Target-induced Conformational Adaptation of Calmodulin Revealed by the Crystal Structure of a Complex with Nematode Ca²⁺/Calmodulin-dependent Kinase Kinase Peptide

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Calmodulin (CaM) is a ubiquitous calcium (Ca²⁺) sensor which binds and regulates protein serine/threonine kinases along with many other proteins in a Ca²⁺-dependent manner. For this multi-functionality, conformational plasticity is essential; however, the nature and magnitude of CaM’s plasticity still remains largely undetermined. Here, we present the 1.8 Å resolution crystal structure of Ca²⁺/CaM, complexed with the 27-residue synthetic peptide corresponding to the CaM-binding domain of the nematode Caenorhabditis elegans Ca²⁺/CaM-dependent kinase kinase (CaMKK). The peptide bound in this crystal structure is a homologue of the previously NMR-derived complex with rat CaMKK, but benefits from improved structural resolution. Careful comparison of the present structure to previous crystal structures of CaM complexed with unrelated peptides derived from myosin light chain kinase and CaM kinase II, allow a quantitative analysis of the differences in the relative orientation of the N and C-terminal domains of CaM, defined as a screw axis rotation angle ranging from 156° to 196°. The principal differences in CaM interaction with various peptides are associated with the N-terminal domain of CaM. Unlike the C-terminal domain, which remains unchanged internally, the N-terminal domain of CaM displays significant differences in the EF-hand helix orientation between this and other CaM structures. Three hydrogen bonds between CaM and the peptide (E87-R336, E87-T339 and K75-T339) along with two salt bridges (E11-R349 and E114-K334) are the most probable determinants for the binding direction of the CaMKK peptide to CaM.

Keywords: calcium binding protein; calcium signaling; EF-hand; molecular recognition; signal transduction

Introduction

Calmodulin (CaM) is a ubiquitous Ca²⁺ sensor protein of a single 148 amino acid polypeptide chain, which consists of two homologous domains, each containing two EF-hand Ca²⁺ binding motifs. CaM activates many proteins and enzymes involved in various cellular functions in a Ca²⁺-dependent manner.¹,² Among numerous CaM target proteins, serine/threonine protein kinases are one of the best characterized protein families in terms of mechanisms underlying CaM-dependent activation. The CaM binding region often occurs near or within an autoinhibitory domain of the enzyme, which usually follows the catalytic domain in the primary sequence.³ The CaM binding to the target enzyme induces a conformational

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Abbreviations used: CaM, calmodulin; CaMKK, CaM kinase kinase; skMLCKp, smMLCKp, skeletal and smooth muscle myosin high chain kinase, respectively.

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change, which relieves the autoinhibition, allowing full enzyme activity.

Recent findings indicated the presence of two kinase-kinase cascades involving different Ca\textsuperscript{2+}/CaM-dependent protein kinases: CaM-dependent protein kinase kinase (CaMKK) has been identified and cloned as an upstream activator of CaM-dependent protein kinase I (CaMKI) and CaM-dependent protein kinase IV (CaMKIV).\textsuperscript{4,5} CaMKK is present in the brain, testis and spleen at high levels, whereas other tissues contain much lower levels of CaMKK. Distinct genes code \(\alpha\) and \(\beta\) isoforms of CaMKK,\textsuperscript{6,7} which exhibit both cytoplasmic and nuclear localization.\textsuperscript{8} Activation of nuclear CaMKIV by CaMKK results in transcriptional activation through phosphorylation of several transcription factors including cyclic AMP-responsive element binding protein both \textit{in vitro} and \textit{in vivo}.\textsuperscript{9–12} In the cytoplasm there is a crosstalk between the CaMKK/CaMKIV cascade and other signaling cascades, including MAP kinases\textsuperscript{13} and protein kinase B. In addition, direct activation of protein kinase B by CaMKKK may play a role in an anti-apoptotic effect upon modest elevation of intracellular Ca\textsuperscript{2+} in NG108 cells.\textsuperscript{14} Recent studies have shown that Ca\textsuperscript{2+}/CaM binding to \(\alpha\) isoform of CaMKKK is absolutely required for operating CaM-kinase cascade as well as Ca\textsuperscript{2+}/CaM binding to downstream CaM-kinases.\textsuperscript{15,16} Examination of the regulatory mechanism of CaMKK by Ca\textsuperscript{2+}/CaM is, therefore, important to understanding the activation mechanism of the CaM-kinase cascade.

