Chapter I

General Introduction

I-1 Calcium Signaling in Eukaryotic Cells

Multicellular organisms developed systems for information exchange between cells. This is achieved by the messenger molecules such as neurotransmitters, hormones, and cytokines, which are released from one cell to transfer the information to their target cells. Such extracellular signals are transduced into the increase of the intracellular concentration of second messenger molecules, leading to a variety of biological reactions.

Calcium ion (Ca^{2+}) was proposed to be one of the second messengers by Rasmussen in 1970 (Rasmussen, 1970). While the Ca^{2+} concentration is about 10^{-3} M in the extracellular fluid and one of the cell organelles, endoplasmic reticulum (ER), it is about 10^{-7} M in the cytoplasm of resting cells. This 10^{4} times difference in Ca^{2+} concentration between inside and outside of cell, which is maintained by Ca^{2+} excluding systems including calcium pump and Na^{+}-Ca^{2+} exchanger on the cell membrane, makes Ca^{2+} flow into cytoplasm upon opening of Ca^{2+}-channels on the cell membrane. Thus, Ca^{2+} concentration in the cytoplasm can rapidly increase in response to the extracellular signals, which enables Ca^{2+} to act as a second messenger.

I-2 Calmodulin as a Signal Transducer

Upon increasing of the intracellular Ca^{2+} concentration, Ca^{2+} binds a variety of Ca^{2+}-binding proteins. Among these proteins, calmodulin (CaM) was discovered as an activator of brain phosphodiesterase (Cheung, 1971; Kakiuchi *et al.*, 1971; Kakiuchi *et al.*, 1973). Since then, CaM has been attentioned as an important signal transducer. CaM is a ubiquitous acidic protein consisting of 148 amino acids and has four Ca^{2+}-binding sites called as EF-hand motif. The C-terminal two EF-hands bind Ca^{2+} with high affinity (K_d = 10^{-7} M), N-terminal two with lower affinity ($K_d = 10^{-6}$ M). Since the binding affinity of CaM to Ca^{2+} is lower than the Ca^{2+} concentration in the resting cells,

CaM binds Ca^{2+} only when the elevation of the Ca^{2+} level is induced by the extracellular signals. This Ca^{2+} binding enables CaM to activate its target enzymes such as myosin light chain kinase (MLCK), Ca^{2+}/CaM dependent kinases (CaMKs), protein phosphatase calcineurin, phosphodiesterase, nitric oxide synthase, Ca^{2+}-ATPase pumps as well as cytoskeletal structural proteins (Figure I-1, Eldik & Watterson, 1998). Thus, CaM mediates a wide range of physiological processes through Ca^{2+}-dependent regulation of target enzymes.

The activation mechanism upon binding of Ca^{2+}/CaM has been revealed for some protein kinases. They have an auto-inhibitory domain that largely overlaps a CaM-binding domain. While the autoinhibitory affinities are usually of the order of 10^{-5}~10^{-7} M although they are quite specific (Smith *et al.*, 1990), most CaM-binding domains have K_ds for Ca^{2+}/CaM ranged from 10^{-7}~10^{-10} M. Therefore, the binding of Ca^{2+}/CaM to the CaM-binding domain of the target enzymes extracts the overlapping auto-inhibitory domain from the substrate-binding site or active site, leading to the activation of the target (Figure I-2).

I-3 Structure of Calmodulin

As mentioned above, Ca^{2+} binding to CaM is a crucial step for Ca^{2+}-signaling pathways. Then, what happens in CaM upon Ca^{2+} binding? The three-dimensional structures of apo CaM and Ca^{2+}/CaM have already been determined by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography (Figure I-3, Babu *et al.*, 1985; Finn *et al.*, 1995; Kretsinger *et al.*, 1986; Kuboniwa *et al.*, 1995; Zhang *et al.*, 1995). A comparison between Ca^{2+}-bound and unbound forms reveals that Ca^{2+} binding to apo CaM changes the interhelical angle of the two helices of each EF-hand such that CaM undergoes a transition from the 'closed' conformation to the 'open' conformation (Figures I-4a,b, Kuboniwa *et al.*, 1995; Zhang *et al.*, 1995). This

Ca^{2+}-induced conformational change results in the creation of a hydrophobic pocket on the surface of each domain (Figure I-4c, Babu *et al.*, 1985; Kretsinger *et al.*, 1986), which in turn is essential for CaM to bind target enzymes in a Ca^{2+} dependent manner (see later, Ikura *et al.*, 1992; Meador *et al.*, 1992; Meador *et al.*, 1993).

