

THE EF-HAND structural motif was first discovered in the crystal structure of parvalbumin by Kretsinger and co-workers in 1973 (Ref. 1). It consists of two perpendicularly placed α -helices (for example, helices E and F in parvalbumin) and an interhelical loop, which together form a single Ca^{2+} -binding site. This relatively simple structural motif has been identified in numerous other proteins since, many of which function as Ca^{2+} sensors in the cell. Ca^{2+} -sensor proteins such as calmodulin (CaM) and troponin C (TnC) enable the cell to detect a stimulatory influx of Ca^{2+} and thereby transduce this signal into a variety of cellular processes that often require a rapid response². The mechanism of this molecular switch lies in the conformational change induced by Ca^{2+} binding. The recent progress of atomic-resolution structural analysis of several EF-hand proteins has provided valuable information about this Ca^{2+} -induced conformational change. Here, the structural aspects of the EF-hand proteins are discussed, and an emerging scheme for the conformational response of the proteins to Ca^{2+} binding is highlighted.

Sensor versus buffer functions

Calcium regulates a wide variety of cellular processes, such as nucleotide metabolism, muscle contraction, cell-cycle control, differentiation and signal transduction. Typically, at low Ca^{2+} concentrations (10^{-7} – 10^{-8} M), EF-hand proteins are inactive, and become active as the Ca^{2+} concentration increases to 10^{-5} – 10^{-6} M. EF-hand proteins with regulatory roles are often termed Ca^{2+} -sensor proteins, whereas those involved in Ca^{2+} buffer and transport functions are termed Ca^{2+} -buffer proteins (Table I). Such classification, first proposed by da Silva and Reinach³, provides a good correlation between their structural and functional characteristics as described below.

The Ca^{2+} -binding affinities of EF-hand proteins vary substantially ($K_d = 10^{-4}$ – 10^{-9} M) and are amino acid sequence-dependent, especially with regard to the 12-residue consensus loop

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Calcium binding and conformational response in EF-hand proteins

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EF-hand proteins undergo conformational changes upon binding of Ca^{2+} . This event is a crucial step in many Ca^{2+} -dependent cellular processes. Recent advances in the three-dimensional structural analysis of various EF-hand proteins have led to new insights into the structure and functional relationship of this large family of Ca^{2+} -binding proteins.

that provides all the amino acids that directly ligate a Ca^{2+} ion⁴. In many proteins, EF hands exist as a pair, with a short anti-parallel β -sheet interaction between the two Ca^{2+} -binding loops. High affinity for Ca^{2+} is frequently a result of cooperative binding to both sites. Furthermore, it has recently become clear that a concerted movement of the helices in these EF-hand pairs is crucial for the cooperativity of Ca^{2+} -binding⁵ (see below). Structural communication can also occur between the two sites across the amino- and carboxy-terminal domains, as exemplified in the case of recoverin⁶. This results in a high Ca^{2+} -binding cooperativity between the two domains.

Binding of a target molecule also allows the amino- and carboxy-terminal domains of CaM to communicate with each other, thereby producing a high cooperativity of Ca^{2+} binding⁷.

Regulatory and structural domains

Most, if not all, EF-hand proteins consist of single, or multiple, pairs of the helix-loop-helix motif (Table I). Typically, a pair of EF-hand motifs forms a globular domain, and a protein containing four such motifs will thus have two domains that can be either structurally independent, as seen in the structures of CaM^{8,9} and TnC¹⁰, or well packed, resulting in a single oval shape, as seen in the structure of

