Crystal structure of a myristoylated CAP-23/NAP-22 N-terminal domain complexed with Ca^{2+} /calmodulin

Mamoru Matsubara^{1,2,*}, Toru Nakatsu^{1,3},
Hiroaki Kato^{1,3} and Hisaaki Taniguchi^{1,4}

¹Membrane Dynamics Project, Harima Institute at SPring-8, RIKEN, Sayo, Hyogo, Japan, ²R&D Laboratories, Carna Biosciences Inc., Kobe, Japan, ³The Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan and ⁴Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan

A variety of viral and signal transduction proteins are known to be myristoylated. Although the role of myristoylation in protein–lipid interaction is well established, the involvement of myristoylation in protein–protein interactions is less well understood. CAP-23/NAP-22 is a brain-specific protein kinase C substrate protein that is involved in axon regeneration. Although the protein lacks any canonical calmodulin (CaM)-binding domain, it binds CaM with high affinity. The binding of CAP-23/NAP-22 to CaM is myristoylation dependent and the N-terminal myristoyl group is directly involved in the protein–protein interaction. Here we show the crystal structure of Ca^{2+} -CaM bound to a myristoylated peptide corresponding to the N-terminal domain of CAP-23/NAP-22. The myristoyl moiety of the peptide goes through a hydrophobic tunnel created by the hydrophobic pockets in the N- and C-terminal domains of CaM. In addition to the myristoyl group, several amino-acid residues in the peptide are important for CaM binding. This is a novel mode of binding and is very different from the mechanism of binding in other CaM–target complexes.

The EMBO Journal (2004) 23, 712–718. doi:10.1038/sj.emboj.7600093; Published online 12 February 2004

Subject Categories: structural biology; signal transduction

Keywords: calmodulin; crystal structure; myristoylation; PKC; protein–protein interaction

Introduction

Since the discovery of protein myristoylation in the catalytic subunit of cAMP-dependent protein kinase (Carr *et al*, 1982), various viral and signal transduction proteins have been shown to be fatty acylated (Johnson *et al*, 1994). This modification is often essential for the proper functioning of the modified protein. For example, the transforming activity of p60^{src} from Rous sarcoma virus is dependent on its

myristoylation (Garber and Hanafusa, 1987). However, the mechanism by which this modification exerts its effects is still largely unknown (Johnson *et al*, 1994; Resh, 1996, 1999). It is generally assumed that hydrophobic acyl groups, such as myristoyl and palmitoyl groups, are often involved in protein–membrane interactions. Due to its intermediate hydrophobicity, myristoylation has been implicated in reversible membrane association of proteins (McLaughlin and Aderem, 1995; Taniguchi, 1999). Studies from our own and others' laboratories have established that such a mechanism, in fact, participates in the phosphorylation-dependent interaction of myristoylated alanine-rich C kinase substrate (MARCKS) with membranes (Taniguchi and Manenti, 1993; Kim *et al*, 1994). In the case of recoverin, the binding of Ca^{2+} induces a drastic conformational change of the protein, causing the myristoyl group hidden inside the protein to protrude, whereupon it can interact with the membrane (Ames *et al*, 1997). The modification has also been shown to affect the stability of cAMP-dependent protein kinase (Yonemoto *et al*, 1993). On the other hand, how protein myristoylation is involved in protein–protein interaction remains unclear, even though it has been extensively studied.

Brain-specific protein CAP-23/NAP-22 was characterized first in chicken brain as a cortical cytoskeleton-associated protein of 23 kDa (Widmer and Caroni, 1990). Its rat homologue was later identified as a neuron-specific acidic protein of 22 kDa (Maekawa *et al*, 1993). Although the physiological function of the protein has not yet been determined, it is known to be involved in spinal axon regeneration and is found in the brain membrane raft fraction (Maekawa *et al*, 1999; Laux *et al*, 2000; Bomze *et al*, 2001). Like the two related proteins GAP-43 and MARCKS, CAP-23/NAP-22 is a prominent substrate of protein kinase C (PKC), and its phosphorylation by PKC is regulated by calmodulin (CaM) binding (Maekawa *et al*, 1994). Using synthetic myristoylated peptides, as well as myristoylated and nonmyristoylated recombinant proteins, we narrowed down the CaM-binding domain of CAP-23/NAP-22 to the myristoylated nine-amino-acid N-terminal peptide (Takasaki *et al*, 1999). Interestingly, the binding of CAP-23/NAP-22 to CaM is dependent on myristoylation; only the myristoylated recombinant protein binds to CaM. Thus, this is the first definitive demonstration that protein myristoylation is involved in protein–protein interaction.