The crystal structures of Ca^{2+}/CaM show a 'dumbbell-shape' structure: the two globular domains (N and C-terminal domains), each of which consists of two EF-hand structures, connected via a domain linker (residues 77-81) in a helix (Figure I-3b, Finn *et al.*, 1995; Kretsinger *et al.*, 1986). Small-angle X-ray scattering (SAXS) studies confirmed that Ca^{2+}/CaM exists as the dumbbell structure not only in crystals but also in solution (Heidorn *et al.*, 1989; Seaton *et al.*, 1985). However, it was indicated by various methods such as mutagenesis (Persechini & Kretsinger, 1988), NMR (Barbato *et al.*, 1992), and theoretical calculation (Spoel *et al.*, 1996) that the domain linker of Ca^{2+}/CaM is highly flexible, suggesting that Ca^{2+}/CaM is not in a single conformation but in an ensemble of many 'elongated' structures in solution. Ca^{2+}/CaM has many glutamic acid residues on the surface of each domains, which may cause electrostatic repulsion between both domains to bring about such 'elongated' structure.

I-4 Molecular Recognition of Ca^{2+}/CaM

A number of three-dimensional structures of Ca^{2+}/CaM complexed with peptides from target enzymes (target peptide) have been determined. The first was an NMR structure of the complex with a 26-residue peptide from skeletal muscle (sk) MLCK (Ikura *et al.*, 1992), which was followed by the crystal structures of complexes with a 20-residue peptide from smooth muscle (sm) MLCK (Meador *et al.*, 1992), and a 25-residue peptide from brain Ca^{2+}/CaM dependent kinase (CaMK) IIα (Meador *et al.*, 1993). Irrespective of the low

homology in the peptide sequences, the peptide adopts a helical conformation and the flexible domain linker of Ca^{2+}/CaM allows its domains to clamp the target peptide in all complexes, leading to a compact globular complex (Figures I-5a,b). The hydrophobic pocket in each CaM domain binds to the bulky hydrophobic residue of the target which seems to anchor the peptide to each Ca^{2+}/CaM domain. In the intermolecular interactions, 80 % are van der Waals contacts and 15 % are electrostatic interactions including hydrogen bonds and ion bridges. Through these interactions, Ca^{2+}/CaM extracts the neighboring auto-inhibitory domain of the target enzyme from its active site, leading to the activation of the target enzyme.

The compounds which inhibit the Ca^{2+}/CaM dependent activation of the target enzymes by the direct interaction with CaM are known as 'CaM antagonists'. Two crystal structures of Ca^{2+}/CaM in complex with one of the CaM antagonists, trifluoperazine (TFP), have also been reported, in which the both domains of CaM collapse like Ca^{2+}/CaM-target peptide complex (Figure I-6, Cook *et al.*, 1994; Vandonselaar *et al.*, 1994). One structure reported by Cook *et al.* (Cook *et al.*, 1994) shows that only one TFP molecule binds to the hydrophobic pocket of CaM C-terminal domain, whereas the other reported by Vandonselaar *et al.* (Vandonselaar *et al.*, 1994) has four TFP molecules: two of them binding to the hydrophobic pocket in each domain of CaM, respectively, and the other two located in the cleft between the CaM domains. In addition, comparisons show that the orientation of the TFP phenothiazine ring binding to one molecule bound to the hydrophobic pocket in each CaM domain and two molecules in the cleft between two domains. From these crystal structures, the CaM inhibition was proposed to be achieved by the TFP induced global structural change of Ca^{2+}/CaM from an 'elongated' to a globular form. However, it is uncertain whether Ca^{2+}/CaM undergoes such change in solution upon the binding of such a small molecule as TFP.

I-5 The Aims of This Thesis

Although the broad specificity of Ca^{2+}/CaM molecular recognition is manifested in the CaM binding regions of these target proteins, which differ significantly in their amino-acid sequences (Table I-1), Ca^{2+}/CaM can bind these regions with affinity of K_d = 10^{-7}~10^{-10} M. Recently, the Ca^{2+}/CaM binding region of the Ca^{2+}/CaM dependent protein kinase kinase (CaMKK) has been identified by the mutagenesis studies. CaMKK regulates the upstream of a cascade that activates the kinases, Ca^{2+}/CaM dependent protein kinase I (CaMKI) and IV (CaMKIV) (Okuno & Fujisawa, 1993; Okuno *et al.*, 1994; Tokumitsu *et al.*, 1994; Tokumitsu *et al.*, 1995). The amino acid sequence of the Ca^{2+}/CaM binding region of CaMKK has low homology with that in the target peptide from MLCK or CaMKII in spite of its high affinity to Ca^{2+}/CaM (K_d = 10^{-9} M), suggesting that their binding to Ca^{2+}/CaM shows various recognition mode among them.

