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MINIREVIEW ARTICLE

# The many structural faces of calmodulin: a multitasking molecular jackknife

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**Abstract** Calmodulin (CaM) is a highly conserved protein and a crucial calcium sensor in eukaryotes. CaM is a regulator of hundreds of diverse target proteins. A wealth of studies has been carried out on the structure of CaM, both in the unliganded form and in complexes with target proteins and peptides. The outcome of these studies points toward a high propensity to attain various conformational states, depending on the binding partner. The purpose of this review is to provide examples of different conformations of CaM trapped in the crystal state. In addition, comparisons are made to corresponding studies in solution. The different CaM conformations in crystal structures are also compared based on the positions of the metal ions bound to their EF hands, in terms of distances, angles, and pseudotorsion angles. Possible caveats and artifacts in CaM crystal structures are discussed, as well as the possibilities of trapping biologically relevant CaM conformations in the crystal state.

**Keywords** Protein conformation · Crystal structure · Complex · Calcium · X-Ray crystallography · Calmodulin

**Abbreviations**

| | |
|---|---|
| CaM | Calmodulin |
| CaMK | CaM-dependent kinase |
| DAPK | Death-associated protein kinase |
| PDB | Protein Data Bank |

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## Introduction

Calmodulin (CaM) is a highly abundant, ubiquitous, small, acidic protein, which plays a major role in the transmission of calcium signals to target proteins in eukaryotes. Hundreds of CaM targets are known, and their respective cellular functions include signaling, metabolism, cytoskeletal regulation, and ion channel regulation, to name but a few. CaM target proteins include, for example, several CaM-dependent protein kinases (CaMK), other enzymes, myosins, receptors, ion channels, and a number of other proteins. The affinity of CaM towards its protein targets varies between the nM and μM ranges.

Most characterized CaM–protein interactions are calcium dependent. The binding of $Ca^{2+}$ to the four EF hands of CaM results in conformational changes that open up a hydrophobic pocket within each of the two lobes of CaM (Zhang et al. 1995). Also other divalent cations can bind to the EF hands (Habermann et al. 1983; Kursula and Majava 2007; Kursula 2014), but their functional relevance has not been assessed in detail. The hydrophobic binding pockets contain several methionine residues, which provide a soft, flexible site for embedding a hydrophobic side chain present in a CaM-binding domain (CBD). In general, target peptide sequences for holo-CaM binding contain an amphipathic α helix with two suitably spaced and oriented hydrophobic anchors, which insert into these methionine-rich pockets. In addition, positively charged residues in the target form salt bridges to CaM and may also be directly involved in determining the binding orientation of the target peptide (Kurokawa et al. 2001) and in inducing structural changes at the central α helix (Kursula 2014).

Interactions between calcium-free (apo) CaM and targets have also been characterized. The best characterized such interactions involve the IQ motifs in target proteins

(Rhoads and Friedberg 1997). Indeed, the conformations of CaM in IQ domain complexes depend on calcium binding to the four EF hands (Black and Persechini 2011).

To understand the function of a protein, it is crucial to obtain high-resolution structural information in different functional states; CaM is one of the most studied proteins from the structural point of view. The Protein Data Bank (PDB) is flooded with structures of CaM; a simple search of the PDB with the CaM sequence and molecule name returns approximately 200 entries. The purpose of this mini-review is to introduce and compare the kinds of different conformations taken by CaM in its various complexes with target proteins. For additional insights into CaM structure and details of target recognition, the reader is directed to a recent review by Villarroel et al. (2014).

## Structure of CaM without bound ligand peptide

The extended, dumbbell-shaped structure of unliganded CaM is supported by the presence of a long central helix (Fig. 1); however, also collapsed conformations have been detected (Fallon and Quiocho 2003; Yamada et al. 2012; Kumar et al. 2013b), suggesting an equilibrium between different conformational states in solution. The atomic resolution structure of the extended conformation also supports significant flexibility and disorder even in the crystal state (Wilson and Brunger 2000).

The middle region of the central helix of CaM is assumed to be relatively disordered in solution, with a propensity to adopt various conformations. Two possible early intermediates on the pathway leading to the central α helix breakage and CaM structural collapse have been crystallized (Fig. 1) (Kursula 2014). An analogous role for amino acid side chains in the vicinal regions of CaM and on target peptides in inducing central helix collapse was, therefore, proposed. The inherent flexibility of the highly charged middle region of the central linker helix, coupled to interactions of this region with target protein positively charged side chains, is likely a very important determining factor in CaM structural collapse. In the two crystal structures of unliganded CaM with more collapsed conformations (Fallon and Quiocho 2003; Kumar et al. 2013b), interactions at the break point of the central helix also include salt bridges between glutamate residues in the middle of the linker and nearby basic residues.