Table I. Some members of the EF-hand protein family			
Proteins	No. of EF hands	M.w. (kDa)	Functions
Ca²⁺ sensors			
Calmodulin	4	17	Modulator of enzymes and proteins
Troponin C	3–4	18	Modulator of muscle contraction
Calcineurin B	4	19	Regulatory subunit of protein phosphatase 2B
Myosin light chains	4	19	Modulator of muscle contraction
Recoverin	4	23	Modulator in retinal rod cells
S-modulin	4	23	Recoverin homologue
Visinin	4	22	Modulator in retinal cone cells
VILIP	4	22	Visinin-like protein
Neurocalcin	4	22	Modulator in brain cortex and cerebellum
Hippocalcin	4	23	Modulator in hippocampus
Frequenin	4	22	Modulator of K ⁺ channel
Caltractin	4	20	Microtubule organization
Calpain large subunit	4	80	Protease catalytic domain
S100 proteins	2	10–12	Modulator of enzymes and proteins
Ca²⁺ buffers			
Parvalbumin	3	12	Ca ²⁺ buffering and transport
Calbindin D _{9k}	2	9	Ca ²⁺ buffering and transport
Calbindin D _{28k}	6	28	Ca ²⁺ buffering and transport
Calretinin	6	32	Ca ²⁺ buffering and transport (calbindin D _{28k} homologue)

recoverin^{10,11}. Some domains have a single, functional Ca^{2+} -binding site, for example, the amino- and carboxy-terminal domains of recoverin and myosin light chains.

A number of recent structural studies have revealed substantial differences in Ca^{2+} -induced conformational changes among the EF-hand domains. This is illustrated in Fig. 1 using the CaM carboxy-terminal domain and calbindin D_{9k} . Both carboxy- and amino-terminal domains of CaM undergo large structural rearrangements upon binding of Ca^{2+} , from the 'closed' conformation (the two helices of each EF-hand are almost anti-parallel) to the 'open' conformation (the two helices are more perpendicular)^{5,12,13}. A concerted movement of helices E and H (coloured red in Fig. 1) with respect to helices F and G (coloured green in Fig. 1) is probably responsible for the high cooperativity of Ca^{2+} binding between the two sites⁵. By contrast, calbindin D_{9k} retains the Ca^{2+} -free 'closed' conformation even after binding Ca^{2+} (Ref. 13). Clearly, some EF-hand domains show large conformational changes and others do not. This is more quantitatively compared using the interhelical angles between the two helices of each EF hand (Table II). On one hand, both amino- and carboxy-terminal domains of CaM^{5,12,13}, as well as the amino-terminal domains of skeletal muscle TnC¹⁴ and recoverin¹⁵, exhibit large changes (up to 60°) in the interhelical angles of the EF-hands. On the other hand, calbindin D_{9k} shows very small conformational changes (less than 12°) upon Ca^{2+} binding¹⁶ (Table II). The carboxy-terminal domain of recoverin also exhibits very little conformational change¹⁵.

Analysis of the diversity in the conformational response to Ca^{2+} binding, together with biological functions, leads to an intriguing finding in the structural and functional relationship of the EF-hand proteins. Protein domains showing large conformational changes are all known to have a trigger function in the activation of target proteins and, therefore, can be termed 'regulatory' domains. Both the

amino- and carboxy-terminal domains of CaM, as well as the amino-terminal domains of TnC and recoverin, are classified as such (Fig. 3). The single domain of calbindin D_{9k} exhibits small conformational changes in response to binding of Ca^{2+} , which may be responsible for the structural stability and function in buffering intracellular Ca^{2+} . Thus, this type of domain can be referred to as a 'structural' or 'buffer' domain, and other examples of this type probably include the carboxy-terminal domains of TnC and recoverin.

The concept of regulatory and structural domains was first introduced for TnC, in which the high-affinity carboxy-terminal domain (structural) always binds Ca^{2+} in the muscle cell, and the low-affinity amino-terminal domain (regulatory) triggers the Ca^{2+} signal leading to muscle contraction¹⁷. The present hypothesis further extends this early notion to other EF-hand proteins, based on recently available structural

information that can predict a physiological role for proteins. For example, it is predicted that calcineurin B, a regulatory subunit of protein phosphatase 2B, should contain at least one regulatory domain. Also, the regulatory myosin light chain should have a regulatory domain, whereas the myosin light chain involved directly in the mechanics of muscle contraction might comprise only structural domains.

