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A structural insight into lead neurotoxicity and calmodulin activation by heavy metals

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Calmodulin is a calcium sensor that is also capable of binding and being activated by other metal ions. Of specific interest in this respect is lead, which is known to be neurotoxic and to have a very high affinity towards calmodulin. Crystal structures of human calmodulin complexed with lead and barium ions have been solved. The results will help in understanding the activation mechanisms of calmodulin by different heavy metals and will provide a detailed view of a putative target for lead neurotoxicity in humans.

1. Introduction

Calmodulin (CaM) is a prototypic EF-hand calcium-binding protein that acts by sensing calcium levels and binding to target proteins in a regulatory manner. Its importance is highlighted by its 100% sequence conservation across all vertebrates. Although CaM is best characterized by its ability to specifically bind Ca^{2+}, a number of studies have indicated that in fact it can also be activated by other metal ions (Habermann *et al.*, 1983; Chao *et al.*, 1984; Richardt *et al.*, 1986; Ouyang & Vogel, 1998; Ozawa *et al.*, 1999). The most studied of these is Pb^{2+}, which has a high affinity towards CaM and is able to activate it even at low concentrations (Habermann *et al.*, 1983; Richardt *et al.*, 1986; Kern *et al.*, 2000). Ba^{2+} and Mg^{2+} have been reported to have significantly lower, if any, affinity towards CaM (Ozawa *et al.*, 1999). For example, the CaM-regulated activation of cerebellar nitric oxide synthase requires an over 200-fold higher concentration of Ba^{2+} than of Ca^{2+} (Yamazaki *et al.*, 1996) and the interaction between CaM and caldesmon is weakened by the exchange of Ca^{2+} for Ba^{2+} (Huber *et al.*, 1996). On the other hand, Mg^{2+} levels in the cellular environment are much higher than those of Ca^{2+} and CaM must be able to function specifically in the presence of an excess of Mg^{2+} ions (Malmendal *et al.*, 1999).

To a large extent, lead seems to be able to substitute for calcium in the regulation of CaM function (Habermann *et al.*, 1983; Fullmer *et al.*, 1985) and many target proteins are indeed activated by Pb-CaM similarly to Ca-CaM (Chao *et al.*, 1995). The effects of Pb have also been reported to present concentration-dependence, such that the activation of CaM by Pb^{2+} peaks in the 30–500 μM range and higher Pb^{2+} concentrations again activate CaM less effectively (Sandhir & Gill, 1994; Weaver *et al.*, 2002).

Heavy metals may accumulate in the body and cause disease states even at low concentrations (Cline *et al.*, 1996; Lidsky & Schneider, 2003). Lead exposure can, for example, cause neurological impairment and it is generally thought that lead is able to interfere with various calcium-dependent processes in the body. In the nervous system, lead affects, among others, CaM-regulated processes, neurotransmitter release and protein kinase C (Simons, 1993; Marchetti, 2003). Low-level lead exposure also affects neuronal growth (Cline *et al.*, 1996). One specific target for Pb poisoning can be CaM; Pb^{2+} binding to CaM may falsely activate it (Verity, 1990; Goering, 1993; Ouyang & Vogel, 1998; Kern *et al.*, 2000), leading to an abnormal response.

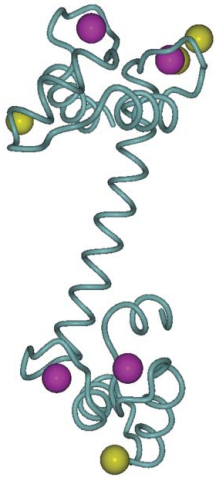

Table 1
Data processing and structure refinement.

Soaked ion	Pb	Ba
Unit-cell parameters (Å, °)	a = 24.6, b = 30.0, c = 53.6, α = 88.6, β = 87.0, γ = 83.0	a = 24.4, b = 29.3, c = 53.3, α = 89.8, β = 85.7, γ = 82.8
Resolution range (Å)	20–2.15 (2.25–2.15)	20–2.2 (2.3–2.2)
Completeness (%)	95.2 (90.2)	94.1 (95.7)
$\langle I/\sigma(I)\rangle$	8.2 (4.3)	12.3 (8.1)
R_{sym} (%)	7.6 (24.6)	4.9 (9.4)
Redundancy	2.2 (2.2)	2.1 (2.1)
R_{cryst} (%)	18.8	24.3
R_{free} (%)	25.3	29.4
R.m.s.d. bond length (Å)	0.011	0.007
R.m.s.d. bond angle (°)	1.3	1.0

We used Ca-CaM in the crystal state to compare the binding of two heavy metals, Pb and Ba, to CaM. Previously, a crystal structure of Pb-CaM has been reported from *Paramecium tetraurelia* and CaM was highly disordered in the crystals obtained (Wilson & Brunger, 2003). We used recombinant human (vertebrate) CaM in order to obtain results that were more relevant to vertebrate biology. The results also have relevance to Pb neurotoxicity, giving a structural insight into how Pb can effectively substitute for Ca in a molecule, specifically CaM, that is central to several regulatory processes.