Many structural studies have focused on determining how CaM can interact specifically with different target proteins, which show very little sequence similarity. So far, three related motifs for Ca\textsuperscript{2+}/CaM binding (1-10, 1-14, and 1-16 motifs) have been characterized structurally on basis of the position of conserved hydrophobic residues (Figure 1a).\textsuperscript{17–21} In the case of skeletal and smooth muscle myosin light chain kinases (skMLCKp and smMLCKp, respectively) and CaM-kinase II (CaMKIIp), bound peptides show a similar helical structure, with two conserved hydrophobic residues located on opposite sides of the helix. In the case of CaM complexed with rat CaM-kinase kinase derived peptide (rCaMKKp), the bound peptide shows unexpected structural features.\textsuperscript{21} First, the bound rCaMKKp consists of two structural segments: an \(\alpha\)-helix and a hairpin-like loop. Second, the binding direction of the rCaMKK peptide relative to the two CaM domains is opposite in orientation when compared with the CaM bound MLCKs and CaMKII peptides. Because of limited precision of the NMR structure, a comparison of the CaM-rCaMKKp structure with other CaM-target complex structures was performed based solely on global structural characteristics.

Here we report the 1.8 Å resolution crystal structure of the CaM complexed with the 27 residue synthetic peptide corresponding to the CaM-binding domain of nematode, \textit{Caenorhabditis elegans}, CaMKK (cCaMKKp).\textsuperscript{22,23} The overall structure retains similar features to the solution structure of the CaM-rCaMKKp complex. A quantitative comparison of the high resolution X-ray structure with other crystal structures of CaM-peptide complexes (CaM-smMLCKp and CaM-CaMKIIp), however, reveals significant changes in the relative orientation of the two CaM domains. Although, the binding orientation of the target peptides to the C-terminal domain is well conserved, major changes occur in the orientation between the target and the N-terminal domain of CaM, involving a movement of the N-terminal helix of CaM with respect to other three EF-hand helices in the N-terminal domain. The current structure also unveils a number of specific contacts between CaM and cCaMKKp, including electrostatic interactions and hydrogen bonding. These interactions identified in the current structure should play a role in determining the binding direction of cCaMKKp relative to the two CaM domains.

### Results and Discussion

#### Structure determination and overall structure

The crystal structure was solved by the molecular replacement method using the coordinates of the CaM-smMLCKp complex\textsuperscript{18} as a search model. The initial rotation and translation search was done using the data of A-form crystal. After the rigid body refinement and simulated annealing refinement,\textsuperscript{24} the electron density map for the helical region of the bound peptide was successfully observed to build an initial model. After several rounds of the manual model building and refinements using X-PLOR,\textsuperscript{24} the C-terminal loop region of the peptide was extended. At this stage, we obtained higher resolution data from the B-form crystal and further refinements were done for the data extended to 1.8 Å. Residues 1 to 3, and 148 of CaM and residues 331 to 333 of cCaMKKp were disordered and not included into the model. The final refined model is basically same as the partially refined model with data of the A-form crystal (the root-mean-square deviation (r.m.s.d.) of C\textsuperscript{\alpha} atoms between them was 0.91 Å). The electron density map around residue Phe352, one of the anchoring residues found in the loop region, is shown in Figure 1(b). The final refinement statistics are shown in Table 1.