The target recognition surface is hydrophobic

Extensive hydrophobic regions found in the crystal structures of Ca^{2+} -CaM^{7,8} (Fig. 2) are essential for their interaction with target proteins. Nuclear magnetic resonance (NMR) and X-ray studies of three Ca^{2+} -CaM and peptide complexes^{18–20} have revealed the significance of hydrophobic interactions of the two lobes of CaM with the α -helical rod of the peptide, using a flexible loop between the two lobes. This results

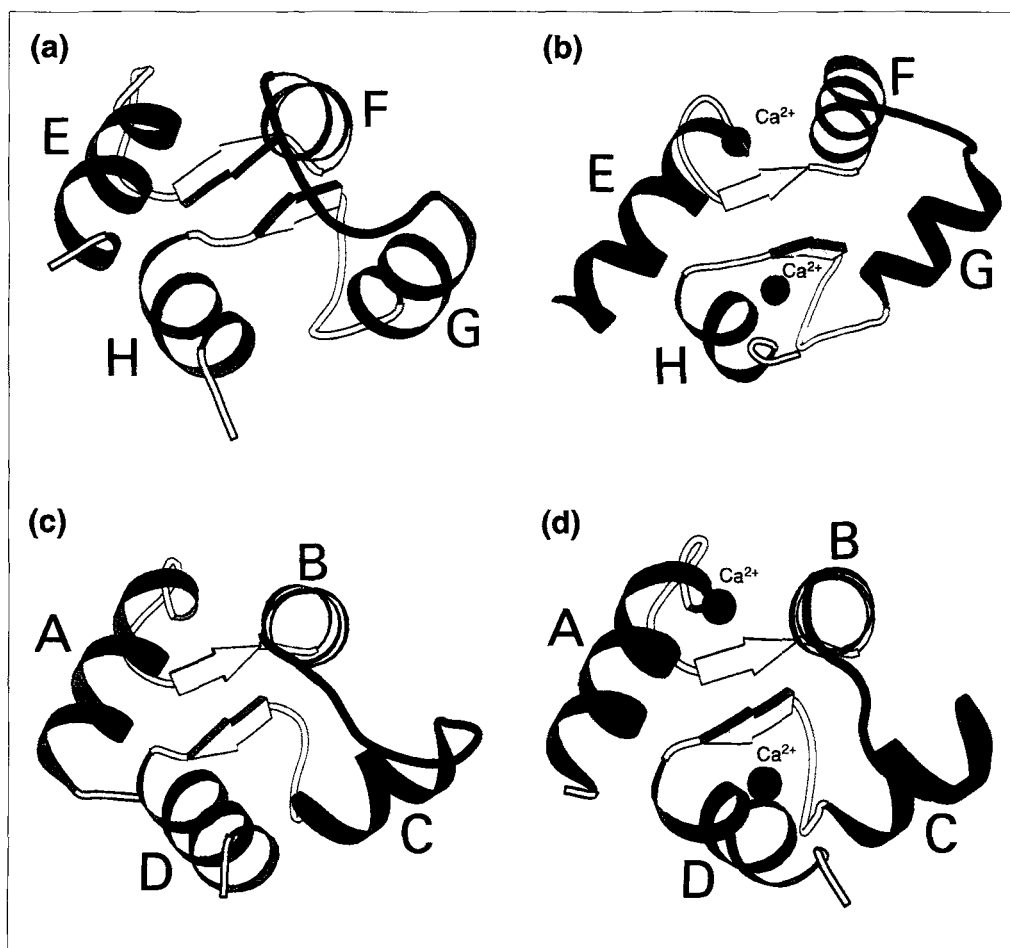


Figure 1

Conformational changes induced by Ca^{2+} binding to a pair of EF hands. The two helices of each EF hand are shown in red and green, respectively. The carboxy-terminal domain of calmodulin (CaM) (a) in the 'closed' Ca^{2+} -free state (Ref. 5) and (b) in the 'open' Ca^{2+} -bound states (PDB code: 4CLN) (Ref. 8). Calbindin D_{9k} in the (c) 'closed' Ca^{2+} -free state and (d) 'closed' Ca^{2+} -bound state (Ref. 15). Helices are labelled from the amino terminus, except in (a) and (b) where helices A, B, C and D of the amino-terminal domain of CaM are not shown.