Here we show the crystal structure of Ca^{2+} -CaM bound to a myristoylated peptide derived from the N-terminal domain of CAP-23/NAP-22. The obtained structure revealed that the myristoyl moiety of CAP-23/NAP-22 enters into a hydrophobic tunnel created by the hydrophobic pockets of CaM. In addition to the myristoyl group, several amino-acid residues in the peptides are important in the binding of CaM. The overall binding mode is distinct from those observed in other protein–protein complexes. These results reveal a unique

*Corresponding author. R&D Laboratories, Carna Biosciences Inc., 5-5-2 Minatojima-minamimachi, Kobe 650-0047, Japan.
Tel.: +81 78 302 7039; Fax: +81 78 302 7086;
E-mail: mamoru.matsubara@carnabio.com

Received: 30 September 2003; accepted: 7 January 2004; Published online: 12 February 2004

structure and demonstrate how protein myristoylation functions in protein-protein interaction.

Results and discussion

Overall structure of the myristoylated CAP-23/NAP-22 peptide-CaM complex

Our previous work demonstrated that the myristoylated nine-amino-acid N-terminal peptide of CAP-23/NAP-22 is sufficient for high-affinity binding to Ca^{2+} -CaM (3.0 nM) and, moreover, that the myristoyl moiety of CAP-23/NAP-22 is required for binding (Takasaki *et al*, 1999). To understand the structural basis of the myristoylation-dependent binding, we attempted to crystallize the complex of CaM and the myristoylated CAP-23/NAP-22 peptide (myr-G₂₀₁GKLSKKKK₂₀₉, mNAP peptide, first N-terminal Gly defined as G201). We

Figure 1 Crystal structure of Ca^{2+} /CaM complexed with the mNAP peptide. Ribbon representation of the complex from the (A) front and (B) side. The CaM N- and C-terminal domains are shown in blue and green, respectively, the mNAP peptide is shown in red, and Ca^{2+} ions are shown as purple spheres. The figure was generated using MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994). In the final model, Ala1, Asp2, Thr79, Asp80, Ala147 and Lys148 of CaM, and Lys207, Lys208 and Lys209 of the mNAP peptide are missing, while Glu11, Asp50, Lys77, Asp78, Lys105 and Glu127 of CaM, and Lys206 of the mNAP peptide, which have weak side chain densities, are modeled as alanines.

Table I Data collection and refinement statistics

Data collection	
Space group	P3 ₂ 21
Wavelength (Å)	1.02
Cell constant (Å)	
<i>a</i> = <i>b</i>	40.6
<i>c</i>	173.6
Resolution (Å)	2.3
Reflections	
Total	39942
Unique	7815
Completeness (%)	96.9
<i>R</i> _{merge} (%)	8.3
Refinement statistics	
Resolution (Å)	20–2.3
Reflections	7799
<i>R</i> _{cryst} (%)	24.7
<i>R</i> _{free} (%) ^a	27.4
RMS deviation	
Bond lengths (Å)	0.006
Bond angles (°)	1.07

^a*R*_{free} was calculated against 5% of the total reflections omitted from the refinement.

successfully crystallized the CaM-mNAP complex and solved the X-ray structure with a 2.3 Å resolution (Figure 1, Table I). Besides a few surface side chains and the residues of the central linker regions, the structure is well defined by the electron density. The overall structure of CaM in the complex is an ellipsoidal compact structure with a cavity, mainly filled with the myristoyl moiety of the mNAP peptide. The conformation of CaM is similar to those in other CaM-peptide or CaM-drug complexes, with the two lobes of CaM folded in such a way that they can grab the target peptide (Ikura *et al*, 1992; Meador *et al*, 1992, 1993; Vondonselaar *et al*, 1994; Osawa *et al*, 1999; Harmat *et al*, 2000).

