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A 1.3Å Structure of Zinc-bound N-terminal Domain of Calmodulin Elucidates Potential Early Ion-binding Step

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Summary

Calmodulin (CaM) is a 16.8 kDa calcium binding protein involved in calcium-signal transduction. It is the canonical member of the EF-hand family of proteins, which are characterized by a helix-loop-helix calcium-binding motif. CaM is comprised of N- and C-terminal globular domains (N-CaM and C-CaM), and within each domain there are two EF-hand motifs. Upon binding calcium, CaM undergoes a significant, global conformational change involving reorientation of the four helix bundles in each of its two domains. This conformational change upon ion binding is a key component of the signal transduction and regulatory roles of CaM, yet the precise nature of this transition is still unclear. Here, we present a 1.3Å structure of zinc-bound N-terminal calmodulin (N-CaM) solved by single wavelength anomalous diffraction (SAD) phasing of a selenomethionyl-N-CaM. Our zinc-bound N-CaM structure differs from previously reported CaM structures and resembles calcium-free apo-CaM despite the zinc binding to both EF-hand motifs. Structural comparison with calcium-free apo-CaM, calcium-loaded CaM, and a crosslinked calcium-loaded CaM suggests that our zinc-bound N-CaM reveals an intermediate step in the initiation of metal ion binding at the first EF-hand motif. Our data also suggests that metal ion coordination by two key residues in the first metal-binding site represents an initial step in the conformational transition induced by metal binding. This is followed by reordering of the N-terminal region of the helix exiting from this first binding loop. This conformational switch should be incorporated into models of either step-wise conformational transition or flexible, dynamic energetic state-sampling based transition.

Keywords

Calmodulin; EF-hand motif; metal ion binding; metal-ion induced conformational switch; X-ray crystallography; SAD phasing

Introduction

The complex processes of intracellular calcium ion (Ca²⁺) signaling rely heavily on members of the EF-hand protein family for signal transduction and regulation¹. These proteins contain a helix-loop-helix calcium-binding EF-hand motif and most undergo a substantial

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Protein Data Bank accession number: The atomic coordinates of Zn-trapped N-terminal Calmodulin have been deposited in the RCSB Protein Data Bank with accession number 2PQ3.

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conformational change upon binding calcium. Examples of this transition between Apo (Ca^{2+} -free) and Ca^{2+} -loaded EF-hands include calmodulin, troponin C, and members of the S100 family². Calmodulin (CaM), a highly conserved 148 amino acid protein, has been extensively studied due to its ability to bind to and modulate a large number of target proteins, in conjunction with its involvement in signal transduction of Ca^{2+} signals. The ubiquitous involvement of CaM in such a diversity of cellular processes is thought to stem from its ability to sense Ca^{2+} with a range of affinities that correlate to intracellular Ca^{2+} levels in most cells ($K_d = 5 \times 10^{-7}\text{M}$ to $5 \times 10^{-6}\text{M}$)^{3; 4; 5}. It is able to selectively bind Ca^{2+} despite the fact that in the resting cell, there are high levels of other cations, especially the divalent cation magnesium (Mg^{2+}) which is present in concentrations between 0.4–1mM (roughly 100–10,000-fold higher than intracellular Ca^{2+} concentrations). CaM also uses many different binding modes to interact with its sequence- and structure-diverse targets⁶. Such binding could lead to the activation of its effectors by mechanisms of the removal of auto-inhibition, dimerization, or catalytic site rearrangement⁷.

Extensive studies both by X-ray crystallography and NMR^{8; 9; 10} have revealed the conformational switch from apo-CaM to Ca^{2+} -loaded CaM in atomic details. CaM possesses two globular N- and C-terminal domains, termed N- and C-CaM, that each contain two EF-hand motifs^{11; 12}. A four-helix bundle in N-CaM is tethered to a four-helix bundle in C-CaM by a flexible linker. Ca^{2+} binding occurs in the loop region of all four EF-hand motifs. Each Ca^{2+} ion is coordinated by the side-chain oxygens of four residues in the DXDXD/NXXXXXXE consensus sequence and one main-chain carbonyl oxygen from the residue at the 7th position. Upon Ca^{2+} binding, CaM undergoes a large conformational change involving reorientation of the helices in each domain. The coordinated movement of the two EF-hand motifs in N- and C-CaM triggers the conformational switch from a compact, “closed” state to an “open” conformation, in the process exposing a large hydrophobic pocket¹³. This hydrophobic pocket, rich in methionines, is thought to contribute to CaM’s sequence-independent recognition of target proteins.

