so essential for formation of the  $\text{CaM}:\text{nNOS}$  complex [1].

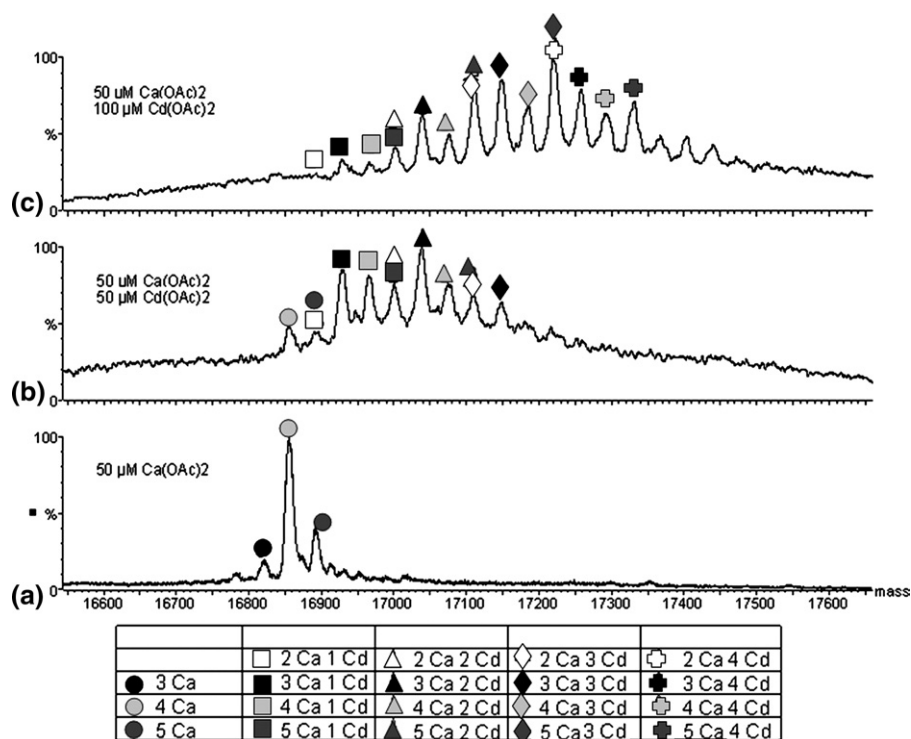
### Calmodulin and Cadmium

Cadmium has few useful biological functions, and is toxic to humans, probably because it competes with zinc for binding sites and can therefore interfere with some of zinc's essential functions. In this way, it may inhibit enzyme reactions and utilization of nutrients.

Cadmium may also be a catalyst to oxidation reactions, which can generate free-radical tissue damage. Although cadmium predominantly competes at zinc binding sites, there are reported effects of its interaction with calcium ion channels [42] and it is known to affect the bones causing bone and joint aches and pains. Cadmium toxicity threatens the health of the body by weakening the immune system by causing a decreased production of T lymphocytes (T cells) [43]. Calmodulin is involved in the signal cascade to produce T cells and therefore possibly elevated levels of cadmium interfere with calcium uptake in calmodulin. These symptoms of cadmium toxicity, and cadmium's similarity in coordination number and size to calcium [44] suggest that it may also compete for calcium binding sites.

*Apo calmodulin and cadmium.* Cadmium was added to calmodulin from which the metal ions had been chelated by EGTA. Increasing concentrations of  $\text{Cd}(\text{OAc})_2$  were added to 8  $\mu\text{M}$  CaM, and analyzed by nanospray on the Q-ToF. No binding of cadmium was seen to apo calmodulin, up to a concentration of 250  $\mu\text{M}$ . It appears from this result that cadmium does not have a strong affinity for either the EF hands or the auxiliary sites at these concentrations.