The current structure retains similar features to the solution structure of CaM in complex with rCaMKKp.\textsuperscript{21} The backbone r.m.s.d. value for C\textsuperscript{\alpha} atoms between the two structures is 0.66 Å for the N-terminal domain of CaM and 0.73 Å for the C-terminal domain. The r.m.s.d. value increases up to 1.48 Å when both domains are used for the superposition. This is probably due to the ill-definition of the relative orientation of the two CaM domains in the NMR-derived structure. The r.m.s.d. value between cCaMKKp (this study) and rCaMKKp\textsuperscript{21} is 1.77 Å. This difference is mainly attributed from the C-terminal loop portion for which the NMR-
derived structure had less distance constraints than the α-helix portion of the peptide molecule.

The complex shows an ellipsoidal compact structure (Figure 1(c)), in which two homologous domains of CaM (N and C-terminal domains) wrap around the target peptide. The large hydrophobic patch of each CaM domain makes contact with the hydrophobic part of cCaMKKp. The complex used in this study is formed by proteins from different sources (CaM from *Xenopus laevis* and CaMKKp from *Caenorhabditis elegans*). *X. laevis* CaM and *C. elegans* CaM should make contact with cCaMKKp in the same way, since side-chains of all three non-conserved residues (Phe99, Thr143, and Thr147 in *C. elegans* CaM, and Tyr99, Gln143, and Ala147 in *X. laevis* CaM) do not participate in

Figure 1. (a) Sequence alignment of Ca$^{2+}$-CaM binding sequences of CaMKK, MLCK and CaMKII. The key hydrophobic residues are boxed. (b) Electron density map of the loop region (residues 349-354) of cCaMKKp at 1.8 Å resolution. (c) Stereo view of CaM (cyan) in complex with cCaMKKp (yellow). Bound Ca$^{2+}$ is shown as a sphere (purple). Figures are drawn by XtalView$^{36}$ for (b), and MOLSCRIPT$^{37}$ and Raster3D$^{38}$ for (c).
interactions with cCaMKKp. The bound peptide consists of two structural segments: the α-helical region (residues 337 to 349) and the hairpin-like loop region at the C terminus (residues 350 to 357). The direction of the peptide with respect to the N and C-terminal domains of CaM is opposite when compared with previously reported CaM-target structures.\(^{17-19}\)

Comparison with other CaM-target complexes

Relative orientation of two CaM domains

One of the important structural features of CaM is its dual domain architecture: the two globular domains are connected by a flexible linker and both interact with the target peptide.\(^{25}\) A comparison of the present structure with other CaM-target structures indicates significant differences in the domain orientation. In order to characterize the relative orientation of the two homologous domains in a quantitative manner, the rotation and translation values along a screw axis, to superimpose the N-terminal domain onto the C-terminal domain, were estimated. The screw axis was chosen to minimize the translation value.\(^{26}\) In the case of the CaM-cCaMKKp complex, the superimposition of the N-terminal domain (residues 5-73) with the C-terminal domain (residues 84-146) requires a rotation of 156° with a translation of \(-5\) Å (Figure 2(a) and (b)). The screw axis is approximately perpendicular to the bound peptide helix (Figure 2(b)), and runs close to Leu342 (the distance between C\(^\beta\) of Leu342 and the screw axis is \(1.0\) Å), a residue located at the center of the helical region of cCaMKKp (Figure 1(a)).

A similar analysis was performed on other CaM-peptide complexes. In the case of CaM-smMLCKp, a 180° rotation along the screw axis yielded a nearly perfect superposition between the N and C-terminal domains (only a 0.1 Å translation was needed), indicating that the complex has a pseudo 2-fold screw symmetry (Figure 2(c) and (d)). The screw axis, perpendicular to the smMLCKp helix, runs close to His805 of smMLCKp, which is located at the center of the helix of smMLCKp (Figure 1(a)). For CaM-CaMKIIp, a rotation of 196° and a translation of 2 Å are needed for the superimposition (Figure 2(e) and (f)). In this case the screw axis runs close to Thr306 of CaMKIIp, which is shifted toward the C terminus of the helical region of the peptide (Figure 1(a)).