However, the structural details for the transition from apo-CaM to Ca^{2+} -loaded CaM remain unclear. The importance of this structural transition has been emphasized across the EF-hand protein family regarding its role in target recognition and calcium signal transduction. Discussion of this structural transition in calmodulin has centered around highly specific, stepwise transitions¹⁴ or models where apo-CaM samples a flexible, dynamic range of conformations at specific sites^{13; 15}. The interaction of zinc ion (Zn^{2+}) with CaM has several unique properties. While Zn^{2+} binds CaM, it does not induce the global conformational change of CaM¹⁶. Here, we report a zinc-bound N-CaM structure at 1.3Å resolution. The comparison with apo-CaM, Ca^{2+} -loaded CaM in its open conformation, and Ca^{2+} -loaded CaM in its closed conformation suggests that our structure may represent an early intermediate step in the metal binding and may provide new structural insights into the transition between apo-CaM and Ca^{2+} -loaded CaM.

Results and Discussion

Structural Determination of N-terminal Calmodulin

Bordetella pertussis, the etiologic agent of whooping cough, secretes a CaM-activated adenylyl cyclase toxin named CyaA. Both N-CaM and C-CaM, respectively can potently activate the catalytic activity of this toxin. We have solved the structure of the C-CaM bound adenylyl cyclase domain of CyaA, providing structural insight into the molecular basis of CaM-mediated toxin activation¹⁷. However, the structural basis by which N-CaM is able to contribute to a 400-fold increase in activation remains elusive. An attempt to crystallize N-CaM bound CyaA yielded instead a crystal containing N-CaM alone, which diffracted to 2.0Å resolution. The lack of a CyaA/N-CaM complex was suspected by the small size of the unit cell and confirmed

by SDS-page analysis of a dissolved crystal. Efforts to solve this structure using molecular replacement with both Phaser and AMoRe¹⁸ and using multiple search models (1J7O13, 1UP5¹⁹, CFC⁹, and 1F54²⁰) failed. We therefore suspected the presence of a previously unreported conformation and proceeded to use selenomethionyl N-CaM to obtain experimental phases through the technique of Single Wavelength Anomalous Diffraction (SAD).

Crystals of selenomethionyl-N-CaM grew at 4 °C in a solution containing 16% PEG8000, 100 mM sodium cacodylate (pH 6.3) and 5 mM ZnCl₂. Crystals diffracted to 1.3 Å and formed a tetragonal lattice with space group *P*4₃2₁2 containing a single molecule in the asymmetric unit. Refinement was performed with REFMAC followed by anisotropic thermal displacement factors using Shelx97. Final data statistics of the model converged at *R*_{work}=14.2%, *R*_{free}=19.6% and are listed in Table 1.

We observe clear electron density for residues 2–76 (Figure 1A), which includes the two helix-loop-helix motifs expected for N-terminal CaM (Figure 1B). Two Zn²⁺ ions as well as a cacodylate molecule, which is involved in Zn²⁺ coordination, are found in this structure (Figure 1B). The identity of these two Zn²⁺ ions and arsenic atom of cacodylate was confirmed by strong anomalous signals from data collected at both the zinc (Figure 2A) and arsenic edges (Figure 2B). The crystal packing is unusual for a CaM structure. Briefly, along one axis, molecules of N-CaM are arranged in a head-to-head fashion in the crystal lattice such that the first ion binding site has direct symmetry contact with its corresponding site in the symmetry related molecule (Figure 2C). This allows cacodylate to coordinate two Zn²⁺ ions from the first EF hand motif within two symmetry related partners.

Noting that Ca²⁺ is the most commonly cited metal ion ligand of calmodulin, we attempted to soak in Ca²⁺ during the crystallization process. However, high concentrations of CaCl₂ (100mM) caused the crystal to shatter. We therefore gradually reduced the Ca²⁺ to a concentration that allowed us to preserve the N-CaM crystal (5mM CaCl₂) and collected data that diffracted to 1.6 Å (Table 1). We then determined this structure and found it to be nearly identical to the non-Ca²⁺-soaked crystal structure. Interestingly, using the Fast Fourier Transform operation in CNS to generate anomalous difference maps from signals collected at the zinc and arsenic edges, we found similar ligand occupation ratios in the non-Ca²⁺-soaked and Ca²⁺-soaked crystals (Supplemental Figure 1). We concluded that this structure still contained Zn²⁺, and therefore from here on, we will use only the non-Ca²⁺ soaked version of N-CaM for our structural analysis.