2. Materials and methods

2.1. Protein expression, purification and crystallization

CaM was expressed from the expression vector pETCM (Hayashi *et al.*, 1998). pETCM was transformed into *Escherichia coli* Rosetta (DE3) and expression was carried out at 310 K in LB medium containing 100 µg ml^{-1} ampicillin. Induction was performed for 12 h with 0.4 m*M* IPTG and the cells were harvested by centrifugation. The cells were suspended in 50 ml cold lysis buffer (10 m*M* HEPES pH 7.5, 1 m*M* EDTA, 1 m*M* NaN_3, 10 m*M* DTT, 10 µg ml^{-1} DNase I) and lysozyme and RNase A were added to 100 and 2 µg ml^{-1}, respectively. Cells were disrupted by sonication and after centrifugation (27 000*g*, 20 min) the supernatant was heated in a 353 K water bath for 10 min and centrifuged again. 5 m*M* $CaCl_2$ was added to the supernatant and the sample was centrifuged once more.

The soluble proteins were applied onto a phenyl Sepharose column (Pharmacia Biotech) equilibrated with 10 m*M* HEPES pH 7.5, 4 m*M* $CaCl_2$. The column was washed with excess equilibration buffer, equilibration buffer supplemented with 500 m*M* NaCl and equilibration buffer again. Elution of CaM was then carried out with 10 m*M* HEPES pH 7.5, 5 m*M* EGTA. The fractions were analyzed by SDS–PAGE and those containing CaM were pooled. The purified protein was dialysed into 50 m*M* HEPES pH 7.5, 20 m*M* $CaCl_2$ and concentrated to 30 mg ml^{-1} by centrifugal ultrafiltration.

Crystals were grown using well characterized CaM crystallization conditions. Briefly, crystals were grown at 277 K using the hanging-drop method over a well of mother liquor consisting of 40–50% MPD and 0.1 *M* sodium acetate pH 4. Drops were prepared by 1:1 mixing of the CaM solution and the well solution, giving a starting concentration of 10 m*M* for Ca ions. Crystals suitable for diffraction studies appeared within a few days.

2.2. Crystal treatment and data collection

Prior to data collection, the crystals were soaked in metal solutions by transferring them to drops of the mother liquor containing either 10 m*M* $BaCl_2$ or 3 m*M* $PbCl_2$. The soaking time was 24 h at ambient temperature.

Owing to the high concentration of MPD in the crystallization solutions, no additional measures were taken for cryoprotection of the crystals. Data were collected at 100 K on beamline I911-2 (Mammen *et al.*, 2004), MAX-lab, Lund, Sweden at a wavelength of 1.0408 Å. The data were processed using *XDS* (Kabsch, 1993) and