In addition, a variety of small organic molecules, antagonists with distinct chemical structures have been found to inhibit the CaM-mediated processes by direct interaction with Ca^{2+}/CaM (K_d = 10^{-5}~10^{-6} M). To understand why Ca^{2+}/CaM can recognize such a variety of molecules, detailed structural analyses of Ca^{2+}/CaM complexes with target peptides and small molecules are crucial.

Thus, I used heteronuclear multidimensional NMR spectroscopy for the solution structure determination of Ca^{2+}/CaM in complex with the target peptide from CaMKK and with one of the CaM antagonists, W-7, and applied small-angle X-ray scattering (SAXS) to analyze the global structure of Ca^{2+}/CaM - W-7 complex in solution. NMR is a powerful method to determine the three-dimensional structure of protein in solution, while SAXS gives the radius of gyration and the distance distribution function, which well describes the global structure of the macromolecule in solution.

These structural studies on Ca^{2+}/CaM in complex with target peptide and CaM antagonist gave the insights into the diversity in Ca^{2+}/CaM molecular recognition. In addition, the structural information obtained here provided an idea for the novel CaM antagonists. A number of new compounds have been synthesized and biochemically tested, resulting in the increase of the inhibition activities. The analysis of their interaction with Ca^{2+}/CaM further revealed the mode of Ca^{2+}/CaM molecular recognition.

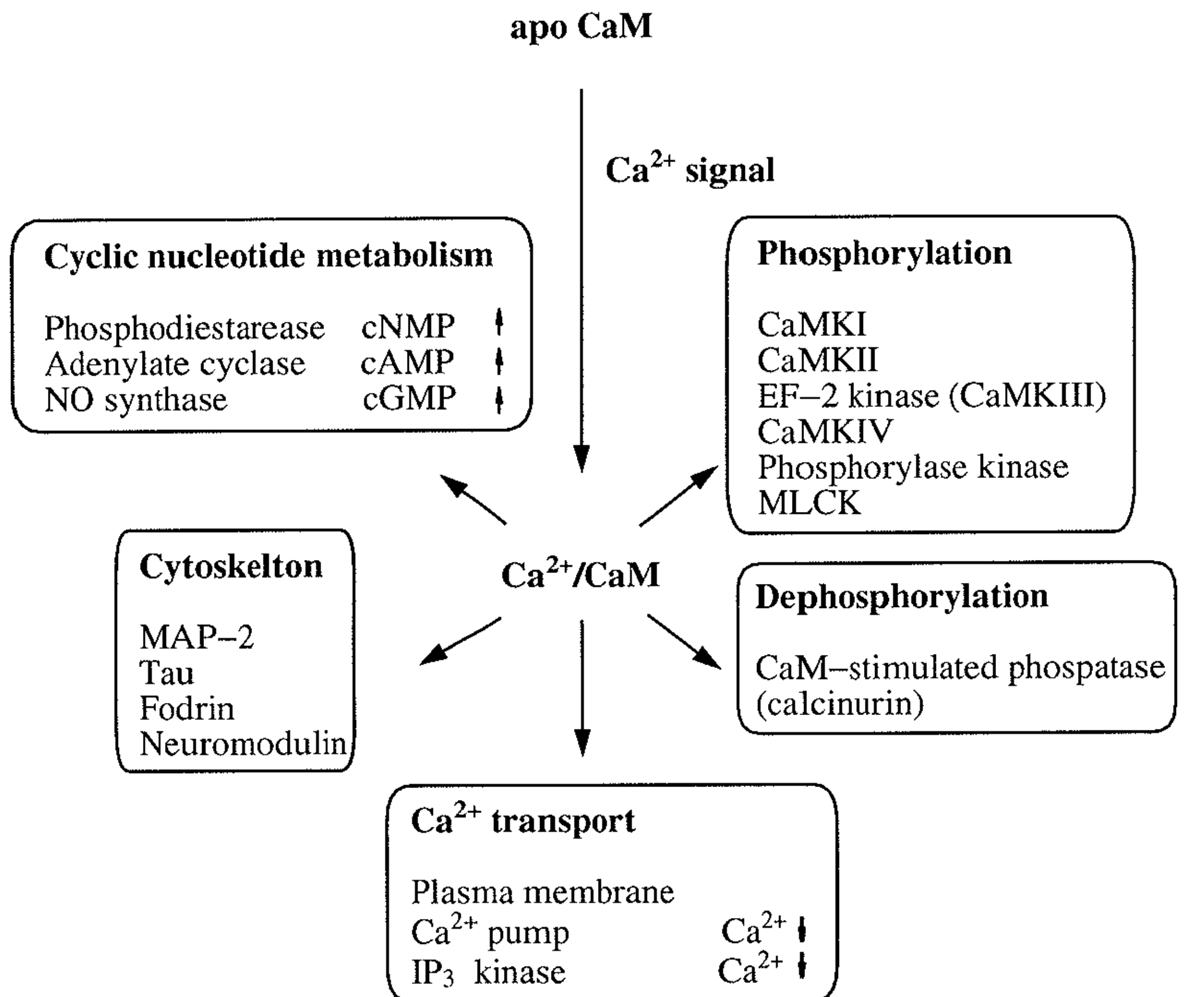


Figure I–1. Summary of the main target proteins of Ca^{2+}/CaM and the effect of Ca^{2+}/CaM binding on signal transduction in the cell.