The three CaM targets compared below have different sequence motifs (Figure 1(a)). The cCaMKKp has α-helical (13 residues, 337-349) and hairpin-like loop regions (eight residues, 350-357), 24 residues totally participating in the interaction with CaM. On the other hand, smMLCKp and CaMKIIp adopt only the α-helix conformation, consisting of 19 and 18 residues, respectively. For optimal binding to these unrelated peptides, CaM adjusts the positions of two domains by changing the degrees of unwinding or expansion of the domain linker region.\(^{19}\) The current analysis defines, in a more quantitative manner, the relative position of two domains of CaM which ranges from 156° to 196° in terms of the rotation angle around the screw axis, and from \(-5\) Å to 2 Å in terms of the translation along the screw axis.

Table 1. Details of data collection and refinement

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Conformational Adaptation of Calmodulin
same treatment against the C-terminal domain shows significantly smaller difference in the peptide helix orientation relative to the domain (Figure 3(b)). The cCaMKKp helix shows large interhelical angles (20° and 35°) with smMLCKp and CaMKIIp, respectively, when the N-terminal domains were superimposed (Figure 3(a)). These are significantly larger than the corresponding values (7° and 9°) obtained when the C-terminal domain was used for superposition (Figure 3(b)).

When comparing the two domains of CaM, the C-terminal domain shows smaller changes upon binding to different targets. The r.m.s.d. value for all Cα atoms between CaM-cCaMKKp and CaM-CaMKIIp19 is 1.18 Å for the N-terminal domains, and 0.61 Å for the C-terminal domains; the r.m.s.d. value between CaM-cCaMKKp and CaM-smMLCKp18 is 0.85 Å for the N-terminal domains and 0.81 Å for the C-terminal domains. For a more detailed comparison, the r.m.s.d values for each residue were analyzed (Figure 3(c) and (d)). In accordance with the marked difference in the peptide orientation relative to the N-terminal domain (Figure 3(a)), helix A (residues 6-19) undergoes a notable conformational change upon binding to different target peptides (Figure 3(c)). Variability in the position of helix A between the three CaM-peptide complexes seems to coincide, at least in part, with differences in the peptide helix orientation with respect to the N-terminal domains (Figure 3(a)), since when helix A was used for superimposition, the cCaMKKp helix shows smaller interhelical angles with smMLCKp (13°) and CaMKIIp (9°). The similar analysis with superimposition of helix H, which also has high r.m.s.d values in the C-terminal domain (Figure 3(d)), shows the corresponding interhelical angle 11° for both peptides. These interhelical angles are larger than the angles when C-terminal domains are used for superimposition (7° and 9°), suggesting no correlation between the motion of helix H and the peptide binding orientation. The loop region which connects two EF-hand motifs of the C-terminal domains also shows significant changes (Figure 3(d)). A previous 15N-relaxation study showed that this loop region is highly dynamic.
and this dynamic feature allows the loop to adopt various conformations. Interestingly, Glu11, located in helix A, forms a salt bridge with the basic residues (Arg349 of cCaMKKp; see Figure 5, below; His805 of smMLCKp, and Arg297 of CaMKIIp) of the target in all three CaM-peptide complexes studied so far. Target-induced motion of helix A, which is probably mediated by the conserved interaction via Glu11, plays an essential role in the adaptation of the different binding orientation of the target helix to the N-terminal domain. The intra-domain helix motion, together with the inter-domain adjustment in the relative orientation of two domains, contribute to the adaptation of the different targets of CaM.