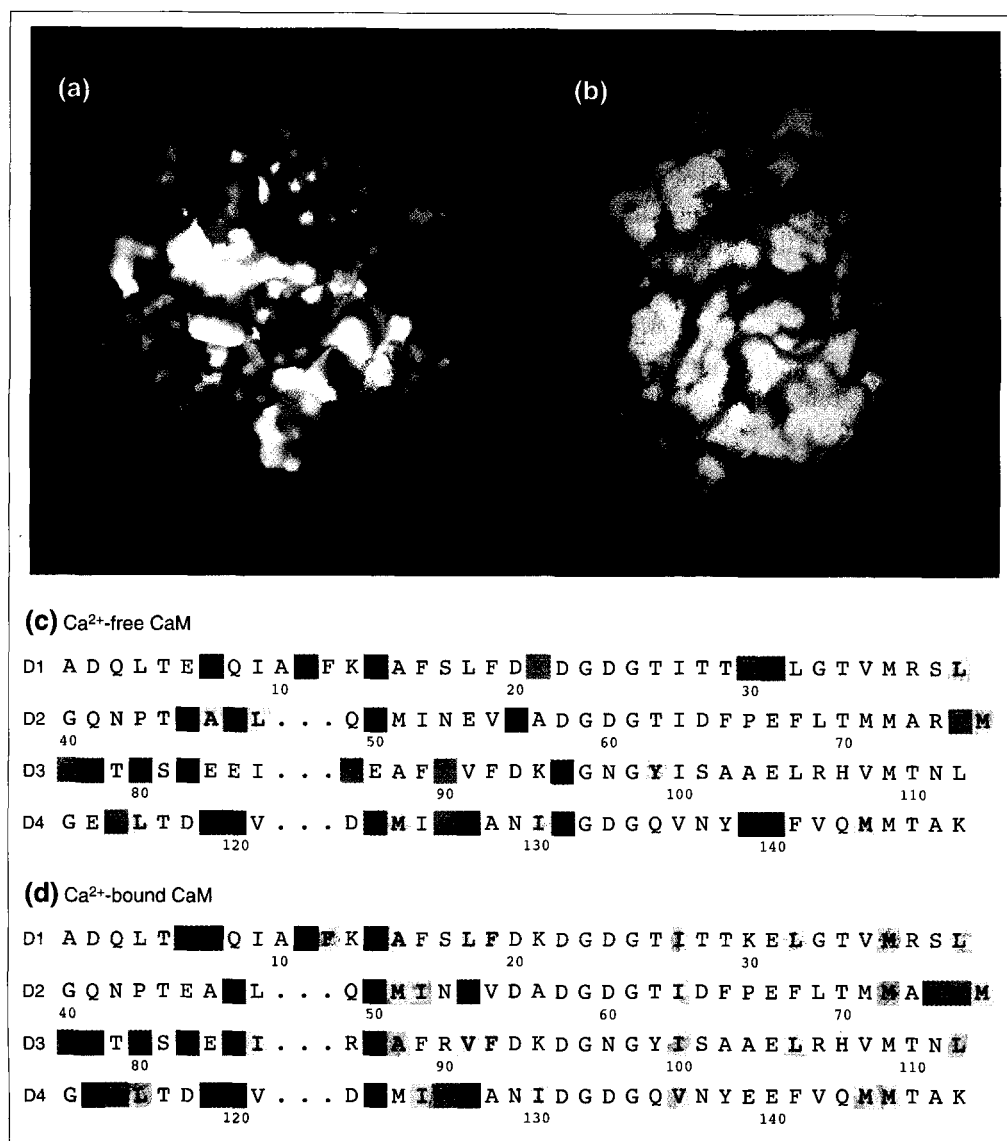


Figure 2

The molecular surface of the carboxy-terminal domain (residues 78–148) of calmodulin (CaM), (a) in the Ca²⁺-free state (Ref. 5) and (b) in the Ca²⁺-bound state (Ref. 8). Hydrophobic residues are shown in yellow, acidic residues in red, and basic residues in purple. The amino acid sequence of CaM highlighting exposed hydrophobic residues (c) in the Ca²⁺-free state and (d) in the Ca²⁺-bound state. The colour codes are the same as those in the panels (a) and (b). EF-hand repeats denoted as D1, D2, D3 and D4; each comprises a helix-loop-helix. D1 contains helix A and B; D2, helix C and D; D3, helix E and F; and D4, helix G and H (see text).