EF–2, elongation factor–2; NO, nitric oxide;

MAP–2, microtuble–associated protein–2;

IP_3 kinase, inositol (1,4,5)– triphosphate 3–kinase

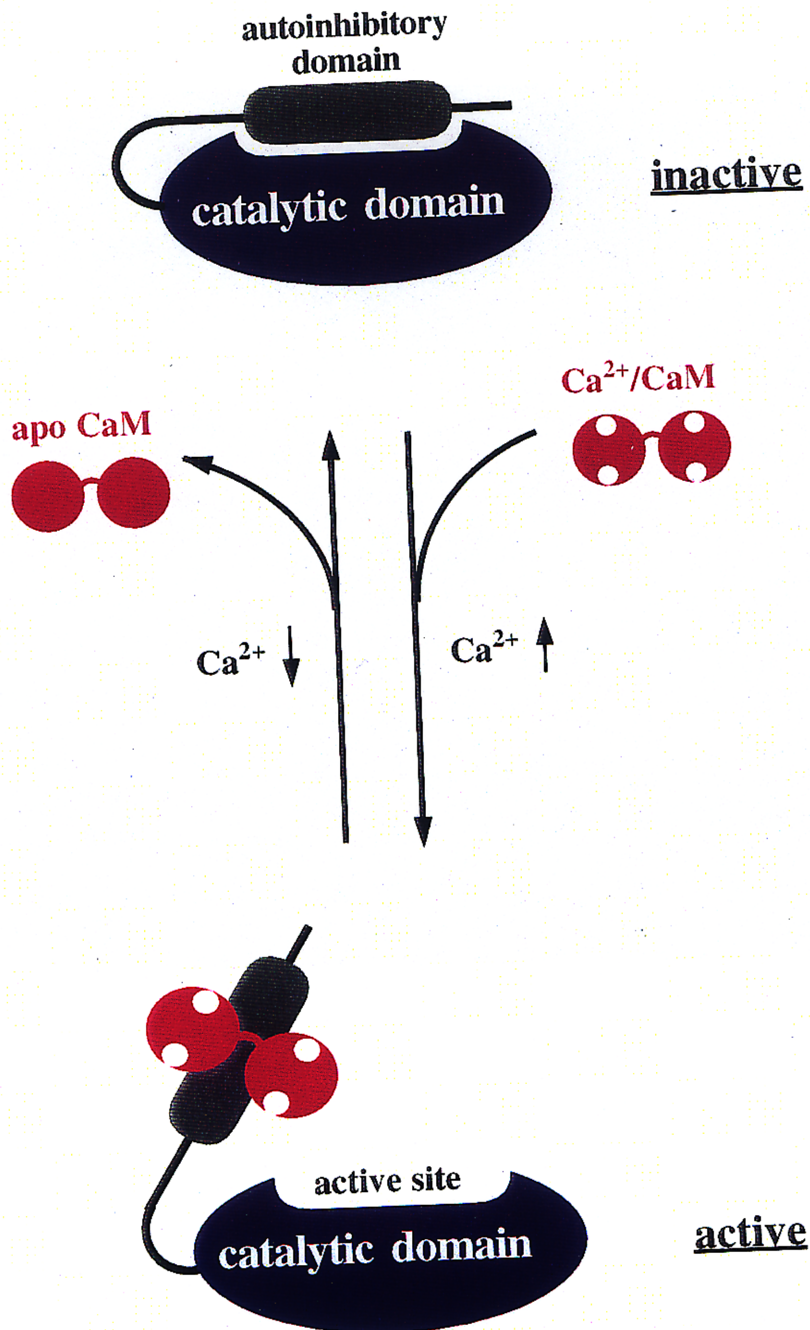


Figure I–2. Schematic drawing of the Ca^{2+}/CaM–dependent activation of some protein kinases including CaMKI, CaMKII, MLCK and so on.