## Classical collapsed CaM–peptide complexes

The first ‘canonical’ CaM–peptide complex structures were determined using CaM-binding peptides from CaM-dependent protein kinases using both X-ray crystallography and solution NMR spectroscopy (Ikura et al. 1992; Meador et al. 1992), and the observed collapsed globular conformation was considered to be a general property of CaM–target protein complexes. Already earlier, a structural collapse of liganded CaM had been detected using small-angle scattering (Heidorn et al. 1989). The collapse of CaM upon CaMK peptide recognition involves the disruption of the

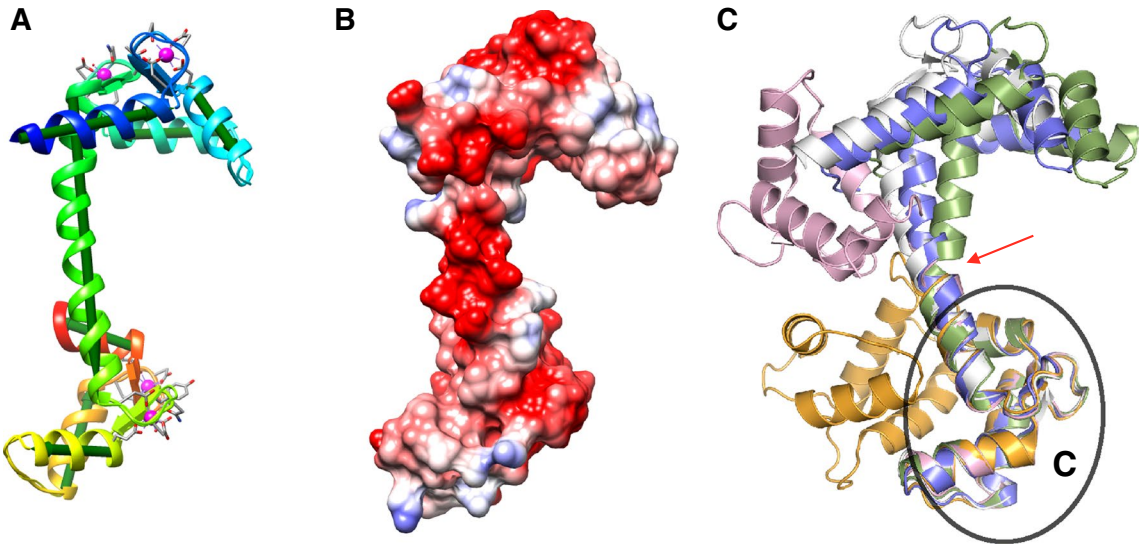


**Fig. 1** Holo-CaM conformations trapped in the crystal state in the absence of bound target peptide. **a** Fully extended, canonical CaM. The four calcium ions in the EF hands are depicted as *magenta spheres*. The cartoon is colored from *blue* (N terminus) to *red* (C terminus). **b** Electrostatic surface potential of holo-CaM. Note that in addition to the lobes, the middle region of the central helix has high negative charge, enabling conformational changes upon interactions with basic residues. **c** Superpositions of CaM structures with kinks in the central helix (*red arrow*). The structures were superposed based on the C-terminal lobe (*circled*) (color figure online)

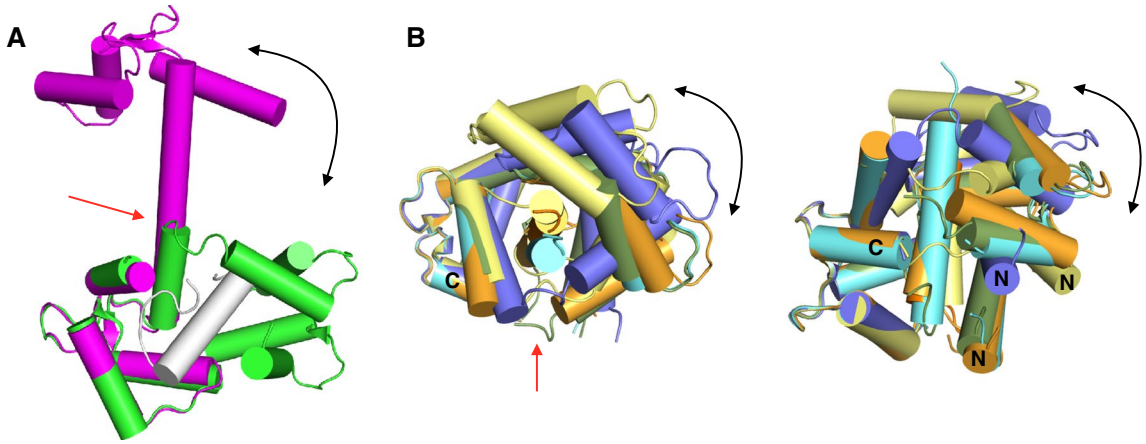


**Fig. 2** Canonical CaM–peptide complexes. **a** A typical conformational change in a CaM–peptide complex. The unliganded CaM is shown in *magenta* and the collapsed conformation in *green*. The bound peptide is *white*, and the hinge is indicated by the *red arrow*. **b** Superposition of five canonical collapsed CaM–peptide complexes. Superposition was done based on the C-terminal lobe. *Left*: view along the peptide axis (in the *middle*); *right*: view turned by 90° about the *horizontal* axis. N- and C-terminal helices of CaM are also indicated (color figure online)