in the 'dumbbell-like' structure of Ca²⁺-CaM collapsing to a compact oval shape. The backbone conformations of the individual domains complexed with the peptide are essentially identical to those of Ca²⁺-CaM, indicating that the interacting surfaces of the two domains are pre-structured prior to target recognition. These surfaces are composed of many hydrophobic residues, mainly from the Ca²⁺-binding loop and the second helix of the EF hand (Fig. 2). However, the first helix of the EF hand provides many surface acidic residues such as Glu and Asp. Myosin light and heavy chains also interact in a manner similar to that of CaM^{21,22}. More recently, the crystal structure of calcineurin

complexed with FKBP12-FK506 showed a 'side-by-side' interaction of the two domains of calcineurin B with the target helix of calcineurin A²³. In all cases, the interaction is predominantly hydrophobic, but is complemented by acidic side chains from the EF hands interacting with basic sidechains of the target molecule.

By contrast, many of the exposed hydrophobic residues on the surface of Ca²⁺-CaM become buried in the Ca²⁺-free state^{5,12,13}. Most striking is the loss of the hydrophobic cavity usually located at the centre of the hydrophobic surface (Fig. 2). This central cavity, in the Ca²⁺-bound state, is responsible for interacting with the

target molecule via an amino acid containing an aromatic or long-alkyl sidechain. The corresponding surface of the Ca²⁺-free state is more hydrophilic, with many acidic and few basic amino acids exposed (Fig. 2). This change enables CaM to act as a molecular switch, converting from the inactive Ca²⁺-free conformation to the active Ca²⁺-bound conformation in response to a stimulatory influx of Ca²⁺ in the cell.

Some proteins, such as neuromodulin²⁴ and myosin I (Ref. 25), can interact with Ca²⁺-free CaM. However, structural information on the interaction between Ca²⁺-free CaM and target molecules is limited. The NMR study by Urbauer *et al.*²⁶ has shown that the overall conformation of Ca²⁺-free CaM does not change upon binding to a neuromodulin-derived target peptide. Thus, it has been suggested that the exposed acidic residues and less hydrophobic character of the Ca²⁺-free CaM structure probably facilitate the interaction with the target proteins. A recent NMR-derived structure of calcyclin, a member of the S100 protein family, provides a further clue in understanding how an EF-hand protein could interact with itself (homodimer) and with other molecules in the Ca²⁺-free state²⁷. The dimer interface is largely hydrophobic, but different from the target recognition surface found in other cases described above.

Conclusions and prospects

It is not surprising that both sensor and buffer functions are strongly coupled with the structural characteristics of EF-hand proteins in response to Ca²⁺ binding. Conformational changes occur only where they are necessary. Ca²⁺-induced exposure of a hydrophobic surface, formed by a pair of EF hands, is critical for the regulatory role of such proteins, whereas only a small conformational change is needed for Ca²⁺-buffer function. It is also notable that the binding affinity for Ca²⁺ is generally higher for buffer proteins than

Table II. Interhelical angle changes of various EF-hand proteins upon Ca ²⁺ binding				
	Interhelical angle change (°) ^a			
	A/B ^b	C/D	E/F	G/H
Calmodulin ^c	34	34	39	36
Troponin C ^d	40	56	e	e
Troponin C HMJ ^f	32	32	e	e
Recoverin ^g	61	7	16	1
Calbindin D _{9k} ^h	12	1	N/A	N/A

^aCalculated using an in-house program written by K. Yap (program available on request). The difference = $\delta_{Ca^{2+}\text{-free}} - \delta_{Ca^{2+}\text{-bound}}$.