Mechanism of the binding mode of the myristoyl moiety

The structure of the myristoylated peptide is exceptional, as expected. In the complex, the peptide consists of a long myristoylated moiety, a hairpin-like loop (residues 201, 202) and an extended region (residues 203–206) (Figure 1, see also Figure 3). The myristoyl moiety of the mNAP peptide is unambiguously defined by electron density (Figure 2A). While the extended region travels along the groove between the two lobes of CaM, the myristoyl moiety goes through the hydrophobic tunnel created by the hydrophobic pockets of the N- and C-terminal lobes of CaM (Figure 2B). Residues of CaM within 5 Å of the myristoyl group are mainly hydrophobic (Figure 2C). In particular, CaM residues Leu39 and Phe92 contribute to binding of the head (C10–C13) of the myristoyl moiety. Residues Phe19 and Leu112 interact with the middle part (C7–C9), while residues Leu18 and Met109

Figure 2 Binding interface of the myristoyl moiety of the mNAP peptide in the complex. (A) Electron density map of the myristoyl moiety. The simulated-annealing electron density map was refined by CNS (Brunger, 1998) and is contoured at 1.0σ. The myristoyl moiety is shown in red. The figure was generated using TURBO-FRODO (Roussel and Cambillau, 1991). (B) Hydrophobic tunnel of CaM-mNAP peptide complex. The space model of the myristoyl moiety is shown in red, and the hydrophobic residues of CaM are shown in yellow. The figure was prepared with SPOCK (Christopher, 1998). (C) Stereo view of CaM residues within 5 Å of the myristoyl group as generated by MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

contribute to binding of the neck (C4–C6) of the myristoyl moiety. The myristoyl group is thus firmly anchored to CaM by multiple hydrophobic interactions.

Until now, few three-dimensional structures of myristoylated proteins have been reported. The NMR structure of recoverin in a Ca^{2+} -free state revealed the direct interaction of the acyl group with its own protein moiety (Tanaka *et al*, 1995). In addition, the crystal structure of myristoylated cAMP-dependent protein kinase has elucidated the structural basis for the effects of acylation on protein stability (Zheng *et al*, 1993). In these structures, the myristoyl moieties are sequestered deeply into hydrophobic pockets formed by many hydrophobic residues. However, these are intramolecular interactions wherein the myristoylated protein interacts with its own acyl group. Our structure is unique because it shows how a protein grabs the covalently bound acyl chain of another protein. Similar myristoylation-dependent protein–protein interactions are thought to play roles in the structural organization or stability of picornavirus capsid protein VP4 (Chow *et al*, 1987) and calcineurin B (Griffith *et al*, 1995). The hydrophobic pocket observed in our structure is reminiscent of that in the isoprenylated Rho-GDI-binding pocket of cdc42 (Gosser *et al*, 1997). However, the myristoyl-binding pocket in our structure is much larger and contains many more hydrophobic residues, suggesting that hydrophobic interactions are highly important for myristoylation-dependent protein–protein binding.

Interaction of peptide amino acids with CaM

We have previously shown that neither myristic acid nor a short myristoylated peptide, myr-GGK, binds to CaM (Takasaki *et al*, 1999). This suggests that the binding of the myristoylated N-terminal peptide is affected not only by a simple hydrophobic interaction between the myristoyl group and the hydrophobic part of CaM but also by other specific interactions. The present structure provides an insight into the structural basis of the interaction between the N-terminal peptide of NAP-22 and CaM (Figure 3A). The two N-terminal Gly residues of the mNAP peptide (Gly201 and Gly202) form a tight hairpin turn, which resembles a hook, positioning the following two amino acids, Lys203 and Leu204, inside of the CaM cavity. Replacing one of the Gly residues with other amino acids greatly reduced the affinity (data not shown), indicating that the presence of two successive Gly residues is essential for binding. The presence of other amino acids makes such a turn energetically unfavorable. The only hydrophobic residue in the N-terminal peptide, Leu204, interacts with the side chains of Phe12 and Met144 of CaM through hydrophobic interactions. Furthermore, it appears that there is an intermolecular electrostatic interaction between the basic residue, Lys203, of the peptide and the negatively charged CaM residue, Glu7. Thus, in addition to the myristoyl group, several N-terminal residues play important roles in the NAP-22–CaM interaction. Unfortunately, no significant electron density is observed for the three C-terminal Lys residues of the peptide. However, many acidic residues of CaM surround the C-terminal basic cluster of the mNAP peptide (Figure 3B), indicating that electrostatic interactions can provide additional binding energy. In fact, successive removal of these Lys residues gradually reduces the affinity of the mNAP peptide for CaM (Takasaki *et al*, 1999).