long central helix, allowing the two lobes to come together and bury the target peptide between them (Fig. 2).

In the collapsed CaM complexes, most often the central part of the linker region, where the long helix has been disrupted, is invisible in electron density maps. This indicates high local entropy induced by target peptide binding. Superposing various CaM–peptide complexes having the collapsed conformation clearly shows that, although each of them has a slightly different conformation, the relative orientation and distance of the N- and C-terminal lobes of CaM with respect to one another is similar (Fig. 2).

Based on the separation of hydrophobic anchor residues in the target peptides, different recognition modes have been identified, with the most common being 1–10 and 1–14. However, e.g. modes 1–11 (Majava and Kursula 2009), 1–16 (Osawa et al. 1999), and 1–17 (Maximciuc et al. 2006) have been identified in crystal structures. Furthermore, the complexes of CaM with peptides from DAP kinases have a combined 1–10–14 mode, where two hydrophobic residues from the peptide lie in the same pocket of CaM in the crystal structures (Kuczera and Kursula 2012; Patel et al. 2011). Also, the complex with a myristoylated peptide from CAP-23/NAP-22 has a classical collapsed conformation, even though the ligand peptide does not form an α helix and the myristoyl tail also binds between the CaM lobes (Matsubara et al. 2004).

## Other CaM–peptide complexes

Originally, the recognition mode of CaM was thought to always involve wrapping around the target peptide in a collapsed conformation. Since the early studies, a number of CaM complexes have been determined, showing various conformations for CaM, in addition to the fully open unliganded conformation and the collapsed, essentially globular, peptide-bound conformation. The increase in structural knowledge has indicated that it is relatively common for CaM to bind its targets also using more open conformations. Such conformations might—due to flexibility and unfavorable crystal contacts—often be unamenable to crystallization. A detailed description of the conformational variability in CaM–IQ complexes has been presented elsewhere (Black and Persechini 2011).

One interesting case of CaM complexed with a target peptide in a non-canonical conformation is presented by the target peptide taken from calcineurin A (Fig. 3a). In all crystal structures of the complex so far, a 2:2 arrangement is observed, in which CaM remains extended and two peptides bind between two CaM molecules (Majava and Kursula 2009; Ye et al. 2006, 2008). This kind of complex was novel, but studies in solution indicated that the corresponding samples were in a collapsed 1:1 complex (Majava and Kursula 2009). For switching between the 1:1 and 2:2 arrangements, a domain swapping event must take place. It is currently not known, which one of these complexes corresponds to the physiologically relevant one, or if both of them are, indeed, related to calcineurin regulation. The 2:2 complex could be an example of a crystallization artifact, where favorable crystal contacts result in the crystallization of a physiologically irrelevant conformation. The conformation of CaM in the 2:2 complex very closely resembles that on unliganded holo-CaM (Majava and Kursula 2009); with only a very slight opening of the lobes—without unfolding of the central α helix.