*Calmodulin, calcium, and cadmium.* To investigate whether cadmium is capable of displacing calcium, a sample of holo calmodulin was analyzed with varying concentrations of cadmium acetate. Figure 4a, shows the spectra



**Figure 4.** Deconvoluted spectra of holo CaM (10  $\mu\text{M}$  CaM 33  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$ ) (a) to which increasing concentrations of  $\text{Pb}(\text{OAc})_2$  have been added, (b) 3  $\mu\text{M}$ , (c) 8  $\mu\text{M}$ , and (d) 16  $\mu\text{M}$ .

of holo calmodulin, formed by the addition of 8  $\mu\text{M}$  CaM and 50  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$ , and Figure 4b and c show the addition of 50  $\mu\text{M}$  and 100  $\mu\text{M}$  amounts of  $\text{Cd}(\text{OAc})_2$  to this sample. Deconvolution of the data shows definite increase in mass of the CaM when exposed to cadmium ions. However interpretation of the spectra is difficult due to the similarities in mass of cadmium (112.41 u) and 3 calciums (120.23 u), which is further complicated by the error in mass introduced by the ‘assumption’ of the transform deconvolution software that all the charges are from protons. The deconvolution process means  $\text{Cd}^{2+}$  addition is shown as a 110.39 Da increase and  $3\text{Ca}^{2+}$  is shown as a 114.19 Da increase. The resolution of the Q-ToF instrument used for these experiments is insufficient to resolve overlapping masses with 4 Da difference when analyzing proteins with relatively high mass and consequently some peaks must be assigned to more than one stoichiometry conformation. Nevertheless, apparently cadmium displaces calcium. The peak at 17,040 Da (dark blue triangle) is assigned as CaM:3Ca:2Cd, and as the majority of the protein is initially in the CaM:4Ca form, it is probable that one of the calcium ions is displaced to form this complex. There is a possibility that this species is formed via the small amount of CaM:3Ca that is present in the initial solution, however the relative intensity of CaM:3Ca is small and is unlikely to account for the large relative intensity of the CaM:3Ca:2Cd peak. The addition of  $\text{Cd}^{2+}$  shifts the charge distributions towards  $-8$  suggesting that the additional  $\text{Cd}^{2+}$  is

filling the vacant EF hands and retaining the conformation found for CaM:4Ca.

Although the deconvoluted spectra give many permutations of  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$ , cadmium is definitely binding to CaM. However this data is somewhat inconclusive as to whether the cadmium is binding to the EF hand or auxiliary sites. For instance the peak at  $\sim 17,190$  could be CaM:1Ca:4Cd, but could also be CaM:4Ca:3Cd, or a superimposition of the two. In the former case, three of the cadmiums may be binding into the EF hands, and the fourth in an auxiliary site, although it is equally possible that the four cadmiums are all binding to auxiliary sites leaving three of the EF hands empty.

*Calmodulin, calcium, cadmium, and nNOS peptide.* To further probe whether CaM exposed to  $\text{Cd}^{2+}$  was forming the tertiary with the linker helix exposed available for binding to target proteins and peptides, nNOS was added to the sample. No change in the charged ion series or mass increase was seen upon adding nNOS to the sample; the deconvoluted spectra showed no increase in mass of 2433 Da, which would indicate binding of nNOS. Upon addition of the peptide the signal is suppressed slightly and poorer resolution is achieved. No CaM:4Ca:nNOS was observed either. In the sample before addition of nNOS, various combinations of CaM:xCa:yCd are observed which are difficult to clearly identify (see Figure 4b and c), and so possibly no CaM:4Ca is present. However even with nNOS concentrations of 100  $\mu\text{M}$  no complex was observed.