Figure 3 Binding interface of specific residues in CaM with the mNAP peptide. (A) Stereo representation of the binding mode in the N-terminal protein domain generated by MOLSCRIPT (Kraulis, 1991) and Raster3D (Merrit and Murphy, 1994). The myristoylated peptide is indicated in purple, and the residues of CaM are shown in yellow. (B) Electrostatic surface of CaM colored by charge (red, negative; blue, positive), with the mNAP peptide shown in ribbon (green) representation. The figure was produced using GRASP (Nicholls *et al*, 1991).

Novel mode of target recognition by CaM

The majority of the CaM-binding domains contain positively charged and hydrophobic residues, and many of them have the potential to form an amphiphilic α -helix (Crivici and Ikura, 1995; James *et al*, 1995). Indeed, previously determined structures show that CaM-bound target peptides form α -helices in the CaM complex. However, in the present structure, the peptide moiety of the mNAP peptide does not form an α -helix in the complex (Figure 1). The present structure was compared with that of a typical target peptide–CaM complex (CaM kinase II peptide–CaM complex) (Figure 4A). In the comparison, the N-terminal domains of CaM were aligned to minimize the differences. Interestingly, the myristoyl moiety of the mNAP peptide almost coincides with the α -helical peptide of CaM kinase II (Figure 4B). However, there are significant differences between them, especially the position of hydrophobic residues involved in the interactions. In the canonical CaM-binding domains, two key hydrophobic residues are positioned 9, 13 or 15 residues apart. These two residues are anchored to the hydrophobic pockets formed in each of the two CaM lobes (Figure 4C). In the hydrophobic pockets of the CaM C-terminal lobe, specific hydrophobic residues of the target peptides can be seen (Leu299 of the CaMKII peptide, Trp800 of the MLCK peptide and Phe157 of the MARCKS peptide), which are located in the same space (Figure 5A). Although our recent structure of the MARCKS peptide–CaM complex shows a novel CaM-binding mode, this hydrophobic pocket of the C-terminal lobe is

Figure 4 Comparison of the CaM-mNAP peptide with other CaM-peptide complexes. Images were generated by MOLSCRIPT (Kraulis, 1991) and Raster3D (Merrit and Murphy, 1994). (A) The CaM-mNAP peptide complex (blue) is superimposed onto the CaM-CaMKII peptide complex (green) to give the maximal alignment of the N-terminal domains. (B) Comparison of the space location of the bound peptides. The α -helix formed by the CaMKII peptide is shown in green, while the mNAP peptide is shown in red. (C) Comparison of CaM-peptide complexes. MLCK peptide (1-14 motif), CaMKK (1-16 motif), CaMKII (1-10 motif), MARCKS (basic motif) and mNAP peptide (novel motif) are shown. Side chains of the two key hydrophobic residues are shown in red.

present (Yamauchi *et al*, 2003). Furthermore, similar hydrophobic pockets in the N-terminal lobe of CaM are formed by the key hydrophobic residues (Leu308 of the CaMKII peptide and Leu813 of the MLCK peptide) (Figure 5B). In contrast, the two key hydrophobic residues in the mNAP peptide-CaM complex are absent in both N- and C-terminal lobes of CaM (Figure 5A and B). Thus, our present structure is distinct from the hydrophobic pockets formed in other CaM complexes because the myristoyl moiety and Leu204 take the place of the two hydrophobic residues. Furthermore, the parts of CaM involved in the interaction are quite different than in other complexes, because many hydrophobic residues are aligned to form a single large hydrophobic cavity to accommodate the more bulky myristoyl moiety.

The recently reported structures of three CaM-target complexes, Ca^{2+} -activated K^+ channel (SK2)-CaM, edema factor (EF)-CaM and plant glutamate decarboxylase (GAD)-CaM, show that CaM can take completely different, nonglobular conformations (Schumacher *et al*, 2001; Drum *et al*, 2002; Yap *et al*, 2003). In these structures, there are unique domain orientations of CaM. This is especially evident when comparing the angles between the helices flanking the linker (Yap *et al*, 2003). The interhelical angles for the classical CaM-peptide complexes range between -114.2 and -123.2° , while the values for SK2, EF and GAD complexes are 78.0 , 47.8 and -26.3° , respectively (Yap *et al*, 2003; Table II). However, the interhelical angle for the mNAP peptide-CaM is -110.1° , suggesting that the domain orientation resembles that of classical CaM-peptide complexes. Furthermore, the distance