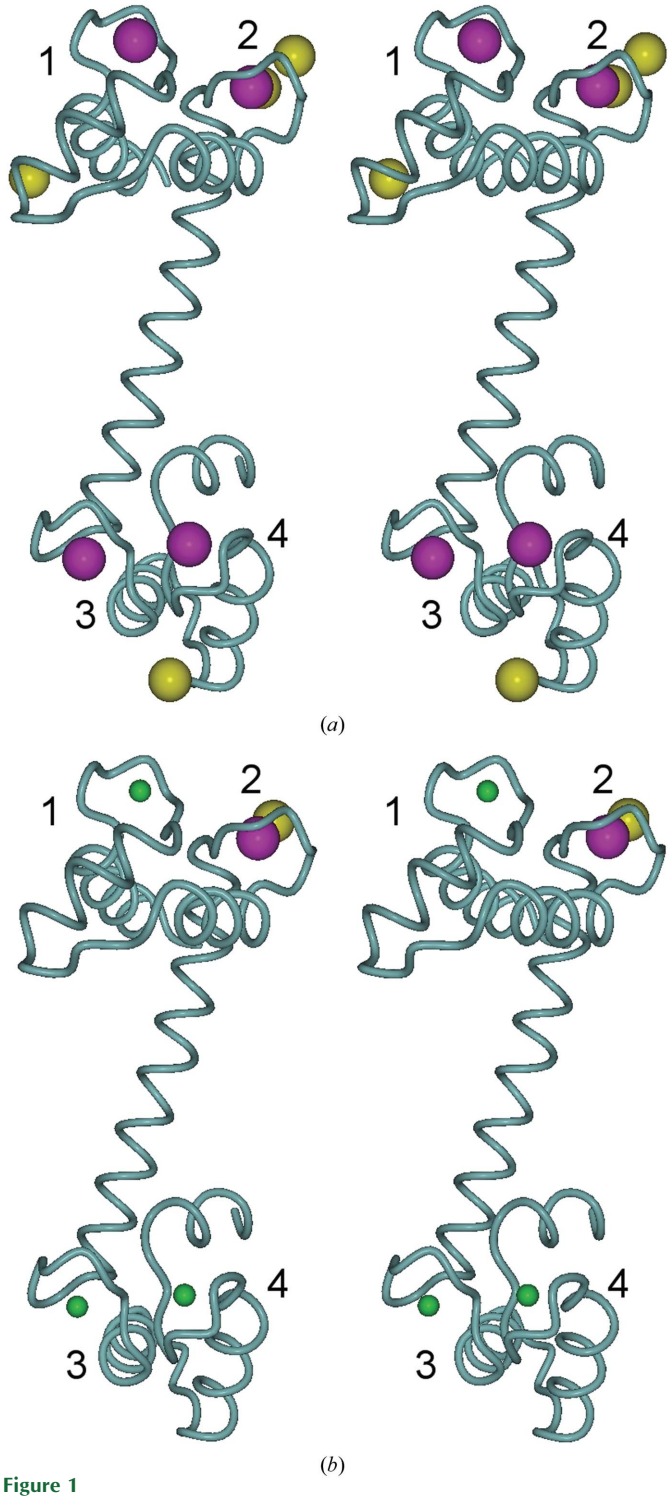

Figure 1
Stereoviews of the overall structures of the complexes and the locations of the bound heavy-metal atoms: (*a*) the Pb complex, (*b*) the Ba complex. Small green spheres indicate Ca atoms, large magenta spheres indicate soaked heavy-metal atoms bound to the EF-hands and yellow large spheres indicate heavy-metal atoms bound outside the EF-hands.

XDSi (Kursula, 2004) and the data-processing statistics are shown in Table 1.

2.3. Structure solution and refinement

The structures were solved by molecular replacement (Vagin & Teplyakov, 1997) using the structure of Ca-CaM (PDB code 1up5) (Rupp *et al.*, 1996) as a template. During refinement, TLS parameters were applied in *REFMAC* (Murshudov *et al.*, 1997). Model building was performed in *Coot* (Emsley & Cowtan, 2004). The Pb and Ba ions were identified by their very strong electron density. Anomalous difference maps were calculated using the phases from the final refined model and the anomalous differences of the measured data in order to validate the locations of the heavy-metal atoms. The refinement statistics are summarized in Table 1.

3. Results and discussion

3.1. The Pb complex

Lead has been reported several times to be the element most able to act like Ca in CaM activation. Thus, it was expected that Pb binding to CaM could be observed even in the Ca-CaM crystal state.

Indeed, all four EF-hands in the Pb-soaked structure have Pb bound instead of Ca (Fig. 1). No significant structural rearrangements occur upon replacement and the coordination geometry is highly similar, further indicating that Pb can act like Ca in binding and activating CaM. The fact that all Ca atoms were substituted by Pb proves that all the Ca-binding sites are accessible in the crystal state. While the general coordination distance between protein O atoms and Ca is around 2.3 Å, the distances in the Pb complex vary between 2.0 and 2.7 Å; the overall coordination does not change significantly when compared with Ca-CaM.

In addition, a total of four Pb atoms were detected in other sites. Interestingly, one of these is located close to EF-hand 2, being coordinated by Asp58 and Asp64. The other sites are scattered around the CaM surface, interacting with negatively charged CaM surface residues. This may be a mechanism for the reported decrease in CaM activation by Pb at higher concentrations. Since no such Ca atoms are observed upon crystallization in Ca-containing solutions, the affinity of Pb for non-EF-hand sites in CaM seems to be higher than that of Ca.