*Cadmium conclusion.* Whether cadmium binds to the calcium binding sites of calmodulin and competes with calcium is inconclusive from the mass spectrometry results. The apo CaM does not appear to bind  $\text{Cd}^{2+}$ , suggesting a low affinity to form the EF hands. When calcium is present,  $\text{Cd}^{2+}$  associates to CaM. The shift in the distribution of charge states to lower charges suggests that the  $\text{Cd}^{2+}$  may be complexing to the protein, reducing the negative charge. This is supported by the apparent displacement of  $\text{Ca}^{2+}$  by  $\text{Cd}^{2+}$  from the holo form of CaM. However when exposed to a proven peptide ligand, CaM exposed to  $\text{Cd}^{2+}$  does not form a complex. Cadmium ions have a very similar radius to calcium ions and the experiments carried out by Chao et al. indicated that this similarity allowed  $\text{Cd}^{2+}$  to bind to CaM and prevent  $\text{Ca}^{2+}$  binding [16].

Unlike calcium which is a hard acid,  $\text{Cd}^{2+}$  is a soft acid and therefore will have different preferential ligating groups. In any given EF hand sequence,  $\text{Cd}^{2+}$  will form different interactions than  $\text{Ca}^{2+}$  which may form outside of the consensus  $\text{Ca}^{2+}$  loop. Cadmium may therefore have a greater affinity than calcium for a non-EF hand site, which in turn prevents  $\text{Ca}^{2+}$  from binding to the EF hand sequence. The interactions it does form, do not allow the structural changes necessary to expose the linker peptide to which nNOS binds.

### *Calmodulin and Lead*

Lead toxicity is historically well documented; the fall of the Roman Empire has been attributed to lead pipes in the intricate plumbing of ancient Rome, and the usage for drinking vessels [45], its certain effect on humans, has prompted widespread removal of lead additives from petrol. One of the toxic pathways may be disruption of calcium mediated and calcium regulated functions. This may happen in a variety of ways: (1) direct interference with calcium transport; (2) indirectly altering cell functions required for calcium homeostasis; (3) by substituting for and mimicking the calcium ion at calcium binding sites [46]. The effects of lead on calmodulin are here investigated by mass spectrometry to probe whether lead will cause the same structural change to apo calmodulin as calcium does, whether lead will displace calcium, and whether lead bound calmodulin will still form complexes.

*Apo calmodulin and lead.* As previously, with magnesium, strontium, and cadmium, lead acetate was added to a sample of calmodulin (15  $\mu\text{M}$ ) from which all ions had been chelated with EGTA at various concentrations up to a ratio of 30:1 lead to calmodulin. No binding of lead to the apo calmodulin is observed until lead is in significant excess to calmodulin (>6:1) as can be seen from Figure 5S in the supplementary data.

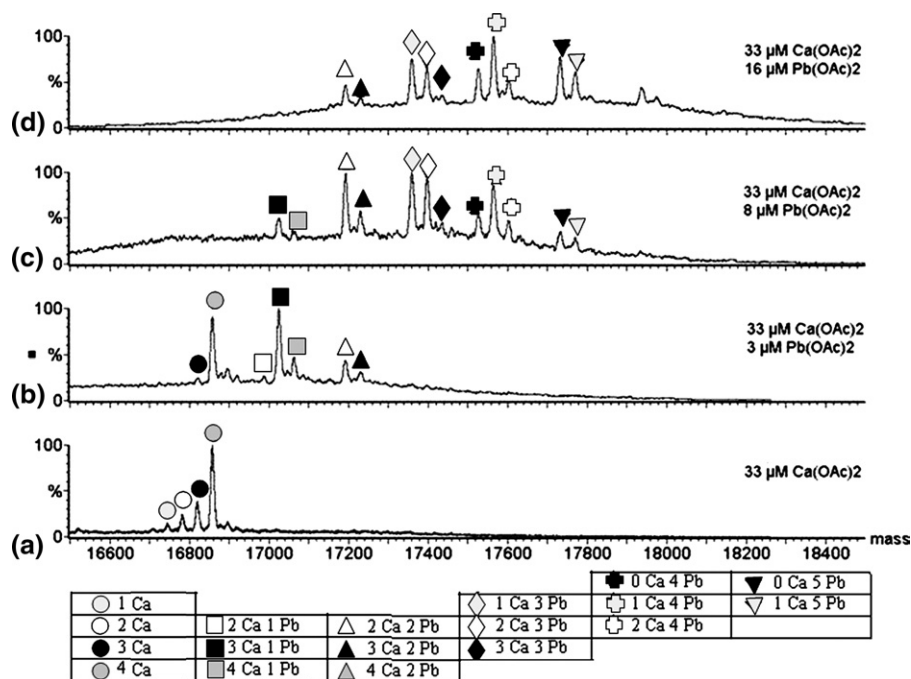
Lead does not appear to have a strong binding affinity for apo calmodulin, however if it is present in large enough excess it will bind to the protein. It is impossible to tell whether lead cations are binding to