Structural Characterization of zinc-bound N-terminal domain of calmodulin

The overall structure of N-CaM is comprised of a four-helix bundle making internal contacts as well as contacts with symmetry related molecules (Figures 1B, 2C). The first Zn²⁺ ion is tetrahedrally coordinated by two aspartate residues (Asp22 and Asp24 of the first calcium binding loop), which are known to coordinate Ca²⁺ ion in the Ca²⁺-loaded CaM structure²¹, and by two cacodylate molecules, the arsenic buffer used in the crystallization conditions. There is also one highly ordered water molecule (*sigma*>6.0 in the Fo-Fc map) in site one coordinated by several residues normally involved in metal ion binding (Thr26 and Asp20 of the first calcium binding loop). The second Zn²⁺ ion is also tetrahedrally coordinated by residues Glu67 (normally involved in coordination) and Asp64 of N-CaM as well as by two residues (Glu7 and Glu11) of the symmetry related molecule (Figure 2C). Thus, four of five residues of the second calcium binding loop normally involved in calcium binding are not liganded to the second zinc ion. This Zn²⁺ ion also shows significant anisotropy and is best modeled as one Zn²⁺ ion with multiple occupancy (Figure 1B).

Within both EF-hand domains, the integrity of the helical secondary structure is intact, noting that there is a slight deviation from alpha-helical structure in the N-terminal region of helix B.

The two metal-binding loops are largely in the un-liganded conformation, with three of five residues from binding site one and four of five from binding site two not actively coordinating metal due to large distance and orientational constraints. Between these pair-mate EF-hands, there is a region containing four hydrogen bonds maintained between the backbone carbonyls and nitrogens of residues Gly25-Phe65, Ile27-Thr63 (CO-N and N-CO), and Thr29-Gly61. The importance of this region in a Ca^{2+} -induced conformational change was proposed as acting to stabilize the calcium-free, compact helix form while allowing a relative rigid structure around which helices B/C rotate during domain opening¹⁴.

Structural comparison of zinc-bound N-CaM with ion-free and calcium-loaded structures

To determine how similar our structure is to other previously reported CaM structures, we first performed structural alignments using each of the individual helices (A–D) and then Helices A and D together (residues 6–18 and 65–74, respectively) or B and C together (residues 29–38 and 45–54) followed by calculating RMSD values. The Zn^{2+} -bound N-CaM structure is globally similar to the NMR structure of the Ca^{2+} -free CaM (C^α RMSD=0.929 Å for residues 4–76, 1CFD⁹), the X-ray crystallographic structure of the Ca^{2+} -free N-domain of CaM alone (C^α RMSD=0.62 Å, 1Q×5⁸) and of 41/75 cross-linked CaM (C^α RMSD=1.161 Å, 1Y6W¹⁴). These low RMSD values indicate that our structure is indeed in a state most closely resembling the native Ca^{2+} -free conformation (Table 2). It is expected that within individual helices there is not substantially large reorientation between closed and open states, and on the level of the individual helix our structure is similar to the calcium loaded structure with the exception of helix B, where there appears to be significant structural difference (C^α RMSD=1.159 Å). A larger RMSD is expected for the anti-parallel β -sheet region due to the fact that different numbers of hydrogen bond pairs are possible in the closed versus open conformations (C^α RMSD=0.434 Å, 1CFD and C^α RMSD=0.932 Å, 1CLL).

The similarity of our Zn^{2+} -bound N-CaM structure to the ligand-free CaM is clearly revealed by the superposition of our structure (opaque) and the Ca^{2+} -free CaM (1CFD) and Ca^{2+} -loaded CaM (1CLL) structures (transparent) (Figure 3). The closed conformation of this Zn^{2+} -bound N-CaM structure can be further confirmed by comparing the inter-helical angles calculated for several high-resolution structures using the program Interhlx (Kyoko Yap, University of Toronto). Again, structural alignments were performed using a combination of helices A and D together (residues 6–18 and 65–74). Theoretical lines can be drawn through each helix, and the calculation of angles between these lines provides an interhelical angle. In addition, we calculated a theoretical center of mass for each helix and found the distances between these centers of mass as another measure of global similarity between structures. Generally speaking, the conformational change that moves CaM from a closed form to an open form involves the “swinging” of helices B and C away from helices A and D. Examining the interhelical angles between helices A/B, A/C, or C/D provides the most obvious examples of this helical movement and values calculated for the structure presented here resemble strongly the closed conformation (Table 3).

Zn^{2+} -coordination Sites

There are two ion-binding sites in the two EF-hands of N-CaM. Anomalous data collected at the Zn-edge confirms that these two sites are occupied by Zn^{2+} (Figure 2A). While these are the same two binding sites as are found in other ion-bound structures of CaM, the ways in which they coordinate the ions differ from previously reported structures. The coordination of Zn^{2+} in binding site one is different from that of previously reported Ca^{2+} -coordinating structures. Here, only two residues (Asp22 and Asp24) coordinate the metal ion, while the other three normally involved in metal coordination are greater than 4 Å away. In particular, Glu31 (the most C-terminal residue of the binding loop) is roughly 10 Å away from the Zn^{2+} ion. This is significant because the glutamate at this position has been shown to be necessary