3.2. The Ba complex

The data from crystals soaked in barium chloride give evidence for the substitution of Ca in EF-hand 2 by Ba, but not in any of the other EF-hands (Fig. 1). This is in line with the reported requirement of a very high Ba concentration for CaM activation. The high number of electrons in a Ba atom makes it very easy to spot Ba atoms in electron-density maps; thus, it can be confidently concluded that in Ca-CaM crystals EF-hand 2 is the only one amenable to substitution by Ba. This may be partly related to the much larger ionic radius of Ba^{2+} (1.35 Å) when compared with Ca^{2+} (0.99 Å) or Pb^{2+} (1.19 Å). The coordination of the Ba^{2+} ion is similar to that of Ca^{2+} and the coordination distances between the ion and the O atoms of CaM EF-hand 2 range between 2.5 and 2.8 Å. The coordination distances for Ca^{2+} in CaM are usually 2.3 Å.

Another Ba atom was detected in the close vicinity of that bound to EF-hand 2, being coordinated by Asp58 and Asp64. It is possible that this site is partially occupied, as evidenced by the weaker electron density and higher temperature factor. The identities of both Ba atoms were confirmed by the presence of high peaks, approximately 12σ and 6σ in height, in the anomalous difference map calculated from the final refined phases and anomalous differences from the measured data (Fig. 2). No anomalous peaks were detectable at the other calcium-binding sites, further confirming that only EF-hand 2 bound Ba in the crystal state.

4. Concluding remarks

In this paper, we have presented the first structural analysis of heavy-metal binding to human (vertebrate) CaM. Since the starting point was Ca-CaM in the crystal state, it was expected that no large structural changes would be seen upon soaking in other metal ions. However, clear differences were seen between Pb and Ba in their ability to substitute for Ca in CaM crystals.

Our results confirm previous studies showing different affinities of various metal ions towards CaM. While Pb has been shown to activate CaM effectively and in fact has a higher affinity than Ca (Fullmer *et al.*, 1985; Richardt *et al.*, 1986; Ouyang & Vogel, 1998), Ba activation requires an around 200-fold higher concentration than Ca for CaM activation (Kreye *et al.*, 1986; Yamazaki *et al.*, 1996). These observations can be explained at least in part by the variations in the ionic radii of these ions and by the differences in metal-ion coordination distances in the EF-hands in the complexes: these would be expected to be reflected in the actual affinity. On the other hand, the coordination geometry for both Ca, Ba and Pb is similar in the EF-hands, indicating similar mechanisms when CaM is activated by different metals.

The Ca affinities of the different EF-hands in CaM vary and the affinities for EF-hands 2 and 4 are the lowest (Ye *et al.*, 2005). Interestingly, these EF-hands have one fewer negatively charged residue coordinating the metal ion than do EF-hands 1 and 3. This may be one reason why Ba binding is preferentially seen in EF-hand 2.

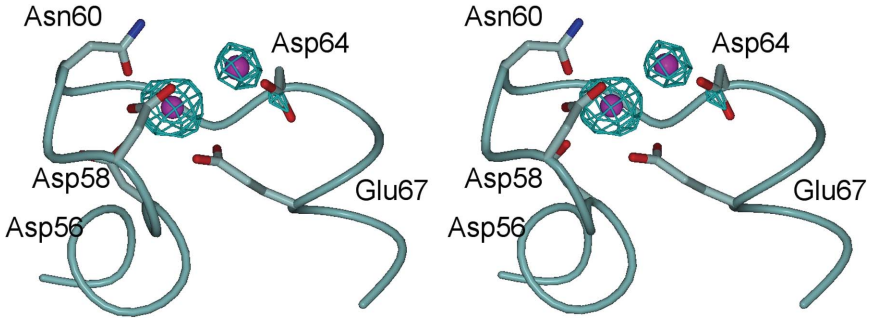

Figure 2
Anomalous difference map (cyan) around the bound Ba atoms (magenta) at EF-hand 2 contoured at 5σ.

In the Pb complex, non-EF-hand binding sites were also observed. These sites are probably involved in the inactivation of CaM at high Pb concentrations. Thus, if CaM is indeed a major target for lead neurotoxicity as has been proposed (Verity, 1990; Goering, 1993; Ouyang & Vogel, 1998; Kern *et al.*, 2000), the toxicity-related activation mechanism of CaM by lead may have two aspects: hyperactivation at low concentrations of Pb and inactivation at high concentrations.

For both Pb and Ba, a heavy-metal ion sits just outside the metal-binding site of EF-hand 2, being coordinated by two of the same residues, Asp58 and Asp64, as the bound metal ion in the EF-hand. Looking at the EF-hand, this ion lies in the direction from which the metal ion should enter its binding pocket; it is possible that this second ion at EF-hand 2 represents a state where an ion is just about to be bound/released.

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