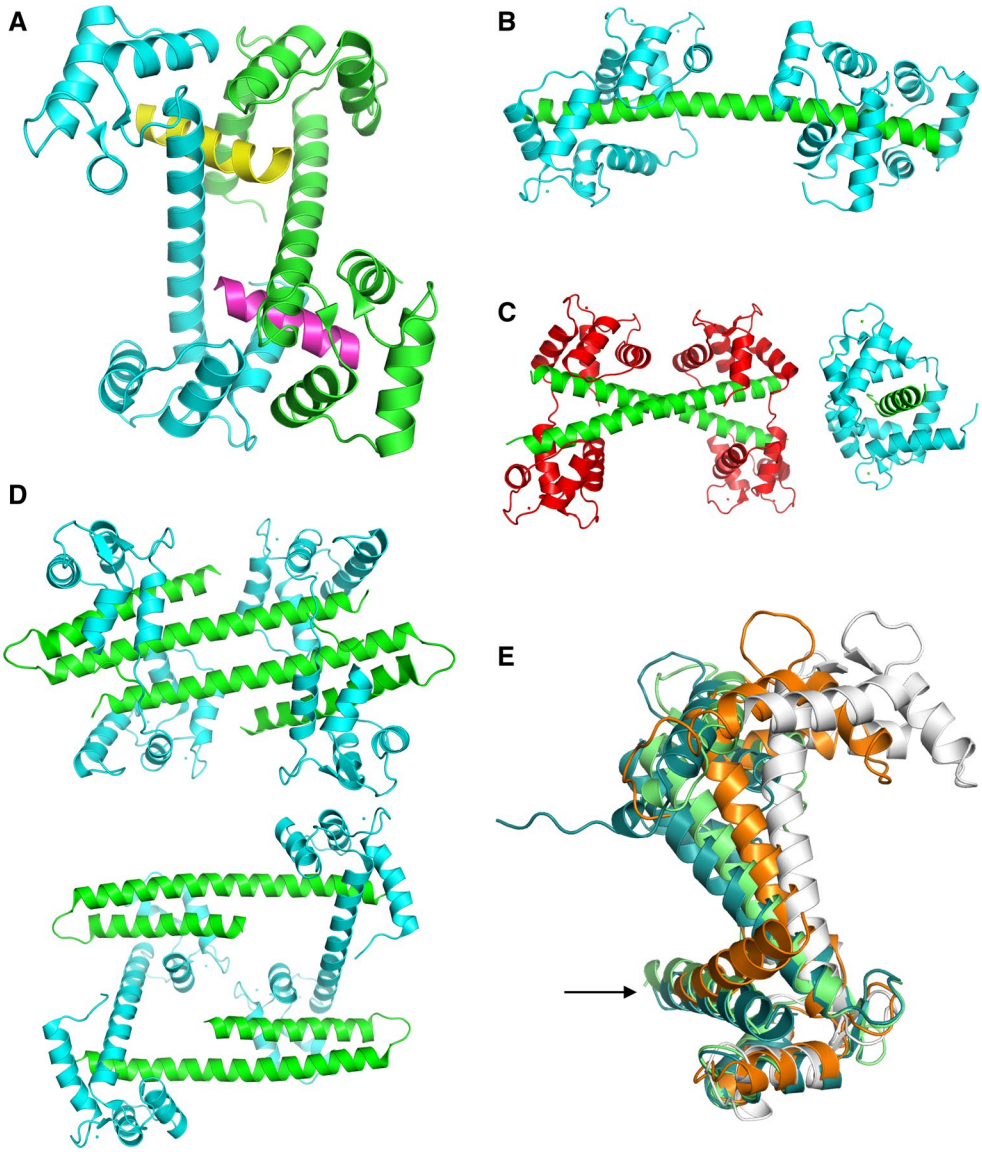


**Fig. 3** CaM–peptide complexes with non-canonical conformations. **a** 2:2 complex between CaM and the CBD from calcineurin A (Majava and Kursula 2009). **b** Two molecules of CaM (*cyan*) bound to a long peptide from the $Ca^{2+}$-ATPase (*green*) (Tidow et al. 2012). **c** CaM bound in a 2:2 complex to the pre-IQ region and in a 1:1 mode to the IQ domain of the Ca(v)1.2 channel (Fallon et al. 2005, 2009). **d** 2:2 complexes of CaM (*cyan*) with CBD segments from two alternatively spliced variants of a potassium channel (Schumacher et al. 2001; Zhang et al. 2012). **e** CaM–IQ complex structures determined using fusion constructs of CaM and peptides from three different IQ domains (Kumar et al. 2013b; Reddy Chichili et al. 2013). CaM remains extended in all complexes, being non-identical to unliganded holo-CaM (*white*). The ligand peptide is indicated by the *arrow* (color figure online)

In addition to $Ca^{2+}$-dependent targets, CaM binds a number of targets in its apo (metal-free) form; such interactions normally involve so-called IQ motifs. The conformations of CaM in these complexes are often dictated by the relative importance of the CaM C-terminal lobe, while the conformation of the N-terminal lobe varies. In fact, most structures of the IQ domain–CaM complexes involve at least partially charged $Ca^{2+}$–CaM, and often these structures present a rather canonical collapsed conformation. Structures of CaM bound to peptides from ion pumps/channels have emerged during recent years; in general, the target sequences carry IQ motifs. These structures include, for example, plasma membrane $Ca^{2+}$-ATPase (Tidow et al. 2012), and sodium, potassium, and calcium channels (Fallon et al. 2005, 2009; Mori et al. 2008; Reddy Chichili et al. 2013; Sarhan et al. 2012; Schumacher et al. 2004; Wang et al. 2012). In the Ca pump structure (Tidow et al. 2012), two molecules of CaM bind to a long peptide of the pump in collapsed 1–14 modes, with somewhat unique conformations, when compared with other CaM–peptide complexes (Fig. 3b). On the other hand, the pre-IQ and IQ domains from the voltage-dependent Ca(v)1.2 channel bind in different modes (Fallon et al. 2005, 2009); the pre-IQ peptide binds as a dimer to an opened CaM, while the IQ domain forms a rather canonical collapsed complex (Fig. 3c). Structural variability in the CaM N-terminal domain was also reported (Van Petegem et al. 2005). In the case of the small-conductance $Ca^{2+}$-activated potassium channels, different splice variants of the CBD apparently give rise to different conformations in the respective CaM complexes (Schumacher et al. 2001; Zhang et al. 2012); CaM remains either fully or partially open, while engaged in interactions with two peptides in a 2:2 complex (Fig. 3d). It cannot be ruled out, however, that the two

conformations may also be caused by the crystallization process.