for Ca^{2+} binding through mutational studies²², and its importance in the mechanism of Ca^{2+} binding was recently outlined¹⁴. In fact, replacement of Glu31 in any of the sites in CaM causes a dramatic decrease in Ca^{2+} affinity and impairs the conformational change^{23; 24}. Because the coordination of our second Zn^{2+} ion involves three of four residues typically not implicated as being important for Ca^{2+} binding (Asp 64 and Glu7, Glu11 of the symmetry related molecule), we conclude that its presence here is to facilitate the crystal packing between two N-CaM molecules. A previous flow dialysis study revealed that CaM has two Zn^{2+} ion sites with 80–300 μM affinity as well as four or five lower affinity Zn^{2+} -binding sites²⁵. It is possible that the Zn^{2+} ion revealed by our N-CaM structure is one of those low affinity sites whose precise positions have not previously been identified.

Effect of Zn^{2+} ion on the activation of bacterial adenylyl cyclase toxins by CaM

Zn^{2+} ion has been shown to be capable of supporting the activation of the CaM effector myosin light-chain kinase (MLCK) by CaM (roughly 18% above baseline), although such activation is significantly less than the activation by other ions such as cadmium (Cd), terbium (Tb), and europium (Eu)²⁶. Consistent with this observation, although Cd, Tb, and Eu can induce a measurable global conformational change in calmodulin based on the studies of Fourier-transform infrared spectroscopy (FTIR) and electron-spin resonance spectroscopy, whereas Zn^{2+} ion cannot induce the same transition²⁶. To further investigate how Zn^{2+} ion affects the activation of CaM effectors, we examined the effect of Zn^{2+} ion on the activation of anthrax edema factor (EF) and pertussis CyaA by CaM (Figure 4).

EF and CyaA are bacterial toxins secreted by pathogenic bacteria that cause anthrax and pertussis, respectively. While both toxins have CaM-activated adenylyl cyclase activity to intoxicate the target cells by raising the intracellular cyclic AMP level, their binding to CaM differs substantially^{17; 27; 28}. We found that in the presence of 10 mM Zn^{2+} , CaM could activate EF but not CyaA. However, the V_{max} for the adenylyl cyclase activity of CaM-activated EF in the presence of Zn^{2+} is approximately 10 fold lower than that in the presence of 1 μM free Ca^{2+} . Together, these data indicate that Zn^{2+} ion is capable of partially substituting the ability of Ca^{2+} ion to modulate the activity of CaM effectors such as MLCK and anthrax EF. These data further suggest that Zn^{2+} -bound CaM is partially able to activate its effectors and may represent a conformational intermediate between apo-CaM and Ca^{2+} -loaded CaM.

Mechanistic aspects of the early stages of metal-ion binding

The Ca^{2+} -binding loop of CaM is 12 residues in length and several key residues are highly conserved across EF-hand Ca^{2+} -binding proteins. In CaM, the loop (N-terminal residue) begins with an aspartate residue and ends with a glutamate residue, both of which coordinate Ca^{2+} . In between, three other highly conserved residues are involved in coordination: the side chain carboxylic acids of two aspartates and the main-chain carbonyl oxygen of a threonine residue. The NMR structures of Apo-CaM and crystallographic temperature factors appear to show a more dynamic N-terminal of the loop region, while the C-terminal part of the loop is highly structured. This is due to main-chain hydrogen bonds of an anti-parallel β -sheet (contacts between residue 25 and 65, 27 and 63, as well as 29 and 61) in Apo-CaM, which significantly constrains the flexibility of C-terminal part of the Ca^{2+} -binding loops of the pair-mate EF-hand²⁹.

In our structure, the residues immediately C-terminal to this region (residues 28–33) maintain a non- α helical secondary structure, and are more consistent with the hydrogen bond patterns of a 3_{10} -helix (Figure 5). This pattern is also seen in NMR and x-ray structures of apo-CaM^{9; 20}; however, this is missing from a recently reported cross-linked CaM (41/75) trapped intermediate structure^{14; 16}. These residues adopt the typical $i, i+4$ hydrogen bonding patterns observed in α -helices in the Ca^{2+} -loaded, open conformation structures^{20; 21}. It follows that

in order to adopt the α -helical, rigid position of these residues which is present in the Ca^{2+} -loaded structures, these residues would need to undergo significant reordering of the main-chain atoms in concert with movement of the bidentate Glu31 into a coordinating position. In the cross-linked intermediate CaM mechanism discussion, this helical region simply moves into a position where this bidentate Glu31 is able to complete Ca^{2+} coordination. There is no discussion of required helical reordering, possibly due to the fact that trapping this state with a crosslinking disulfide bond created an α -helical structure that is not necessarily present in the initial stages of metal ion binding.