^bHelices are named A to H going from the amino-terminus to the carboxyl terminus.

^cThe structures of Ca²⁺-free calmodulin (CaM) (Ref. 5) and Ca²⁺-bound CaM (Ref. 6) are used.

^dThe structures of Ca²⁺-free and Ca²⁺-bound amino-terminal troponin (TnC) fragment 1–90 (Ref. 16) are used.

^eNot reported owing to the lack of the structure or the model of the Ca²⁺-free state of these EF-hands.

^fThe crystal structure of half Ca²⁺-saturated TnC (the amino-terminal domain is free for binding Ca²⁺) and a model for the fully Ca²⁺-loaded form reported by Herzberg and James (Ref. 10) are used.

^gThe structures of Ca²⁺-free myristoylated recoverin (Ref. 12) and single-Ca²⁺-bound unmyristoylated recoverin (Ref. 11) are used.

^hThe structures of Ca²⁺-free and Ca²⁺-bound calbindin D_{9k} (Ref. 15) are used.

N/A, not applicable.

for regulatory proteins. Several major questions still remain to be answered.

First, it is difficult to predict from amino acid sequence data alone whether the EF-hand protein of interest has a regulatory or structural domain, and thus a sensor or buffer function,

respectively. A useful test, at this time, is to check the ability of the protein to bind to a hydrophobic column, such as phenyl-sepharose, in a Ca²⁺-dependent manner. Only Ca²⁺-sensor proteins possessing regulatory domain(s) have the ability to bind to such a column.

Second, does the structural analysis of CaM bound to three different target molecules cover all possible modes of the CaM interactions with the targets? The answer is likely to be no, since CaM can recognize more than 30 different proteins and enzymes^{28,29}. The 'side-by-side' interaction of two EF-hand domains with a target molecule, similar to that found in the calcineurin structure²³, might also be possible for CaM and some target proteins. Finally, how does Ca²⁺-free CaM recognize neuromodulin, myosin I and others? The recent crystal structures of myosin light chains^{21,22} could be used to model the interaction of a Ca²⁺-free EF-hand molecule, such as CaM, with a target protein (M. Swindells, unpublished). It is hoped that these questions will be solved over the next few years owing to the rapidly increasing number of crystal and NMR structures of EF-hand proteins being elucidated.

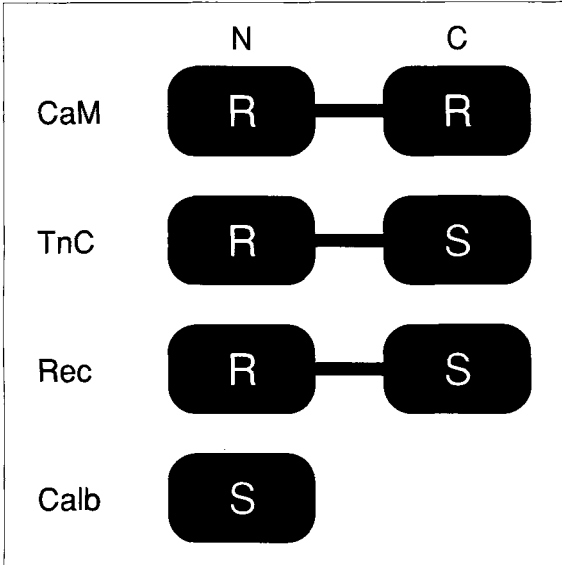


Figure 3

EF-hand proteins consist of regulatory and/or structural domains. The regulatory domain is shown in red and the structural domain in purple. The amino- and carboxy-terminal domains are labelled as N and C, respectively. Calmodulin, troponin C, recoverin and calbindin D_{9k} are labelled as CaM, TnC, Rec and Calb, respectively. Structural data for both Ca²⁺-free and Ca²⁺-bound forms are available for all domains of the proteins listed, except for the carboxy-terminal domain of TnC and the amino-terminal domain of Rec. Note that the crystal structure of recoverin has only one Ca²⁺ bound to the third site in the carboxy-terminal domain, although the second site in the amino-terminal domain binds Ca²⁺ in solution.

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