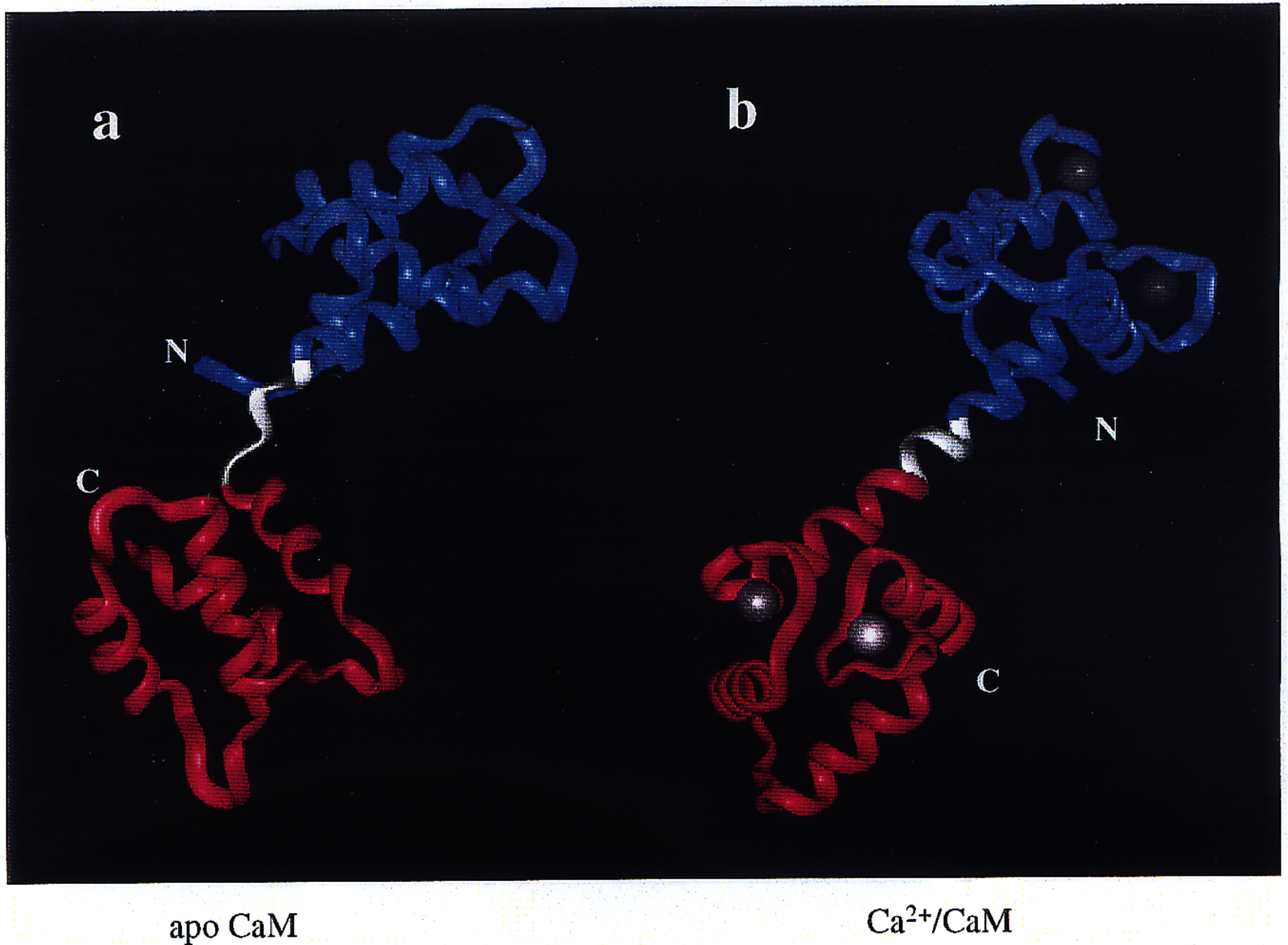


Figure I–3. Ribbon drawing of the solution structure of apo CaM (a, Zhang *et al.*, 1995; Kuboniwa *et al.*, 1995) and the crystal structure of Ca^{2+}/CaM (b, Babu *et al.*, 1985). N (residues 1–76) and C–terminal (residues 82–148) domain of CaM and the domain linker (residues 77–81) are shown in blue, magenta, and white, respectively. N and C–termini of CaM are labeled. Calcium ions are drawn as white spheres.