The structural comparison with apo- and Ca^{2+} -loaded CaM leads to the hypothesis that our structure might represent a trapped intermediate binding state. We propose that metal ion coordination may begin with several N-terminal loop region aspartates, and that initially the ligand site is occupied by a highly coordinated water molecule ($\sigma > 6.0$ in 1Fofc). In order for full metal-ion coordination to occur, several steps must take place: the coordinated water molecule must be displaced. To do so, there must be reordering and therefore flexibility of the region immediately C-terminal to the binding loop. In addition, the remaining aspartate, carbonyl main-chain oxygen, and bidentate glutamate must also move into position. In our Zn^{2+} -bound N-CaM structure, the metal ion in binding site I is coordinated by only two aspartate residues, leaving the possibility that this represents one of the first steps in metal ion coordination. Here, these two residues would be able to maintain first contacts with the metal ion, followed by neighboring side chains of Asp20 and main chain carbonyl of Thr26. The highly ordered water molecule, which in our structure makes contacts with the main chain carbonyl of Thr26, must be displaced before coordination between these four residues together could take place. In addition, the main chain residues 28–33 of the N-terminus of the exiting helix need to rearrange to an α -helical orientation in conjunction with the movement of Glu31 into a coordinating position.

While we describe these conformational changes in a discrete, step-wise manner, it is also possible that the intermediate we have trapped represents one of the energy states that the N-terminal domain may sample between the canonical closed and open conformations. Consistent with lower temperature factors and less flexibility in NMR data of the C-terminal region after the binding loop, this suggests that reordering of this region may happen only after binding of a metal ion by more than two of the four N-terminal coordinating residues. This would explain why the N-terminal region of helix B, which is located at the C-terminal region of the first binding loop, maintains the 3_{10} -helical structure although we do see coordination of a metal ion by two of five residues at the first binding loop. In a recently reported structure of a trapped intermediate formed by a crosslinking disulfide bond between residues 41/75, the author suggests that exiting helix B pivots around residue Ile27 in a locked, α -helical form as a step in the conformational switch. In this manner, reordering of this region and movement of Glu31 into a coordinating position could then cause the observed “swinging” of helices B/C relative to A/D that takes place during the conformational change. Our structure implies an earlier stage during which this α -helical region has not yet formed, and therefore our structure may represent an earlier step in metal ion binding that is a prerequisite for helical reordering and movement. Taken together, these steps describe the potential mechanism by which the motion of the four-helix bundle changes N-CaM from a closed to an open conformation upon binding a metal ion.

Ca^{2+} regulates a diverse and vast number of cellular processes via transient increases from roughly $0.1 \mu\text{M}$ in a resting cell to $1\text{--}10 \mu\text{M}$ in an activated cell. However, the proteins largely responsible for primarily responding to this increase (CaM or other EF-hand proteins) accomplish this in the presence of $100\text{--}10,000$ -fold excess Mg^{2+} with very high selectivity for Ca^{2+} . The question of how EF-hand proteins are able to accomplish this remains one of the more intriguing aspects of this family of proteins, especially because it is thought that, in the

absence of signaling or other events that produce large influxes of calcium, Mg^{2+} can at least partially populate EF-hand binding sites^{30; 31}. Through NMR shift experiments^{30; 31; 32}, it has been postulated that Mg^{2+} can bind to the N-terminal portions of the N-CaM metal binding loops without inducing a conformational change. Interestingly, it was specifically shown that the bidentate glutamates in loop position 12 (Glu31 and Glu67) do not coordinate Mg^{2+} at all. In addition, the shift experiments conclude that Mg^{2+} only induces local conformational changes in and immediately around the binding loops, but does not induce the global conformational change from the closed to open state as observed with Ca^{2+} . We also do not observe a large, global conformational change although we do observe local shifts in conformation induced by Zn^{2+} binding. This may be related to the binding mode of these two divalent cations. In the EF-hand protein recoverin, the seven-coordinate Ca, Sr, Ba, and Cd divalent cations can induce large conformational changes, while the six-coordinate Mg cannot trigger a signaling response.