Three open CaM conformations bound to peptides were recently reported using CaM–peptide fusion proteins (Fig. 3e). The peptides in question came from neurogranin (Kumar et al. 2013b), neuromodulin (Kumar et al. 2013b), and a neuronal voltage-gated sodium channel (Reddy Chichili et al. 2013). All these peptides belong to the IQ motif class. It is possible that the affinity of the isolated peptides to open CaM is not high enough to provide a stable complex for crystallization without the use of fusion protein techniques. On the other hand, the fusion assembly will largely dictate the orientation of the peptide with regard to CaM, and the structures should, hence, be considered with care. The conformational differences in these cases relate to a hinge motion at the C-terminal end of the long linker helix.

Open CaM conformations bound to target peptides have also been observed in solution using small-angle X-ray scattering, when the complexes cannot be crystallized. As one example, a complex between a peptide from the myelin basic protein (MBP), a major multiple sclerosis autoantigen, and CaM was studied in solution, and CaM was observed to remain open, with only slight conformational changes (Majava et al. 2008). The same kind of CaM–MBP complex, with a small compaction upon peptide binding, was observed using peptides from two different species that differ slightly in sequence (Majava et al. 2010), but all crystallization attempts of these complexes failed. This can be explained by the significant structural heterogeneity that was observed for the same complex (Nagulapalli et al. 2012). Also, a peptide from phosphorylase kinase, Phk13, was suggested to bind to an open conformation of CaM, in contrast to another peptide from the same kinase (Trewhella et al. 1990).

## CaM–protein complexes

In addition to short CaM-binding peptides, CaM has also been studied in the context of larger protein–protein complexes. As far as CaMK family kinases—the classical targets of CaM regulation—are concerned, the crystal structures of two complexes have been determined for kinases bound to CaM. In the crystal structure of CaMK2 bound to calmodulin (Rellos et al. 2010), the CaM–CBD unit is located far from the kinase core, connected by a disordered linker (Fig. 4a). In this case, CaM adopts the classical collapsed conformation, which is very similar to that seen in the crystal structure of the corresponding CaM–peptide complex. Similar results in solution have also been reported for myosin light chain kinase (Krueger et al. 1997, 2001). On the other hand, in the death-associated protein kinase (DAPK)–CaM complex (de Diego et al. 2010), CaM bound to the CBD also interacts with the kinase small lobe; it has apparently been pushed away from its predicted binding