the EF hands. At higher charge states there is an obvious preference for CaM:1Pb and CaM:2Pb, which suggests that there are sites which preferentially bind the lead cations, perhaps in one or other lobe of calmodulin. However at lower charges states, where the structure may have a more compact conformation, there are five or six atoms of lead bound to the calmodulin. This may be lead binding to auxiliary sites as suggested by Milos et al. [13, 14] or possibly to other unknown lead specific sites.

*Calmodulin, calcium, and lead.* Calmodulin from which calcium had not been chelated was exposed to lead acetate to probe the competition between calcium and lead (Figure 5). Figure 5a shows the spectra of holo calmodulin, formed by the addition of 10  $\mu\text{M}$  CaM and 33  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$ , and Figure 5b, c, and d show the addition of 3  $\mu\text{M}$ , 8  $\mu\text{M}$ , and 16  $\mu\text{M}$ , amounts of  $\text{Pb}(\text{OAc})_2$  to this sample, respectively. Unlike apo CaM, calciated CaM binds lead readily, and apparently also rapidly displaces calcium, since the peak intensities attributable to CaM: $x\text{Ca}$  decrease with addition of  $\text{Pb}(\text{OAc})_2$ . At equal concentrations of lead and calmodulin, several stoichiometries of lead calcium calmodulin complexes can be seen. Additions of higher concentrations of lead result in more of lead binding to the complex. Up to 6 metal ions are seen bound as shown in Figure 5. Above four metal ions, lead must be binding to sites other than the EF hands; of course it is plausible that even the first four lead ions do not bind to EF hands, although, as shall be shown below, the protein is configured to a form that allows nNOS binding. Lead calcium CaM spectra are easier to interpret than those of Cadmium, given that the larger mass of lead provides greater resolution between the peaks.

*Calmodulin, calcium, lead, and nNOS peptide.* To further probe the structure of the CaM:Pb:Ca complex, the nNOS peptide was added to a sample of calmodulin, which had previously been exposed to calcium and lead. A mass increase equivalent to the mass of the peptide is seen for a calmodulin sample exposed to lead and nNOSpep (Figure 6). This clearly shows the presence of the species CaM:4Pb:nNOSpep. Binding of the peptide to CaM: $x\text{Ca}$ : $y\text{Pb}$  pertains to the retention of the structure and exposed hydrophobic regions. This suggests that lead ions are capable of displacing calcium completely from any or all of the EF hands whilst maintaining the overall tertiary structure of holo CaM. This complex was only observed when calcium was initially present. The  $\text{Ca}^{2+}$  ions appear to configure the protein to allow the  $\text{Pb}^{2+}$  to bind to form a conformation that, has a preference for the CaM:4Pb form, and also allows chelation of the nNOS peptide.