The specificity of EF-hand proteins for calcium has been proposed to depend on three things: the protonation state of the binding cavity which gives rise to a preference for divalent cations; constrained cavity size as determined by specific side chains in the loop that have been referred to as “gatekeepers”^{33; 34}; and the binding mode, referring here to the fact that calcium in proteins usually is 6–8 coordinated and prefers harder ligands (such as the oxygens of carboxyl groups) and bidentate coordination³⁵. As a divalent cation, zinc tends to prefer “softer” ligands (the sulfurs on cysteines or the nitrogens on histidines). This is shown in its various coordination modes of the well-studied zinc finger family of structural proteins, involving 2Cys and 2His (cellular or transcription factor type zinc fingers), 3Cys and 1His (the retroviral type zinc fingers), or 4Cys (the steroid receptor type zinc finger). In zinc-finger proteins and other enzymes, zinc is usually tetrahedrally coordinated, as we observe in our structure here. Interestingly, the harder ligand aspartates and glutamine are involved in our Zn^{2+} -bound N-CaM structure. This likely reflects the low affinity of such interactions since a high concentration of Zn^{2+} ion is required to stabilize this crystal. Our structure can provide further evidence for the importance of the bidentate glutamate in position 12 because, in conjunction with the NMR shift studies proposing that Mg^{2+} can coordinate the N-terminal residues in the binding loop but not the bidentate glutamate, we also do not observe a large, global conformational change although we do observe local shifts in conformation induced by Zn^{2+} binding. Our structure provides further knowledge of the structural basis for the conformational transition in calmodulin that is consistent with what we know about EF-hand protein metal ion coordination, calcium specificity, and transitional mechanism.

Materials and Methods

Protein expression and purification

N-terminal CaM was expressed from pProEx-N-CaM cloned from rat full-length CaM with a stop codon introduced after residue Lys78. This was transformed to a competent *E. coli* BL21 (DE3)pUBS520 strain and grown in 4L of T7 LB media at 30 °C overnight. Protein over-expression was induced by the addition of 0.2mM IPTG when O.D. at 595nm reached 0.6–0.8. After overnight incubation, 0.1M PMSF was added and cells were harvested by centrifugation (10,000 rpm for 10min at 4°C). Pellets were frozen and thawed before lysing with 0.1 mg/ml lysozyme lysis buffer, followed by sonication and ultracentrifugation to separate cell debris (35,000 rpm for 30 minutes). 5mM $CaCl_2$ was added to collected supernatants and this lysate was loaded onto a phenyl sepharose column then eluted with 1mM EGTA as described³⁶. Peak fractions were collected, applied to a DEAE column, and eluted with a salt gradient. Peak fractions as identified by SDS-PAGE were subjected to gel filtration (Superdex 75) and stored at –80°C. This entire process was repeated for the purpose of growing seleno-methionyl N-CaM, except that cultures were grown in minimal media as described³⁷.

Crystallization

Crystals of N-CaM and seleno-methionyl N-CaM were grown using the hanging drop vapor diffusion method at 4 °C with reservoirs containing 1 mL of 100mM Na Cacodylate, 5–10mM Zn Acetate, and 16% PEG8000. Crystals appeared after roughly three days and grew to a size of 400×500×100 μ m over the course of one week. Crystals were frozen in liquid nitrogen cryo solutions containing 100mM Na Cacodylate, 5–10mM Zn Acetate, 16%PEG8000, and 20% Glycerol.

X-ray data collection, structure determination and refinement

The structure was solved using single-wavelength anomalous dispersion (SAD) data of the seleno-methionyl substituted protein. Data were collected at the Advanced Photon Source at Argonne National Laboratories (Argonne, IL). One complete data set representing peak parts of the selenium scattering spectrum was collected at 100 K at the radiation wavelength $\lambda_1=0.97929\text{\AA}$ (peak). The data were processed with DENZO and scaled with SCALEPACK³⁸. Six anomalous site positions were found by SOLVE³⁹, including 3 Se, 2 Zn and 1 As. The presence of these latter two metals were confirmed by collecting data at the Zn and As edges and finding two and one anomalous sites, respectively. The structure was refined with SHARP⁴⁰. The initial electron density map was obtained using the “remote” data set ($\lambda_3=0.97929\text{\AA}$). These experimentally obtained phases were then applied to a native N-CaM data set with very high resolution (approximately 1.3 $\text{\AA}$) to generate a first model.

Segments of the model including individual helices were adjusted manually within the electron density map using the program Coot⁴¹ followed by rigid body fitting with CNS. Side-chains that had no clear representation in the electron density map were omitted from the model and the model was refined using the simulated annealing protocol in CNS⁴². After several cycles of manual model rebuilding, it became clear that additional electron density was present in one of the Zn²⁺-binding sites. The extra density in site I could be modeled adequately with a cacodylate group that was present in the crystallization buffer. The cacodylate group coordinates and dictionary files for Refmac were built by CCP4⁴³. After restrained and TLS refinements in Refmac reached values of $R_{\text{work}}=0.146$ and $R_{\text{free}}=0.192$, SHELX97 was used to include anisotropic refinement parameters. The quality of the structure was validated with the program PROCHECK⁴⁴. Using this first model as a search model for the Ca²⁺-soaked data set followed by a similar refinement protocol yielded $R_{\text{work}}=0.138$ and $R_{\text{free}}=0.190$. The model statistics are given in Table 1.