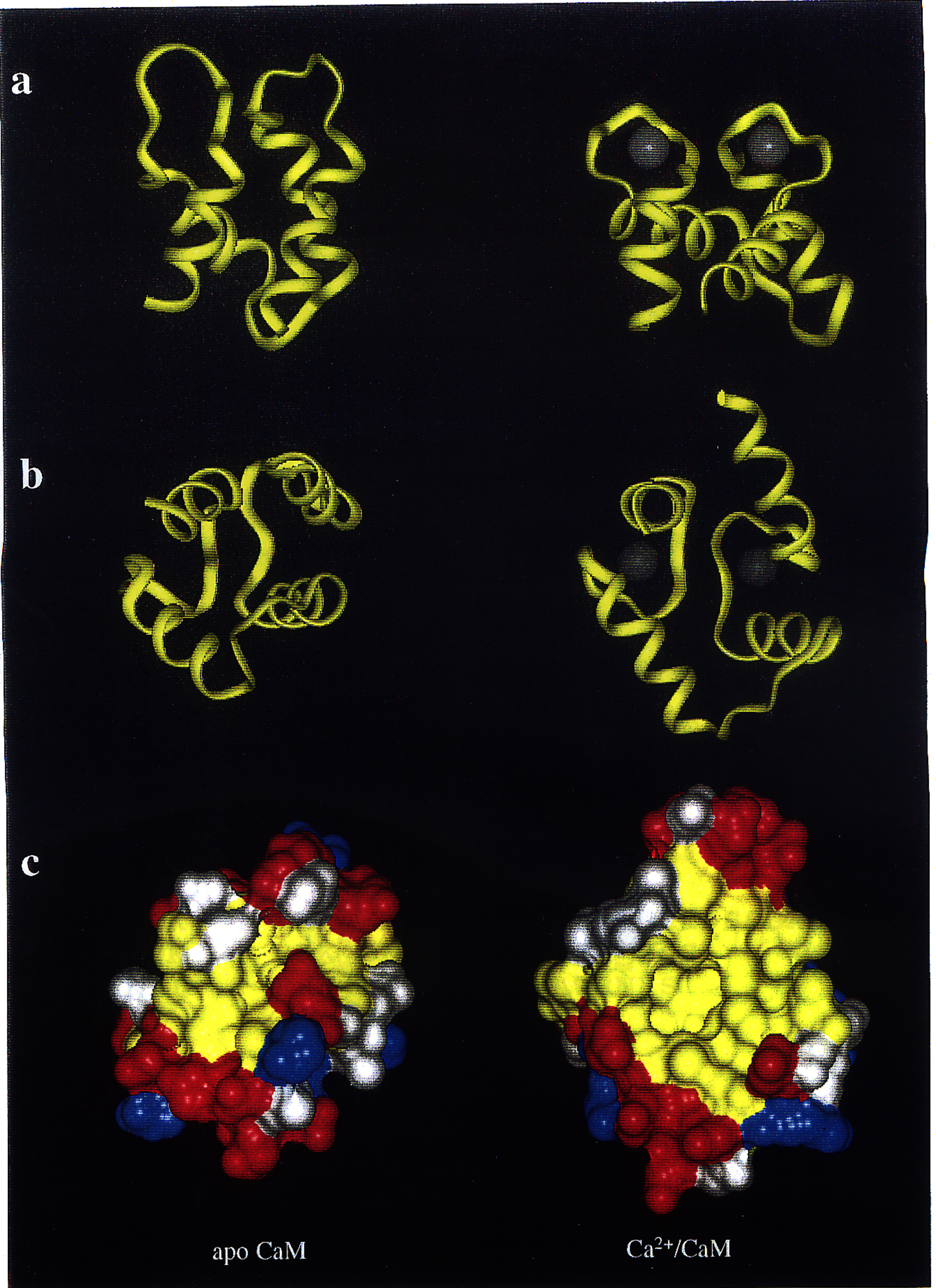
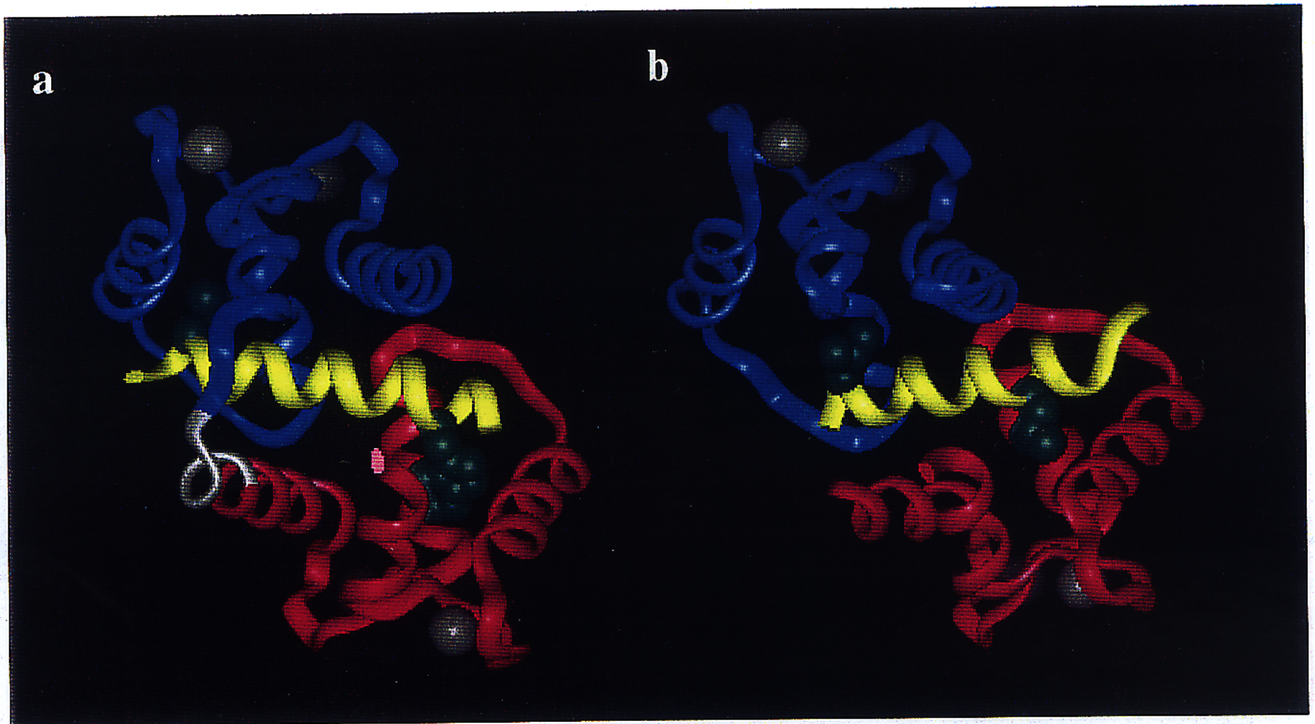