Figure 5 (A) Interactions between the CaM C-terminal lobe and the bound peptides. The CaM-MLCK peptide (red, left side), the CaM-CaMKII peptide (green, left side) and the CaM-MARCKS peptide structure (yellow, left side) were superimposed onto the CaM-mNAP peptide structure (blue, right side) with respect to the C-terminal domain of CaM. The hydrophobic residues (Trp800 in MLCK, Leu299 in CaMKII and Phe157 in MARCKS) occupied the space in the hydrophobic pockets of the CaM C-terminal lobe. In contrast, the hydrophobic pocket was absent in CaM-mNAP peptide structure. (B) Interactions between the CaM N-terminal lobe and the bound peptides. The CaM-MLCK peptide (red, left side), the CaM-CaMKII peptide (green, left side) and the CaM-MARCKS peptide structure (yellow, left side) were superimposed onto the CaM-mNAP peptide structure (blue, right side) with respect to N-terminal domain of CaM. The hydrophobic residues (Leu813 in MLCK and Leu308 in CaMKII) occupied the space in the hydrophobic pockets of the CaM N-terminal lobe. In contrast, the hydrophobic pocket was absent in CaM-mNAP peptide structure.

between residues 72 and 85 for the mNAP peptide-CaM complex ($19.3 \text{ \AA}$) is almost identical to that for classical CaM-peptide complexes (Table II). Thus, the present structure shows that the globular form of Ca^{2+} -CaM can be generated when it is complexed with the mNAP peptide, because the myristoylated target uses a broad hydrophobic interaction instead of the conventional hydrophobic pocket. Overall, the CaM-mNAP peptide complex represents a novel mode of target recognition for CaM and demonstrates the surprising versatility of this important signaling molecule.

Implications for myristoylation-dependent interaction

CAP-23/NAP-22 may not be the only example where a myristoyl group is involved in a protein-protein interaction. Recently, we demonstrated that the N-terminal regions of the HIV-1 Nef protein and MARCKS bind to CaM (Hayashi *et al*, 2002; Matsubara *et al*, 2003). The N-terminal regions of these proteins share structural features, including one hydrophobic residue surrounded by a few basic residues that are preceded by the myristoyl group. This raises the possibility that the protein-bound myristoyl group is directly involved in protein-protein interactions. Recent biochemical evidence has shown that CAP-23/NAP-22 and MARCKS, which share many

Table II Comparison of CaM–mNAP peptide complex with other CaM–target complexes

CaM target	Motif	CaM structure	Target structure	Interhelical angle ^a	Distance ^b
mNAP	Myristoylation dependent	Collapsed	Myristoylation + no helix peptide	–110.1	19.3
MARCKS	Basic	Collapsed	Short helix + no helix peptide	–112.4	18.9
MLCK	1–14	Collapsed	Helix	–114.2	17.6
CaMKII	1–10	Collapsed	Helix	–123.2	20.1
CaMKK	1–16	Collapsed	Helix + hairpin loop	–115.3	18.6
GAD	Nonclassical	Extended	Two-helix peptide	–17.2	12.5
EF	Nonclassical	Extended	Complicated	47.8	25.1
SK2	Nonclassical	Extended	Three helices, dimer	78	28.9

^aInterhelical angle between CaM helices 4 (residues 65–72) and 5 (85–92) calculated as described (Yap *et al*, 1999, 2002, 2003).

^bDistance between CaM residues 72 and 85 (Å) calculated as described (Yap *et al*, 1999, 2002, 2003).

structural features, accumulate in a neuronal membrane raft fraction (Maekawa *et al*, 1999; Laux *et al*, 2000; Khan *et al*, 2003). In the membrane rafts, these proteins are regulated by CaM binding and PKC-mediated phosphorylation in the N-terminal domain. In the case of HIV-1 Nef, the protein also exists in the membrane rafts after infection by the HIV-1 virus (Wang *et al*, 2000; Zheng *et al*, 2001). The myristoylation may help recruit these proteins to the membrane raft structures by means of both protein–protein and protein–lipid interactions.

Owing to the hydrophobicity and the structural resemblance to the lipids, the roles of fatty acylation in the protein–lipid interaction are easy to understand. The roles of fatty acylation in protein–protein interactions, on the other hand, have remained controversial. Our previous biochemical and present structural studies clearly establish the direct involvement of fatty acylation in protein–protein interaction. The present structure of the CaM–mNAP peptide complex explains how protein myristoylation mediates protein–protein interaction. Comparison with other structures such as recoverin and cAMP-dependent protein kinase, in which proteins bind to their own myristoyl groups, should provide a framework for understanding the molecular basis of lipid modification recognition by proteins.