*Lead conclusion.* Lead cannot bind to apo calmodulin unless present at high concentrations. Whether it binds to the EF hands of apo calmodulin cannot be discerned from this data, however we do not see a preference for

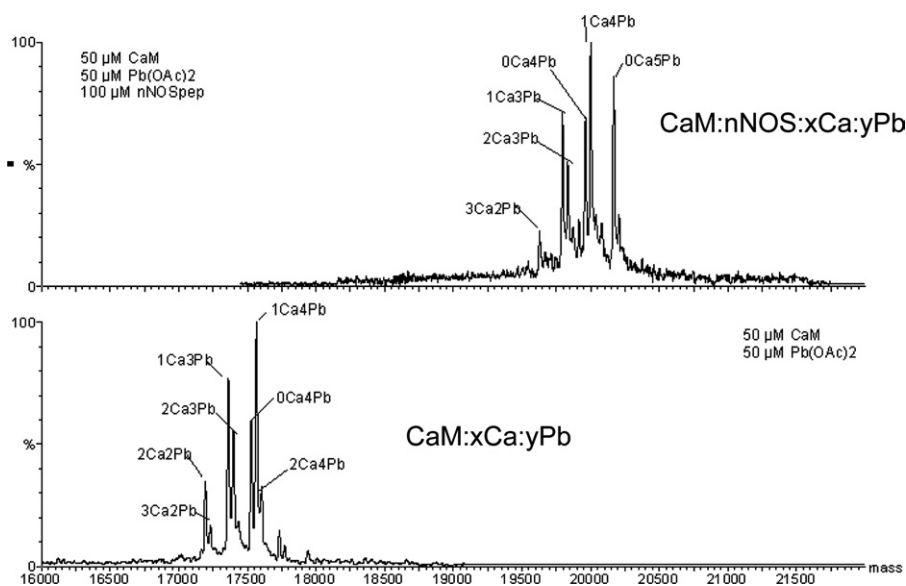


**Figure 5.** Holo CaM at 10  $\mu\text{M}$  CaM 33  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$  (a) to which increasing concentrations of  $\text{Pb}(\text{OAc})_2$  has been added, (b) 3  $\mu\text{M}$ , (c) 8  $\mu\text{M}$ , and (d) 16  $\mu\text{M}$ .

the CaM:4Pb form by titrating  $\text{Pb}(\text{OAc})_2$  into apo CaM. In contrast, lead binds to calcium bound calmodulin at significantly lower concentrations than is required to bind apo calmodulin, and will displace calcium from the EF hands even when free lead is at a lower concentration than calcium. There are other sites on calmodulin to which lead has an affinity when present in high concentration. Although lead has displaced calcium from the EF hand, the tertiary structure of holo calmod-

ulin is retained, as seen by the preservation of a complex with the specifically binding nNOS peptide, and indeed the observation of this complex proves that lead must be binding to the EF hands. In summary, divalent lead is not able to complex effectively to an EF hand by itself; however it has the ability to displace calcium once the EF hand has been formed.

The divalent lead cation has an ionic radii 25% larger than that of calcium. This does not seem to affect its



**Figure 6.** Deconvoluted spectra of 15  $\mu\text{M}$  CaM with 50  $\mu\text{M}$   $\text{Ca}(\text{OAc})_2$  and 50  $\mu\text{M}$   $\text{Pb}(\text{OAc})_2$  (a). (b) Shows the same solution with the addition of the nNOS peptide at 100  $\mu\text{M}$ . All of the protein in this sample has the nNOS peptide bound to it.

**Table 1.** Properties of divalent anions analyzed here by ESI-MS

Cation	Coordination geometry [44]	Ionic radii [44]	Hard or soft acid [50]
Ca <sup>2+</sup> MWt 40.08	VI, VII, VIII, IX, XI, XII	VII, 1.06 Å	Hard acid
Mg <sup>2+</sup> MWt 24.30	IV, V, VI, VIII	VI, 0.72 Å VIII, 0.89 Å	Hard acid
Sr <sup>2+</sup> MWt 87.62	VI, VII, VIII, IX, X, XII	VII, 1.21 Å	Hard acid
Cd <sup>2+</sup> MWt 112.41	IV, V, VI, VII, VIII, XII	VII, 1.03 Å	Soft acid
Pb <sup>2+</sup> MWt 207.2	IV, VI, VII, VIII, IX, X, XI, XII	VII, 1.23 Å	Borderline hard/soft acid