Adenylyl cyclase activity assay

Edema factor (EF) and adenylyl cyclase domain of CyaA were expressed and purified as previously described^{27; 45}. The activities of EF and CyaA were measured at 30°C in the presence of 10mM MgCl₂, 5mM ATP and a trace amount of [α -32P] ATP for 10 minutes. The reaction was buffered by 50mM HEPES, pH 7.2 and free calcium concentration was controlled by 10mM EGTA to 1 μ M based on calculations using the MAXC program. 10mM ZnCl₂ and 10mM MgCl₂ were used to test the activity of EF. cAMP was separated from ATP by Dowex and alumina columns as described previously²⁷. Initial velocities were linear with time and less than 10% of the ATP was consumed at the lowest substrate concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaM	Calmodulin
N-CaM	N-terminal calmodulin
SAD	single wavelength anomalous diffraction

Figure 1.

The overall structure of zinc bound N-CaM. (A) The 2Fo-Fc density map of N-CaM contoured at 1.2σ. (B) The ribbon representation of N-CaM. The two helix-loop-helix EF hand Zn²⁺ binding sites are shown in different colors: EF-hand I (helices A and B)- rose red; EF-hand II (helices C and D)- lime green. The spheres show the two Zn²⁺ ions present in these binding sites. The zinc ion in site two has double occupancy. There is also a cacodylate molecule present from the crystal growth buffer with Arsenic shown in purple. The side-chains of the residues involved in binding are shown in stick representation with different colors: Carbon- gray; Nitrogen- blue; Oxygen- red. Residue T26 shows alternative conformation.

Figure 2.

Structural details in the metal binding sites, ligand identity, and molecular packing. (A) 2Fo-Fc map calculated from data collected at the zinc-edge ($\lambda=1.28237\text{\AA}$). (B) 2Fo-Fc map calculated from data collected at the arsenic-edge ($\lambda=1.04393\text{\AA}$). (C) Molecule packing in the asymmetric unit of N-CaM. Transparent molecules are symmetry related molecules. The two helix-loop-helix EF hand Zn^{2+} binding sites are shown in different colors: EF-hand I (helices A and B)- rose red; EF-hand II (helices C and D)- lime green. The spheres show the two Zn^{2+} ions present in these binding sites. The zinc ion in site two has double occupancy. There is also a cacodylate molecule present from the crystal growth buffer with arsenic shown in purple. The side-chains of the residues involving the binding are shown in stick representation with different colors: Carbon- gray; Nitrogen- blue; Oxygen- red.

Figure 3.

Structure alignment of N-CaM with N-terminal domain of the NMR structure of Apo-CaM (left, 1CFD) and the X-ray structure of Ca²⁺-CaM (right, 1CLL). The residues involved in ion coordination are shown as sticks and labeled. These global alignments were performed using helices A and D, and show that our structure is predominantly in the closed conformation. 1CFD and 1CLL are presented as transparent. The two helix-loop-helix EF hand Zn²⁺ binding sites are shown in different colors: EF-hand I (helices A and B)- rose red; EF-hand II (helices C and D)-lime green. The spheres show the two Zn²⁺ ions present in these binding sites. The zinc ion in site two has double occupancy. There is also a cacodylate molecule present from the crystal growth buffer with Arsenic shown in purple.

Figure 4.

The effect of zinc ion on the activation of two bacterial adenylyl cyclase toxins, EF and CyaA, by CaM. Assays were performed at 30 °C for 10 minutes in the presence of 1 nM EF or CyaA with the indicated concentration of CaM either with 10 mM ZnCl₂ or 1 μM free calcium ion buffered by EGTA and data is a representative of two experiments.

Figure 5.

Comparison of the N-terminal region of Helix B of N-CaM (A) and Ca²⁺-loaded CaM (1CLL). The segments of the helix that are in a traditional alpha-helical configuration are colored rose red, while the most N-terminal portion of the helix in our structure displays a 3₁₀-helical conformation and is colored magenta. The oxygen and nitrogen of residues are colored red and blue, respectively.