We recently reported the crystal structure of the MARCKS effector domain complexed with CaM (Yamauchi *et al*, 2003). MARCKS and CAP-23/NAP-22 belong to the same protein family of acylated hydrophilic and acidic proteins (McLaughlin and Aderem, 1995; Taniguchi, 1999). In contrast to CAP-23/NAP-22, MARCKS has a domain in the middle of its structure that binds various cellular components, including CaM and membrane phospholipids. The structure of a peptide derived from this domain complexed with CaM was novel, because it does not form a conventional α -helix (Matsubara *et al*, 1998; Yamauchi *et al*, 2003). The structure of the MARCKS peptide/CaM complex clearly lacks one of the two hydrophobic pockets (Yamauchi *et al*, 2003). This and our present work illustrate the versatility of CaM in recognizing a wide variety of target structures. This degree of structural versatility is beyond that previously predicted for CaM (Crivici and Ikura, 1995; James *et al*, 1995). Myristoylated proteins have diverse functions and intracellular destinations, and these are clearly regulated by this modification due to alterations in both protein–lipid and protein–protein interactions. Finally, our structure may help in understanding intracellular signaling and pathological processes, because protein myristoylation not only regulates the function and

localization of signaling molecules but also has been implicated in cancer, viral disorders and infections. In this way, these studies may assist in the future development of therapeutic agents for cancer and infectious diseases.

Materials and methods

Crystallization, data collection and structure determination

A myristoylated peptide of nine amino acids, based on the N-terminal sequence of CAP-23/NAP-22 (myr-GGKLSKKKK) and synthesized using standard Fmoc chemistry, was purchased from Research Genetics. Recombinant human CaM was expressed in *Escherichia coli* and purified to homogeneity by column chromatography on phenyl-sepharose. CaM was dialyzed against H₂O and lyophilized. For crystallization (hanging-drop vapor diffusion), 0.9 mM CaM and 2.7 mM peptide in 10 mM CaCl₂ were mixed 1:1 with reservoir solution (36% PEG1000, 20 mM cacodylate buffer, pH 5.5) and equilibrated against 1 ml of reservoir solution at 4°C. The crystal growth took 2–3 weeks. Glycerol (20%) was used as the cryoprotectant. Reflections from the structure were collected from a single crystal at 90 K using an image plate detector and a SPring-8 BL45XU beamline. Data processing was carried out using DENZO and Scalepack (Otwinowsky and Minor, 1997). The space group is P3₂21 with cell constants $a = b = 40.6$ Å and $c = 173.6$ Å, wherein there is one CaM–peptide complex in the asymmetric unit. The structure was determined by molecular replacement using the AMoRe program of the CCP4 package (CCP4, 1994) and the protein portion of the CaM–arylalkylamine complex as a model for the CaM structure (Harmat *et al*, 2000).

Model refinement

The model-building steps were carried out with the TURBO-FRODO (Roussel and Cambillau, 1991) program and the resulting model was refined with CNS (Brunger *et al*, 1998). We used rigid body refinement followed by torsion-angle-simulated annealing at a starting temperature of 4000 K (slow cooling) to 2.3 Å. The progress of model building and refinement was monitored using R_{free} . After several rounds of model building and refinement, the refinement converged to $R = 24.7\%$ and $R_{\text{free}} = 27.4\%$. The final structure consists of 144 CaM residues, four Ca²⁺ ions and one myristoylated peptide, with an RMS deviation of 0.006 Å in bond length and 1.07° in bond angles. PROCHECK showed that the final model lacked residues in disallowed regions. Interhelical angles and distances were calculated by VGM (Yap *et al*, 1999, 2002).