The ionic radii listed have been quoted from Shannon's 1976 paper [44], different sources do quote slightly different radii. For example in Chao et al.'s paper on activation of calmodulin by metal cations, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Sr<sup>2+</sup>, Cd<sup>2+</sup>, and Pb<sup>2+</sup> are quoted as 0.99, 0.65, 1.13, 0.97, and 1.20 Å, respectively; however it does not mention what coordination geometry these radii are for [16].

ability to fit into the EF hands, suggesting the EF hands are flexible and can expand to fit the larger ion. Vogel et al. used NMR to examine lead 207 binding to calmodulin [47], in this work they observe that the chemical environment occupied by the Pb<sup>2+</sup> ions in the presence of CaM is similar to that found for Ca<sup>2+</sup>, interestingly they also find that Pb<sup>2+</sup> ions bind simultaneously to the EF hands in apo calmodulin. The binding of lead to apo CaM contrasts slightly with our findings, although we are unable to perform experiments at the high concentrations they employed (0.1 M Pb(NO<sub>3</sub>)<sub>2</sub>). The fact that both methods indicate a similar chemical environment for Pb<sup>2+</sup> within the EF hands as for Ca<sup>2+</sup> can be explained by considering the acid/base characteristics of the metal ion protein system [50]. Lead cations are borderline acids, and share similar ligating properties to both hard and soft acids [50]. Therefore possibly Pb<sup>2+</sup> forms similar interactions as both Ca<sup>2+</sup> and Cd<sup>2+</sup>, maximizing the number of associations that can be made between the Pb<sup>2+</sup> ion and the EF hand. These interactions may prove stronger than those formed by calcium, evidenced by the fact that with the holo CaM system, very small levels of lead acetate can compete for the EF hands in CaM (Figure 5b and c) although not strong enough to initially shape the EF hand, Pb<sup>2+</sup> can displace Ca<sup>2+</sup> once it has formed.

## Conclusions

Along with divalency, the cations chosen here have at least one similar property to calcium, which may explain their ability to compete to some extent for calcium [16] These are summarized in Table 1. Despite this, all the metals examined here exhibit different behavior

when competing for the EF hands in calmodulin, and our major findings from this nano-ESI MS study can be summarized (Table 2):

1. The order of affinity for apo CaM is Ca<sup>2+</sup> >> Sr<sup>2+</sup> ~ Mg<sup>2+</sup> > Pb<sup>2+</sup> ~ Cd<sup>2+</sup>
2. In the presence of calcium the order of affinity alters to Pb<sup>2+</sup> > Ca<sup>2+</sup> > Cd<sup>2+</sup> > Sr<sup>2+</sup> > Mg<sup>2+</sup>
3. Sr<sup>2+</sup> appears to mimic the co-operativity of Ca<sup>2+</sup> binding to CaM; Mg<sup>2+</sup> does not
4. Pb<sup>2+</sup> and Ca<sup>2+</sup> form CaM:4M<sup>2+</sup> complexes with nNOS, the other metals will not

Several factors contribute to affinity between a binding loop and a metal ion including the loop size, which influences bond distance and bond strength to the encapsulated metal ion; the kind and number and positioning of donor groups in the loop; hydrophobicity of the loop; and metal ion coordination geometry. In addition, an exact consideration of competition between calcium and another metal ion would of course have to consider the k<sub>on</sub> and k<sub>off</sub> rates for the binding of each different metal. The results presented here provide a qualitative insight into calmodulin's affinity for divalent metal ions studied using direct infusion nano ESI-MS. Comparison of our major findings (Table 2) with the metal ion parameters in Table 1 does not reveal a single prevalent factor that dictates cation affinity for CaM. Chao and coworkers [48] suggest that ionic radii may be principally responsible for an ability to bind to CaM; however our results suggest that other factors such as coordination geometry and preferential ligating groups must play an equally important role in selectivity and affinity.