Table 1

Crystallographic statistics

Data Collection	Crystallographic statistics			
	Se-N-CaM	Native-N-CaM	Native-N-CaM	As-N-CaM
Beamline	APS 19ID	APS 19-BMC	APS 19-ID	APS 19-BMC
Wavelength (Å)	0.97929	0.97951	0.97929	1.28237
Space group	P4 ₃ -2 ₁ -2	P4 ₃ -2 ₁ -2	P4 ₃ -2 ₁ -2	P4 ₃ -2 ₁ -2
Cell dimension(Å)				
a	35.2	35.3	35.2	35.3
b	35.2	35.3	35.2	35.3
c	142.6	143.2	143.3	142.5
Resolution (Å)	50–2.0	32–1.3	30–1.6	50–2.2
Rsym (%) ^a	12.4(36.2)	7.0(31.5) ^e	7.6(48.5) ^e	16.3(25.3)
I/sigma	41.2(10.1)	46.1(3.8) ^e	27.2(3.2) ^e	45.8(12.0)
Redundancy ^b	29.3(30.1)	12.5(8.6) ^e	13.2(9.1) ^e	24.6(26.2)
Completeness (%)	98.3(97.8)	99.0(92.1) ^e	99.8(99.4) ^e	99.9(99.9)
FOM(Figure of Merit)	0.87			
Unique reflections	36347	12687	12125	33915
Refinement				
R _{work} ^c		0.142	0.174	
R _{free} ^d		0.196	0.215	
No. atoms				
Protein		612	612	
Water		126	117	
B-factors				
Protein		9.5	9.6	
Water		10.9	11.2	
r.m.s. deviations				
Bond lengths (Å)		0.008	0.009	
Bond angles (°)		1.17	1.21	
Ramachandran plot (%)				
Favorable region		95.4	95.4	
Allowed region		4.6	4.6	
Generously allowed region		0	0	
Disallowed region		0	0	

^a $R_{\text{Sym}} = \sum |I_j| / \langle I \rangle - I_j / \langle I \rangle$ where I_j is the intensity of the j th reflection and $\langle I \rangle$ is the average intensity.

^b $N_{\text{Obs}} / N_{\text{Unique}}$.

^c $R_{\text{work}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$.

^d R_{free} , calculated the same as for R_{work} but on the 5% data excluded from the refinement calculation.

^e the outer resolution shell.

Table 2
Comparison of the N-CaM with Apo and Ca²⁺-bound structures of CaM

Protein/segment ¹	N-CaM	Helix A	Helix B	Helix C	Helix D	Helices A+D	Helix B+C	EF-hands scaffold
Apo-CaM	0.929	0.324	0.299	0.295	0.363	0.598	0.452	0.434
Ca ²⁺ -loaded CaM	4.512	0.561	1.159	0.297	0.273	1.474	0.276	0.952
CaM 45/71	1.161	0.302	0.735	0.440	0.200	0.545	0.950	0.454

¹ Accession numbers for Apo-CaM, calcium-loaded CaM, and calcium-loaded crosslinked CaM41/75 are 1CFD, 1CLL, and 1Y6W, respectively. Definition of helices in CaM: helix A (aa 6–18), helix B (aa 29–38), helix C (aa 45–54), helix D (65–74). N-CaM includes amino acid residues 4–76 and the EF-hand β -sheet scaffold includes amino acid residues 25–27 and 61–63. The C $^{\alpha}$ root mean square deviation (RMSD) values are listed for the alignment of various segments. For the EF hand β -sheet scaffold, all backbone atoms are used for the alignment. Values for RMSD were obtained from the align function of the program Coot.

Table 3

Spatial relation between α -helices in CaM

Protein/Helices ¹	A-B	A-C	A-D	B-C	B-D	C-D
N-CaM	133(12.6) ²	-84(17.6)	133(10.8)	113(8.6)	-46(13.3)	143(12.0)
Apo-CaM	136(12.9)	-93(21.2)	126(10.6)	124(11.9)	-49(12.9)	129(14.1)
CaM41/75	129(13.4)	-97(21.4)	129(10.7)	122(12.6)	-50(12.6)	127(13.0)
Ca ²⁺ -loaded CaM	88(18.9)	-161(22.8)	107(10.5)	112(11.2)	-41(18.0)	87(16.2)

¹ Definition of helices in CaM: helix A (aa 6–18), helix B (aa 29–38), helix C (aa 45–54), helix D (65–74). Accession numbers for zinc-bound N-CaM, Apo-CaM, calcium-loaded CaM, and calcium-loaded crosslinked CaM41/75 are 2PQ3, 1CFD, 1CLL, and 1Y6W, respectively.

² The interhelical angles (deg.) and distances (Å) between centers of mass (in parentheses). These were calculated with the program Interhix. The angles are defined as described by Drohat *et al.*⁴⁶ as follows: (1) the molecule is oriented in such a way that the helices of interest (*i, j*) are in planes parallel with the screen; (2) the first helix (*i*) is in front of the second helix (*j*), and the first helix (*i*) is aligned vertically (0°) with its N→C vector pointing upward. An imaginary vector aligned vertically (0°) with its tail at the N terminus of the second helix (*j*) is rotated (by ≤180°) clockwise or counterclockwise until it is aligned with the N→C vector of the second helix (*j*). This angle of rotation defines the interhelical angle Ω with a clockwise rotation giving a positive Ω value and a counterclockwise rotation giving a negative Ω value.