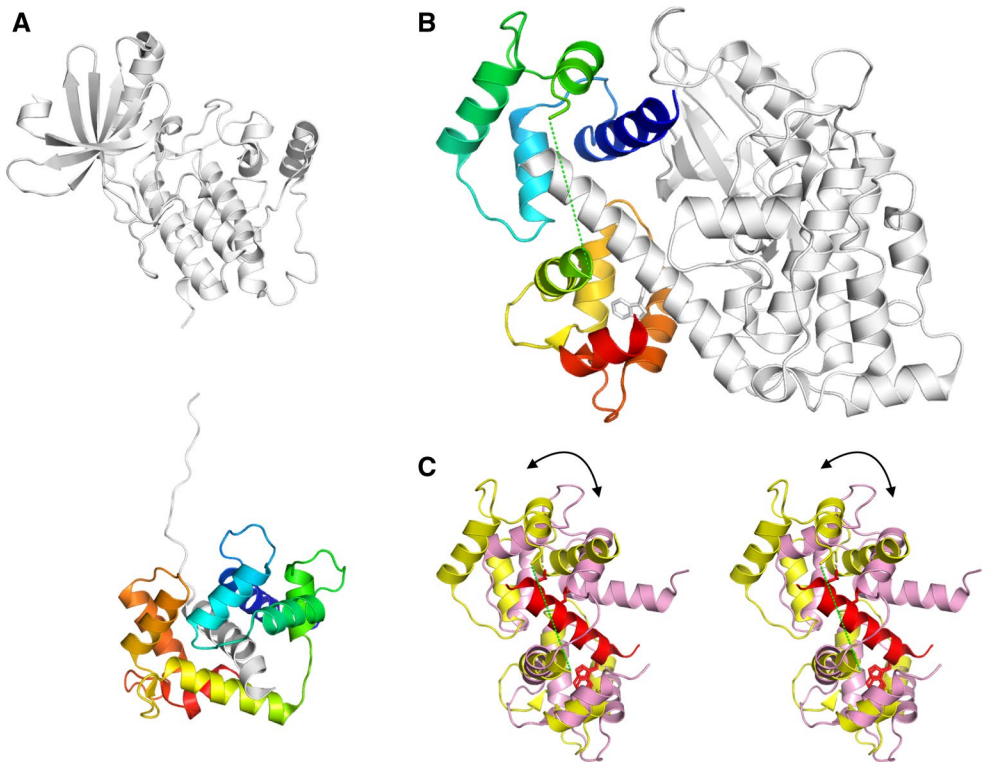


**Fig. 4** CaM–CaMK protein complexes. **a** CaM (*rainbow*) bound to CaMK2 (*white*) shows how the autoinhibitory region has been completely detached from the kinase core by CaM binding (Rellos et al. 2010). **b** CaM (*rainbow*) bound to DAPK1 (*white*) (de Diego et al. 2010). The N-terminal helix of CaM (*blue*) binds to the surface of the kinase small lobe. **c** Comparison of CaM conformation (in stereo) bound to the same sequence (*red*) in the context of the protein–protein complex (*yellow*) and the corresponding protein–peptide complex (*magenta*) (color figure online)

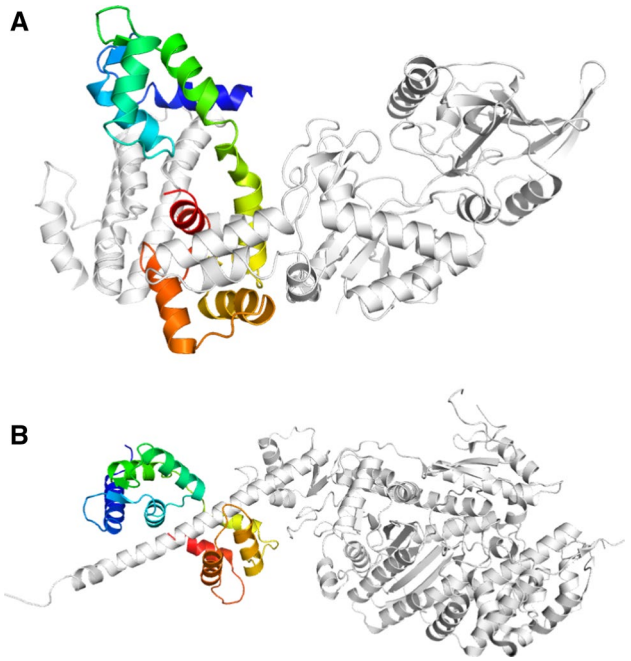


**Fig. 5** CaM–protein complexes involving IQ domains. **a** CaM bound to the anthrax edema factor (Drum et al. 2002). **b** CaM bound to the myosin 1b motor domain (Shuman et al. 2014) (color figure online)

site. For DAPK, complexes of CaM with peptides or the kinase protein show significantly different conformations (Fig. 4b, c). It is possible that the structure of the CaM–DAPK complex is a snapshot on the activation pathway towards a more open/disordered conformation, such as that seen for CaMK2 (Rellos et al. 2010).

Another example of a CaM–protein complex is provided by the anthrax edema factor, which traps CaM in an *extended* conformation (Drum et al. 2002). The interactions between the two proteins are much more complex than could be deduced from simple CaM–peptide complexes. The edema factor is an adenylyl cyclase important in the pathogenesis of anthrax. The conformation of CaM in the complex (Fig. 5a) shows the C-terminal domain bound to the target sequence, but also being embraced by other parts of the edema factor. The N-terminal domain hydrophobic pocket is turned away and does not interact with the target.

Very recently, the structure of the myosin 1b motor domain in complex with CaM was published (Shuman et al. 2014), and the corresponding myosin 1c motor domain bound to CaM was also reported (Munnich et al. 2014). In these complexes, the binding pocket of the C-terminal lobe is occupied, while the pocket of the N-terminal lobe is not used and turned away from the binding partner (Fig. 5b). The conformation of CaM is however compact. The EF hands also do not have bound calcium, which is related to the sequences being of the IQ motif type. All these features are reminiscent of the complex between CaM and a peptide from myosin V, in which two molecules of CaM bind to the extended peptide in a similar conformation (Houdusse et al. 2006). In general, this is a typical binding mode of IQ motifs to CaM, where the C-terminal lobe of CaM seems to be the driving force.