**Table 2.** Summary of the effect of the five different divalent cations on binding CaM

Divalent metal	Binds apo CaM	Replaces Ca <sup>2+</sup>	Auxiliary site binding	Holo form complexes to nNOS
Ca <sup>2+</sup>	High	—	Yes	Yes
Mg <sup>2+</sup>	High	No	Yes	No
Sr <sup>2+</sup>	High	No	Yes	No
Cd <sup>2+</sup>	Low	Yes	Yes	No
Pb <sup>2+</sup>	Low	Yes	Yes	Yes

$\text{Ca}^{2+}$  binding to CaM is here shown to be cooperative which agrees with several previous studies [30, 33, 49] and similar cooperativity is seen when  $\text{Sr}^{2+}$  binds to apo CaM. This strongly indicates that  $\text{Sr}^{2+}$  will mimic calcium ion binding in the absence of calcium. If calcium is available to CaM, then it will bind preferentially over strontium, and strontium will not displace calcium. This said, none of the other metals studied appear to mimic as calcium as effectively as strontium. Magnesium ions do not appear to bind in a cooperative manner, and there is no propensity for the CaM:4Mg stoichiometry. Even if  $\text{Mg}^{2+}$  do bind to the EF hands this does not cause the same structural change as with  $\text{Ca}^{2+}$ .

The heavy metals  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$  will not bind easily to apo CaM but act similarly when exposed to CaM:4Ca. They both are able to displace  $\text{Ca}^{2+}$  from an EF hand if present in excess over  $\text{Ca}^{2+}$  although lead does this with much more alarming rapacity. This may be one of their routes of toxicity, particularly at suboptimal concentrations of  $\text{Ca}^{2+}$ , which would provide an alternative explanation to the view that heavy metal toxicity affects cellular reactions by interacting with essential sulfhydryl groups of target enzymes [16]. The fact that lead and cadmium cannot chelate to the EF hands of apo CaM points to their softer acidity. These metals prefer to form longer weaker covalent bonds and presumably do not interact effectively as individual ions with the harder bases presented by the carboxylic groups in the Calcium binding loops. Once the site has been formed by  $\text{Ca}^{2+}$  then the higher coordination environment appears amenable to both metals.

Evidence of up to six auxiliary cations bound to CaM have been reported by several groups [13, 14, 18] by a variety of methods including mass spectrometry [13], where up to 10 cation sites are reported on calmodulin. Calas and coworkers [13] suggest that the discrepancy in the number of auxiliary sites between their results and that reported by Loo and coworkers [22], may be related to differences in ionization, and the use of negative mode and deprotonation of the sample. They propose that the protein tertiary structure can be modified by protonation and deprotonation and this can influence the non covalent binding of the ligand. It follows that the auxiliary sites are unable to bind  $\text{Ca}^{2+}$  at basic pH. Despite this, the allosteric effect of these auxiliary metal binding sites on calmodulin has been widely reported. Here we have analyzed calmodulin at near neutral pH, close to physiological conditions, and therefore optimal conditions if the auxiliary sites are of physiological importance. Auxiliary binding occurs for every metal examined, as with the competition for calcium sites the subtleties of this are metal dependent.

The caveat to the trends revealed here is that these experiments use direct infusion ESI-MS, which can affect the nature of the observed complexes and does not necessarily reflect conditions in solution. However, we have here demonstrated a method that can provide a relative scale in vacuo of affinity in vitro. These experiments take place in a controlled model environ-

ment and, of course, intercellular conditions are vastly more complicated. Mammalian cells contain millimolar glutathione, which is capable of chelating heavy metals such as cadmium and lead and metallothioneins, whose synthesis is induced by the presence of heavy metals also effectively bind heavy metals as if to protect the cell [16]. However, this may also be applied to the findings of any biophysical technique and we believe that the approach taken here shows promise for a rapid initial screen of the affect of unusual and in particular toxic metals in calmodulin and other protein systems.

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## Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2009.02.008.

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