Figure I–4. a, Side and b, front view of the C–terminal domain of apo CaM in closed conformation (left) and Ca^{2+}/CaM in open conformation (right). c, Surface drawing of the C–terminal domain in the same orientation as b. Acidic, basic, hydrophobic residues are colored in red, blue, and yellow, respectively. Hydrophilic and neutral residues are shown in white.



Ca^{2+}/CaM – MLCK

Ikura *et al,* 1992.
Meador *et al*., 1992.

Ca^{2+}/CaM – CaMKII

Meador *et al*.,1993.

c

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skMLCK   : KKRWKKNFIAVSAANRFKKISSSGAL
smMLCK   : RRKWQKTGHAVRAIGRLSSS
CaMKII   : RRKLKGAILTTMLATRNFS
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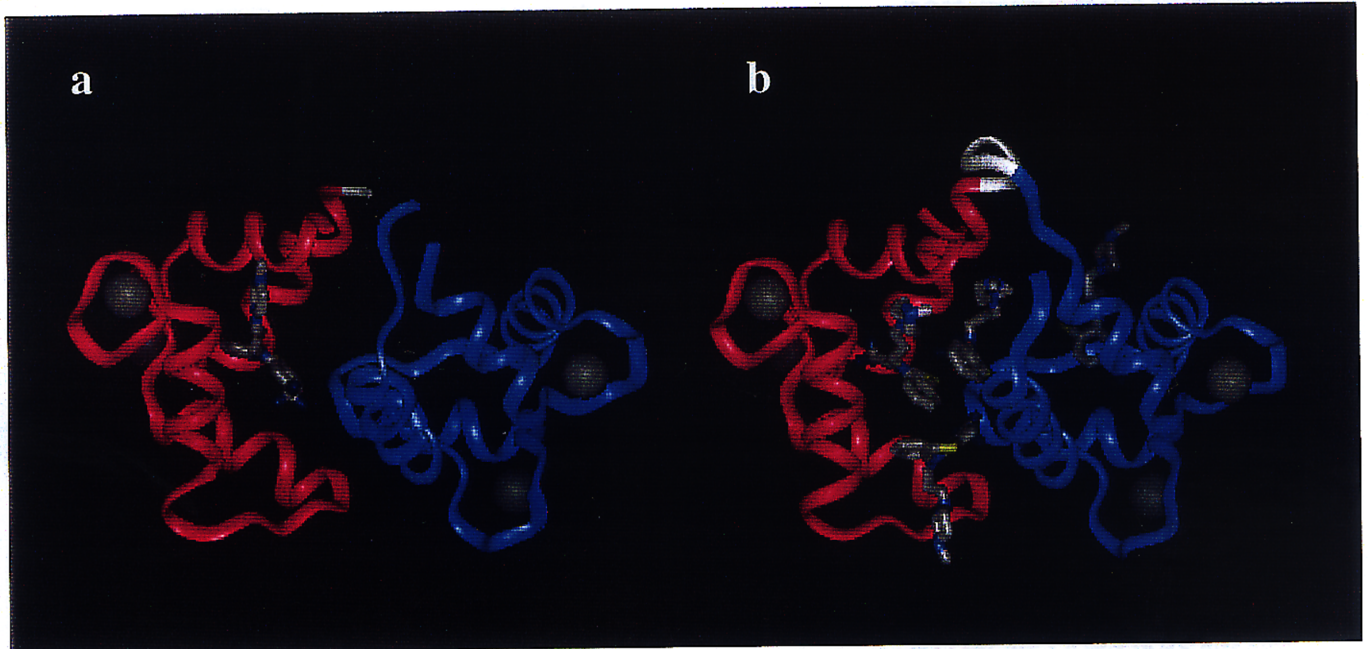
Figure I–5. Ribbon drawing of the structure of the Ca^{2+}/CaM–MLCK peptide complex (a, Ikura *et al*., 1992; Meador *et al*., 1992), and Ca^{2+}/CaM–CaMKII complex (b, Meador *et al*., 1993). The target peptide and its residues anchoring to the hydrophobic pocket of each CaM domain are shown in yellow and green, respectively. N and C–terminal domain of CaM and the domain linker are shown in blue, magenta, and white, respectively. c, Amino acid sequences of the target peptide with anchor residues shaded.