PDB accession code

The coordinates and structure factors have been deposited in the Protein Data Bank under ID code 1L7Z.pdb.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- Ames JB, Ishima R, Tanaka T, Gordon JI, Stryer L, Ikura M (1997) Molecular mechanics of calcium-myristoyl switches. *Nature* **389**: 198–202
- Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JHP (2001) Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat Neurosci* **4**: 38–43
- Brunger A, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL (1998) Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D* **54**: 905–921
- Carr SA, Biemann K, Shoji S, Parmelee DC, Titani K (1982) n-Tetradecanoyl is the NH₂-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. *Proc Natl Acad Sci USA* **79**: 6128–6131
- CCP4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D* **50**: 760–763
- Chow M, Newman JFE, Filman D, Hogle JM, Rowlands DJ, Brown F (1987) Myristoylation of picornavirus capsid protein VP4 and its structural significance. *Nature* **327**: 482–486
- Christopher JA (1998) SPOCK: The Structural Properties Observation and Calculation Kit (Program Manual). College Station, TX, USA: The Center for Macromolecular design, Texas A&M University
- Civici A, Ikura M (1995) Molecular and structural basis of target recognition by calmodulin. *Ann Rev Biophys Biomol Struct* **24**: 85–116
- Drum CL, Yan SZ, Bard J, Shen YQ, Lu D, Soelaiman S, Grabarek Z, Bohm A, Tang WJ (2002) Structural basis for the activation of anthrax adenyl cyclase exotoxin by calmodulin. *Nature* **415**: 396–402
- Garber EA, Hanafusa H (1987) NH₂-terminal sequences of two src proteins that cause aberrant transformation. *Proc Natl Acad Sci USA* **84**: 80–84
- Gosser YQ, Nomanbhoy TK, Aghazadeh B, Manor D, Combs C, Cerione RA, Rosen MK (1997) C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. *Nature* **387**: 814–819
- Griffith JP, Kim JL, Klm EE, Slntohak MD, Thomson JA, Fltzglbbn MJ, Fleming MA, Caron PR, Hsiao K, Navia MA (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **82**: 507–522
- Harmat V, Bocskei Z, Naray-Szabo G, Bata I, Csutor AS, Hermecz I, Aranyi P, Szabo B, Liliom K, Vertessy BG, Ovadi J (2000) A new potent calmodulin antagonist with arylalkylamine structure: crystallographic, spectroscopic and functional studies. *J Mol Biol* **297**: 747–755
- Hayashi N, Matsubara M, Jinbo Y, Titani K, Izumi Y, Matsushima N (2002) Nef of HIV-1 interacts directly with calcium-bound calmodulin. *Protein Sci* **11**: 529–537
- Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A (1992) Solution structure of calmodulin-target peptide complex by multi-dimensional NMR. *Science* **256**: 632–638
- James P, Vorherr T, Carafoli E (1995) Calmodulin-binding domains: just two faced or multi-faceted? *Trends Biochem Sci* **20**: 38–42
- Johnson DR, Bhatnagar RS, Knoll LJ, Gordon JI (1994) Genetic and biochemical studies of protein N-myristoylation. *Annu Rev Biochem* **63**: 869–914
- Khan TK, Yang B, Thompson NL, Maekawa S, Epand RM, Jacobson K (2003) Binding of NAP-22, a calmodulin-binding neuronal protein, to raft-like domains in model membranes. *Biochemistry* **42**: 4780–4786
- Kraulis PJ (1991) MOLSCRIPT—a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* **24**: 946–950
- Kim JB, Blackshear PJ, Johnson JD, McLaughlin S (1994) Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. *Biophys J* **67**: 227–237
- Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P (2000) GAP43, MARCKS, CAP23 modulate PI(4,5)P₂ at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J Cell Biol* **149**: 1455–1471
- Maekawa S, Maekawa M, Hattori S, Nakamura S (1993) Purification and molecular cloning of a novel acidic calmodulin binding protein from rat brain. *J Biol Chem* **268**: 13703–13709
- Maekawa S, Murofushi H, Nakamura S (1994) Inhibitory effect of calmodulin on phosphorylation of NAP-22 with protein kinase C. *J Biol Chem* **269**: 19462–19465
- Maekawa S, Sato C, Kitajima K, Funatsu N, Kumanogoh H, Sokawa Y (1999) Cholesterol-dependent localization of NAP-22 on a neuronal membrane microdomain (raft). *J Biol Chem* **274**: 21369–21374
- Matsubara M, Titani K, Taniguchi H, Hayashi N (2003) Direct involvement of protein myristoylation in myristoylated alanine-rich C kinase substrate (MARCKS)–calmodulin interaction. *J Biol Chem* **278**: 48898–48902
- Matsubara M, Yamauchi E, Hayashi N, Taniguchi H (1998) MARCKS, a major protein kinase C substrate, assumes non-helical conformations both in solution and in complex with Ca²⁺-calmodulin. *FEBS Lett* **421**: 203–207
- McLaughlin S, Aderem A (1995) The myristoyl-electrostatic switch: a modulator of reversible protein–membrane interactions. *Trends Biochem Sci* **20**: 272–276
- Meador WE, Means AR, Quirocho FA (1992) Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin–peptide complex. *Science* **257**: 1251–1255
- Meador WE, Means AR, Quirocho FA (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of X-ray structures. *Science* **262**: 1718–1721
- Merrit EA, Murphy MAP (1994) Raster3D version 2.0. A program for photorealistic molecular graphics. *Acta Crystallogr D* **50**: 869–873
- Nicholls A, Sharp K, Honig BH (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins Struct Funct Genet* **11**: 281–296
- Osawa M, Tokumitsu H, Swindells MB, Kurihara H, Orita M, Shibamura T, Furuya T, Ikura M (1999) A novel target recognition revealed by calmodulin in complex with Ca²⁺-calmodulin-dependent kinase kinase. *Nat Struct Biol* **6**: 819–824
- Otwinowsky A, Minor W (1997) Processing of X-ray diffraction data in ascillation mode. *Methods Enzymol* **276**: 307–325
- Resh MD (1996) Regulation of cellular signaling by fatty acid acylation and prenylation of signal transduction proteins. *Cell Signal* **8**: 403–412
- Resh MD (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta* **1451**: 1–16
- Roussel A, Cambillau C (1991) TURBO-FRODO. *Silicon Graphics Geometry Pattners Directory*, Vol. 86 Mountain View, CA: Silicon Graphics
- Schumacher MA, Rivard AF, Bachinger HP, Adelman JP (2001) Structure of the gating domain of a Ca²⁺-activated K⁺ channel complexed with Ca²⁺/calmodulin. *Nature* **410**: 1120–1124
- Takasaki A, Hayashi N, Matsubara M, Yamauchi E, Taniguchi H (1999) Identification of the calmodulin-binding domain of neuron-specific protein kinase C substrate protein CAP-23/NAP22. *J Biol Chem* **274**: 11848–11853
- Tanaka T, Ames JB, Harvey TS, Stryer L, Ikura M (1995) Sequestration of the membrane-targeting myristoyl group of recoverin in the calcium-free state. *Nature* **376**: 444–447
- Taniguchi H (1999) Protein myristoylation in protein–lipid and protein–protein interactions. *Biophys Chem* **82**: 129–137
- Taniguchi H, Manenti S (1993) Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. *J Biol Chem* **268**: 9960–9963
- Vandonselaar M, Hickie RA, Quail JW, Delbaere LTJ (1994) Trifluoperazine-induced conformational change in Ca²⁺-calmodulin. *Nat Struct Biol* **1**: 795–801
- Wang JK, Kiyokawa E, Verdin E, Trono D (2000) The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc Natl Acad Sci USA* **97**: 394–399
- Widmer F, Caroni P (1990) Identification, localization, and primary structure of CAP-23, a particle-bound cytosolic protein of early development. *J Cell Biol* **111**: 3035–3047
- Yamauchi E, Nakatsu T, Matsubara M, Kato H, Taniguchi H (2003) Crystal structure of a MARCKS peptide containing the calmodulin-binding domain in complex with Ca²⁺-calmodulin. *Nat Struct Biol* **10**: 226–231