## Simple geometric characteristics for CaM

The positions of the four calcium (or other metal) ions bound to the EF hands in CaM can be used to derive simple geometric parameters to characterize CaM conformation in various structures. The values that were derived here include the pseudo-torsion angle EF1–EF2–EF3–EF4, the angles between EF1–EF2–EF3 and EF2–EF3–EF4, and the distance between Ca ions in EF hands 2 and 3. These parameters are highly indicative of CaM conformation, and they are very sensitive markers for the orientation of the two lobes with respect to one another. The values calculated for some of the representative structures discussed in this paper are given in Table 1.

One obvious observation is that in holo-CaM, the C-terminal lobe is more tightly bent towards the central helix, while the angle for EF hands 1–3 is a right angle, and the N-terminal lobe is more open. In the slightly bent structures of $Ca^{2+}$–CaM and $Sr^{2+}$–CaM (Kursula 2014), the values are close to those of the canonical extended conformation, but especially the pseudo-torsion between the lobes is sensitive to even small conformational differences.

In the various complexes, different conformations have characteristic values for these geometric parameters. Closed compact conformations can clearly be classified, and even extended CaM structures in complexes are usually markedly different from the unliganded holo-CaM conformation.

## How reliable are crystal structures of CaM?

What can be said about the physiological relevance of the crystal structures of CaM in isolation, or in complex with peptides and proteins? The variations in the crystal structures of unliganded CaM can be thought of as examples of CaM plasticity also in solution; of course, the view obtained from them is severely limited, due to restrictions imposed by crystal packing. CaM–peptide complexes might sometimes give quite accurate details on interactions at the protein level, but in some cases, they might actually be biologically non-relevant, as the conformations could be driven by the crystallization process. In addition, the isolated peptide target is not equivalent to the full-length protein. CaM–protein complexes could be thought of as

**Table 1** Geometric characteristics of representative CaM crystal structures based on the EF hand-bound metal ion positions

| Structure/complex | PDB code | Reference | Distance EF2–EF3 (Å) | Pseudo-torsion (°) | Angle EF1–EF2–EF3 (°) | Angle EF2–EF3–EF4 (°) |
|---|---|---|---|---|---|---|
| Holo-CaM | 3CLN | Babu et al. (1988) | 46.2 | −134.5 | 89.8 | 66.3 |
| Bent 1 ($Sr^{2+}$) | 4BW7 | Kursula (2014) | 43.8 | −143.1 | 88.4 | 60.6 |
| Bent 2 | 4BW8 | Kursula (2014) | 44.9 | −131.7 | 90.3 | 60.0 |
| Calcineurin A peptide | 2W73 | Majava and Kursula (2009) | 46.2 | −134.8 | 93.3 | 63.4 |
| DAPK protein | 2X0G | de Diego et al. (2010) | 37.2 | 90.8 | 45.7 | 130.5 |
| DAPK peptide | 1YR5 | Bertini et al. (2009) | 39.0 | 116.9 | 48.5 | 102.1 |
| DAPK peptide (NMR) | 2K61 | Bertini et al. (2009) | 39.1 | 110.3 | 51.4 | 105.9 |
| CaMK2 protein | 2WEL | Rellos et al. (2010) | 37.5 | 106.0 | 52.5 | 94.8 |
| CaMK2 peptide | 3GP2 | – | 37.3 | 102.9 | 55.2 | 91.5 |
| Collapsed holo-CaM | 1PRW | Fallon and Quiocho (2003) | 35.9 | 35.9 | 50.7 | 76.4 |
| Trans holo-CaM | 4HEX | Kumar et al. (2013a) | 33.7 | 178.5 | 138.4 | 79.2 |
| $Ca^{2+}$-ATPase | 4AQR | Tidow et al. (2012) | 38.3 | 92.7 | 58.1 | 99.4 |
| | | | 38.4 | 81.5 | 57.3 | 104.9 |
| Ryanodine receptor | 2BCX | Maximciuc et al. (2006) | 40.1 | 54.4 | 66.2 | 103.0 |
| Dimeric Ca(v)1.2 peptide (pre-IQ) | 3G43 | Fallon et al. (2009) | 46.5 | 68.7 | 80.7 | 89.6 |
| Ca(v)1.2 IQ | 2F3Y | Fallon et al. (2005) | 35.2 | 108.4 | 61.9 | 114.5 |
| eNOS | 1NIW | Aoyagi et al. (2003) | 37.7 | 107.0 | 46.7 | 103.7 |
| CaMKK | 1IQ5 | Kurokawa et al. (2001) | 35.2 | 118.8 | 63.8 | 114.9 |
| NMDA receptor | 2HQW | Ataman et al. (2007) | 36.2 | 124.7 | 59.3 | 86.4 |
| SK2 variant 2 | 3SJQ | Zhang et al. (2012) | 46.3 | −139.1 | 91.2 | 69.1 |

The analysis was not carried out for structures with empty EF hands

the most accurate representations of the actual biological complexes, but in fact, CaM-regulatory regions are often very flexible, and the exact conformations seen in crystals might be affected by crystallization. In any case, it must be remembered that both CaM and its binding motifs on targets are generally very dynamic, and any crystal structure is just a snapshot of a certain energetic minimum.