Cook *et al.*, 1994. Vandonselaar *et al.*, 1994.

Figure I–6. Ribbon drawing of the crystal structure of the Ca^{2+}/CaM–TFP complexes.

a, Ca^{2+}/CaM:TFP = 1:1 complex reported by Cook *et al.* b, Ca^{2+}/CaM:TFP = 1:4 complex reported by Vandonselaar *et al.* N and C–terminal domain of CaM and the domain linker are shown in blue, magenta, and white, respectively.

Table I–1. Alignment of the Ca^{2+}/CaM binding sequences of the CaM targets

Protein	Source	Sequence
MLCK	rabbit sk	KRRWKKNFIAVSAANRFKKI
MLCK	chicken sm	RRKWQKTGHAVRAIGRLSSM
CaMKI	yeast	RQKFRNSVERVRLNMKIQKL
CaMKII	yeast	GFSLRKKLRDAIEIVKLNMR
CaMKIV	mouse	RRKLKAAVKAVVASSRLGSA
CaMK	*E.nidulans*	RSRLKRGIEIIKLANRIEAL
CaMK	*M.domestica*	DAEIVSRLQSFNARRKLRAA
Calspermin	rat	RRKLKAAVKAVVASSRLGSA
Calcineurin A	human	KEIIRNKIRAIGKMARVFSV
Calcineurin A	*N.Crassa*	RRAIKNKILAIGRLSRVFQV
DAP kinase	human	RKKWKQSVRLISLCQRLSRS
Fodrin	human	ASPWKSARLMVHTVATFNSI
NO synthase	rat brain	AIGFKKLAEAVKFSAKLMGQ
Titin	human	HTLIKKDLNMVVSAARISCG
Ryanod.receptor	rabbit brain	ASLFCKLAALVRHRISLFGS
Na^+/Ca^{2+} exchanger	human	SYEFKSTVDKLILLTNLALV
Myosin myr4	rat	NVLFSCHVRKVNRFSKVEDR
Caldesmon	human	AEGVRNIKSMWEKGNVFSSP
Calcium pump	human	QILWFRGLNRIQTQIRVVNA
Adenylyl cyclase	*B.anthracis*	ADAFKKIARELNTYILFRPV
Adenylyl Cyclase	*B.pertussis*	RIDLLWKIARAGARSAVGTE
Utrophin	human	MDVLQRKLREVSTKFQLFQK
Mastoparan X	wasp	INWKGIAAMAKKLL
Mastoparan		INLKALAALAKKIL
TRPL	*D.melanogaster*	EDSLRHSLSRVNIYRALCSP
GNRP	rat	IKKVQFSLRGWLCRRKWKNI
Vesicle-associated protein	rat	CAQIEKNFARAKWKKAVRVT
Myosin NinaC	short-form	VIKVQSMMRALLARKRVKGG
Myosin NinaC	long-form	VIKVQSMMRALLAKKKVKGG
CDPK	*A.thaliana*	GDIVRSRLKQFSMMNRFKKK
CDPK	soybean	GDIVLSRLKQFSAMNKLKKM
CDPK	maize	DPAVLSRIKQFSAVNKLKKM
CaMKII	rat	FNARRKLKGAILTTMLVSR
CaMKII	*D.melanogaster*	FNARRKLKGAILTTMLATR
CaMKI	bovine brain	NFAKSKWKQAFNRTAVVRH
MARCKS protein	mouse macrophage	KKKRFSFKKSFKLSGFSFK
Synapsin	1A,2A,1E	DEPHTDWAKYFKGKKIHGE
Heat shock 84kDa	human	QVANSAFVERVRKRGFEVV

The sequences (Rhoads & Friedberg, 1997) are aligned based on the conserved hydrophobic residues (white with shade). Acidic and basic residues are shown in red and blue, respectively.