Target binding interface

In all complexes, a channel is formed by the two domains of CaM, with an extensive hydrophobic surface responsible for direct contact with the target (Figure 4). More than 60% of the entire surface of the target molecule becomes shielded from the solvent by CaM. The total area of the binding interface of the cCaMKKp with CaM (3140 Å²) is significantly larger than that of CaM-smMLCKp (2790 Å²) and that of CaM-CaMKIIp (2500 Å²). The larger interface of cCaMKKp comprises a total of 24 residues, which form the z-helical and hairpin-like loop regions. In contrast, smMLCKp and CaMKIIp use only 19 and 18 residues, respectively, to form an interacting z-helix.

Within the hydrophobic cavity of which 76% of the total area is non-polar for CaM-cCaMKKp, lies a pocket that is just right for a long hydrophobic side-chain to bind to (Figure 4). In the complexes of known structures, this pocket has been identified consistently as a key anchor point for bound peptides, being able to bind even the largest Trp and Phe side-chains and thereby stabilize the CaM-peptide complex. The cavities in the N and C-terminal domains are both hydrophobic in nature, and each contains four Met residues (Met36, 51, 71, 72 in the N-terminal domain, Figure 4(a), (c) and (e); Met109, 124, 144, 145 in the C-terminal domain, Figure 4(b), (d) and (f)). Recent dynamics studies of the CaM-smMLCKp complex have shown that these four Met side-chains of Ca²⁺-CaM are uncommonly dynamic. The Met side-chains are highly exposed, contributing to 46% of the accessible area of the hydrophobic patches on the N and C-terminal domains. Upon binding with smMLCKp, the internal mobility of these Met side-chains becomes significantly reduced, with Met72 and Met124 becoming most rigid. In the present structure of CaM-cCaMKKp, Met72 of CaM packs against Ile341 of cCaMKKp, while Met124 interacts with the side-chain of Phe352 of cCaMKKp. These and other Met residues undergo certain conformational changes, in order to best fit the shape and nature of the target peptide; however, the lack of
precision especially in side-chain atomic positions prohibited a detailed comparison between this and other structures.

**Charge interactions between CaM and cCaMKKp**

Osawa *et al.* suggested that an electrostatic attraction at the channel outlets formed by the two domains plays a key role in determining the binding direction of the target protein; however, no specific charge interactions could be elucidated due to technical limitations. The present crystal structure supports this notion and reveals several specific charge interactions and hydrogen bonds, enriching our understanding on the electrostatic interactions between CaM and CaMKKp. Near the cCaMKKp N terminus, two hydrogen bonds are formed between CaM and cCaMKKp, Glu87-Arg336 and Glu87-Thr339 (Figure 5(a)). Another hydrogen bond, which is mediated via a water molecule, is present between Lys75 and Thr339. At the other channel outlet (Figure 5(b)), two hydrogen bonds (Leu112-Lys344 and Glu114-His348) and two salt bridges (Glu11-Arg349 and Glu114-Lys334) can be readily identified. The C-terminal region of the cCaMKKp helix has four basic residues (His348, Arg349, Lys350 and Arg351), and His348 and Arg349 make interactions with CaM acidic residues, Glu114 and Glu11, respectively. Comparing this with the primary sequence of rat CaMKK (Figure 1(a)), we see that Thr339 in the N-terminal region is conserved and the two basic residues, His348 and Arg349 in the C-terminal region, are replaced with other basic residues (Arg and Lys, respectively), indicating that most of the hydrogen bonds and salt bridges described above should be present in the CaM-rCaMKKp complex.

A full picture of CaM-cCaMKKp interactions is illustrated in Figure 5(c), which contains all bound water molecules observed as well as van der Waals contacts between the two polypeptides. As Osawa *et al.* suggested, the basic residues at the C-terminal region of CaMKKp (Lys344, His354 and Arg349) indeed interact with CaM acidic residues (Glu11 and Glu114) and fewer charge interactions are found at the N terminus, confirming the importance of the distribution of basic residues in the target peptide to determining the binding direction to CaM. Further understanding of the role of charge in the interaction surfaces will be achieved by a determination of the complex structure of CaM with other targets such as melittin, which binds to CaM in the same direction as cCaMKKp.