Studies on unliganded CaM in solution using small-angle scattering and other methods have indicated the presence of a mixture of conformations, thereby validating and extending the information obtained from crystal structures (Bertini et al. 2010; Heller 2005; Yamada et al. 2012). It is clear that the middle region of the CaM central helix is unstable and tends to unfold and bend also in the absence of ligand peptides. This behavior has also been seen in computer simulations as well as experimental NMR (Baber et al. 2001; Barbato et al. 1992; Ikura et al. 1991; Spera et al. 1991; van der Spoel et al. 1996; Yang et al. 2001). The central region of the linker α helix is also poorly defined in many CaM crystal structures (Kursula 2014).

A simplified method for CaM conformational analysis by paramagnetic NMR, involving the use of lanthanides binding to EF hands, was developed (Bertini et al. 2003, 2004) and further used to validate CaM–peptide complex crystal structures (Bertini et al. 2009). The latter study provided data to directly compare the conformations of CaM–peptide complexes in solution and the crystal state. Some rearrangements were observed for the complexes of CaM with target peptides from the kinases DAPK1 and DAPK2; the crystallized collapsed conformation was also present in solution, but the relative orientations of the CaM lobes changed (Bertini et al. 2009). It is highly likely that the conformational variability seen in CaM–peptide complexes represents only the tip of the iceberg, when considering CaM bound to regulatory domains of full-length proteins in the cytoplasm.

Based on solution studies, it was suggested that the conformations of CaM bound to a recognition peptide and the corresponding full-length protein (CaMKI) are remarkably similar (Kranz et al. 2002). It is likely that the conclusions are valid for the family of CaMKs, where the regulatory region is pulled out of the autoinhibitory cleft during CaM activation (Rellos et al. 2010). For other CaM target proteins, however, this kind of a relationship remains unclear. The overall flexibility of CaM and its target proteins makes studies on such issues in the crystal state very difficult. More data from CaM–protein complexes will be required to assess the relevance of CaM–peptide complex crystal structures with regard to physiological conditions. One example of this is the above-mentioned CaM–calcineurin

A complex, for which the structure of the CaM–peptide complex presents domain swapping and different oligomeric states between crystalline and solution forms. A recent paper also claimed to present the crystal structure of a protein–protein complex for CaM–calcineurin, but unfortunately, the entire CaM molecule, as well as its target region, was invisible in the crystal (Ye et al. 2013). This is especially surprising, since CaM was elsewhere shown to induce folding of the calcineurin regulatory domain also outside the CaM-binding site (Rumi-Masante et al. 2012).

## Conclusions and perspectives

One question that clearly remains is how much of CaM conformational dynamics in solution and in vivo can actually be trapped using crystallography, especially when using short peptide targets. In crystals, the protein molecules are confined in a rigid state, with highly limited flexibility. It is possible that key functional conformations cannot be crystallized due to their short lifetime and/or inability to form suitable crystal contacts. Interestingly, for CaM in the unliganded state, a number of conformations, possibly also linked to conformational changes upon ligand binding, have been crystallized.

On the other hand, the case of calcineurin complexes shows that the structures of CaM–peptide complexes may vary hugely between solution and the crystal state. Whether such examples actually represent two physiologically relevant complexes, or if at least one of them is an artifact, is not known at this date.

It is clear that for a full understanding of CaM–protein interaction, complexes of full-length proteins must be structurally studied. Here, too, lies a caveat. It is perfectly possible to obtain a crystal structure of a complex, which is actually not in the activated conformation. The crystal structures of protein kinase–CaM complexes are indicative of such a situation. The conformations of CaM, or even the stoichiometry, trapped in the crystal may not be physiologically relevant, as has also been suggested in the case of the CaM–SK2 channel peptide complexes (Villarroel et al. 2014). The regions surrounding a CaM-binding site in a protein are often very flexible and can attain various conformations. In this respect, validation of crystal structures using other methods, such as structural studies in solution and structure-based mutagenesis coupled to cellular assays, is highly warranted. Electron microscopy also provides a viable alternative, as recently shown for the aquaporin–CaM complex (Reichow et al. 2013). In any case, the view provided by crystal structures, easily taken as well-defined, single, static conformations of CaM target complexes, is incomplete and, under physiological conditions, these complexes are likely to present a range of conformations and significant molecular flexibility.

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**Conflict of interest** The author declares that he has no conflict of interest.

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