- Yap KL, Ames JB, Swindells MB, Ikura M (1999) Diversity of conformational states and changes within the EF-hand protein superfamily. *Proteins: Struct Funct Genet* **37**: 499–507
- Yap KL, Ames JB, Swindells MB, Ikura M (2002) Vector geometry mapping. A method to characterize the conformation of helix–loop–helix calcium-binding proteins. *Methods Mol Biol* **173**: 317–324
- Yap KL, Yuan T, Mal TK, Vogel HJ, Ikura M (2003) Structural basis for simultaneous binding of two carboxy-terminal peptides of plant glutamate decarboxylase to calmodulin. *J Mol Biol* **328**: 193–204
- Yonemoto W, McGlone ML, Taylor SS (1993) N-myristylation of the catalytic subunit of cAMP-dependent protein kinase conveys structural stability. *J Biol Chem* **268**: 2348–2352
- Zheng J, Knighton DR, Xuong NH, Taylor SS, Sowadski JM, Ten Eyck LF (1993) Crystal structure of the myristoylated catalytic subunit of cAMP-dependent protein kinase reveal open and closed conformations. *Protein Sci* **2**: 1559–1573
- Zheng YH, Plemenitas A, Linnemann T, Fackler OT, Peterlin BM (2001) Nef increases infectivity of HIV via lipid rafts. *Curr Biol* **11**: 875–879