**Concluding remarks**

The versatility of CaM in activating numerous protein kinases as well as other enzymes and proteins originates from its ability to bind a large number of distinct targets by changing its conformation. It has been demonstrated that the linker connecting two CaM domains plays a key role in adjusting the relative position of the domains. The current study allows a better analysis of the relative position of the two domains of CaM, which ranges from 156° to 196° rotation and from −5 Å to 2 Å translation in terms of screw axis. This allows CaM to recognize various types of target sequences such as 1-16 (CaMKK), 1-14 (MLCK) and 1-10 (CaMKII) types. In addition to the domain linker, the present structure determination has elucidated the importance of helix A, which undergoes significant movement relative to other three EF-hand helices in the N-terminal domain of CaM upon binding of cCaMKKp. No effect of similar magnitude was found in the C-terminal domain. Three hydrogen bonds and four electrostatic interactions, found in this crystal structure, are at least partly responsible for target binding specificity, directing the surprising reversed orientation of CaMKKp in contrast with smMLCKp and CaMKIIp. In these charge contacts, the interaction through Glu11, which is conserved in all three
CaM-peptide complexes studied so far, may play a critical role in target recognition by CaM. This and other high-resolution structures along with detailed comparisons between each structure have helped our comprehensive understanding of CaM’s functional versatility.

Materials and Methods

Sample preparation

A 27 residue synthetic peptide corresponding to the CaM binding domain of C. elegans CaMKK was synthesized by Peptide Institute Inc. Recombinant X. laevis CaM was expressed in Escherichia coli and purified to homogeneity as described.31 CaM was dissolved in the unbuffered solution containing 0.1 M KCl and 10 mM CaCl₂ (pH 6.7) and concentrated to 1.5 mM. cCaMKKp was then added to the protein solution with a molar ratio of 1.25:1.

Crystallization and data collection

Two types of crystals (A-form and B-form) were obtained using vapor diffusion at room temperature. A-form crystals were grown by mixing equal volumes of protein solution and crystallization buffer of 0.1 M citrate-NaOH (pH 5.0) and 30% (w/v) PEG 8000 over two to three weeks. In contrast B-form crystals appeared when a crystallization buffer of 0.1 M citrate-NaOH (pH 5.0), 2.0 M ammonium sulfate and 0.2 M sodium tartrate was used. Diffraction data for both crystals were measured at BL-6B at the Photon Factory (Tsukuba). Data reduction, merging and scaling were carried out with programs DENZO32 and SCALA.33 Crystal data and the quality of data sets are summarized in Table 1.

Structure determination and refinement

The N-terminal (Ala5-Ala73) and C-terminal domains (Glu84-Thr146) of CaM (PDB code: 1CDL, molecule A) were used as the initial models for a molecular replacement search with the program AMoRe34 with the data of A-form crystal. Since the presence of a pseudo C-centered lattice were indicated from the diffraction pattern in the a*b*c plane, C222₁ space group was assumed instead of P222₁ to reduce the number of search molecules during the calculations. In the first step, the rotation and translation of the N-terminal domain was determined. The position of the C-terminal domain was then searched with the coordinates of N-terminal domain model fixed.

The model was refined with simulated annealing using X-PLOR.24 The electron density corresponding to the bound peptide was good enough to identify the amino acid residues in the α-helical region. At this stage we succeeded in obtaining higher resolution data of the
B-form crystal. The partially refined model of the CaM-ccA MKKp complex using data between 10.2.5 Å resolution of A-form crystal (R-factor of 0.315) was used for molecular replacement search. Several cycles of refinement and manual rebuilding with the program O35 were performed. The final R-factor is 0.217 (Rfree 0.248) and refinement statistics are summarized in Table 1.

Data Bank accession code

The atomic coordinates have been deposited in the RCSB PDB with the entry code 1IQS.

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References


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