

REVIEW ARTICLE

Structures and metal-ion-binding properties of the Ca²⁺-binding helix–loop–helix EF-hand motifsJessica L. GIFFORD*, Michael P. WALSH† and Hans J. VOGEL*¹

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The 'EF-hand' Ca²⁺-binding motif plays an essential role in eukaryotic cellular signalling, and the proteins containing this motif constitute a large and functionally diverse family. The EF-hand is defined by its helix–loop–helix secondary structure as well as the ligands presented by the loop to bind the Ca²⁺ ion. The identity of these ligands is semi-conserved in the most common (the 'canonical') EF-hand; however, several non-canonical EF-hands exist that bind Ca²⁺ by a different co-ordination mechanism. EF-hands tend to occur in pairs, which form a discrete domain so that most family members have two, four or six EF-hands. This pairing also enables communication, and many EF-hands display positive co-operativity, thereby minimizing the Ca²⁺ signal required to reach protein saturation. The conformational effects of Ca²⁺ binding are varied, function-dependent and, in some cases, minimal, but can lead to the creation of a protein target

interaction site or structure formation from a molten-globule apo state. EF-hand proteins exhibit various sensitivities to Ca²⁺, reflecting the intrinsic binding ability of the EF-hand as well as the degree of co-operativity in Ca²⁺ binding to paired EF-hands. Two additional factors can influence the ability of an EF-hand to bind Ca²⁺: selectivity over Mg²⁺ (a cation with very similar chemical properties to Ca²⁺ and with a cytoplasmic concentration several orders of magnitude higher) and interaction with a protein target. A structural approach is used in this review to examine the diversity of family members, and a biophysical perspective provides insight into the ability of the EF-hand motif to bind Ca²⁺ with a wide range of affinities.

Key words: calbindin D_{9K}, calcium ions (Ca²⁺), calmodulin, co-operativity, EF-hand, magnesium ions (Mg²⁺).

INTRODUCTION

As an intracellular signalling ion, Ca²⁺ is involved in an array of cellular functions from fertilization, contraction, cell differentiation and proliferation, to apoptosis and, in the case of dysregulation, cancer. At a basic level the action of this cation is simple. In response to stimuli such as membrane depolarization, extracellular signalling molecules, or intracellular messengers, the cytoplasmic concentration of this metal ion increases from ~10⁻⁷ M in the resting cell to ~10⁻⁵ M in the activated cell. This increase is the result of either the influx of extracellular Ca²⁺ or the release of this cation from internal stores, particularly the ER/SR (endoplasmic/sarcoplasmic reticulum) (Figure 1A) (reviewed in [1,2]).

The Ca²⁺ that flows into the cytoplasm during the 'on' reaction does not remain free. Instead it becomes bound to a wide variety of CaBPs (Ca²⁺-binding proteins), many of which belong to a homologous family that binds this cation using a characteristic helix–loop–helix structural motif termed the 'EF-hand' (see Supplementary Table 1 at <http://www.BiochemJ.org/bj/405/bj4050199add.htm>). Functionally, EF-hand proteins can roughly be divided into two general classes: the Ca²⁺ sensors and the Ca²⁺ buffers. The Ca²⁺ sensors translate the chemical signal of an increased Ca²⁺ concentration into diverse biochemical responses.

This signal transduction is accomplished predominantly through a Ca²⁺-induced conformational change, as illustrated by the classic examples of CaM (calmodulin) and recoverin (Figure 1B). Ca²⁺ binding to the ubiquitous sensor CaM elicits a structural response through which CaM binds its target proteins, in many cases removing enzymatic autoinhibitory domains, thereby activating these enzymes in a Ca²⁺-dependent manner. In the case of the vision-associated protein recoverin, the binding of Ca²⁺ results in the extrusion of a hydrophobic myristoyl group, allowing recoverin to associate with the membrane and subsequently its membrane-bound protein target. The Ca²⁺ buffers are a smaller subset of the EF-hand protein family. Exemplified by calbindin D_{9K} and parvalbumin, these proteins help to modulate the Ca²⁺ signal both spatially and temporally as they bind the free Ca²⁺ to transmit the signal throughout the cell or to remove the potentially harmful ion from the cytoplasm.

Owing to the physiological importance of the EF-hand-containing proteins, much is known about this family. Here we will attempt to review the structural consequences and energetic driving factors of Ca²⁺ binding. We first discuss how the EF-hand binds Ca²⁺ via both canonical and non-canonical co-ordination mechanisms. This is followed by an analysis of the EF-hand domain in isolation and how this domain is organized in EF-hand-containing proteins. We will then examine the Ca²⁺-induced

Abbreviations used: ALG-2, apoptosis-linked gene-2; AtCBL2, *Arabidopsis thaliana* (thale cress) calcineurin B-like protein; CaBP, Ca²⁺-binding protein; CaM, calmodulin; cTnC, cardiac-muscle troponin C; CaVP, Ca²⁺ vector protein; CIB, Ca²⁺- and integrin-binding protein; EF1, EF-hand 1; EH, Eps15-homology; ELC, essential light chain; ER/SR, endoplasmic/sarcoplasmic reticulum; GCAP-1, guanylate cyclase activating protein-1; NCS, neuronal Ca²⁺-sensor; NCXs, Na⁺/Ca²⁺; PEF, penta-EF-hand; RLC, regulatory light chain; rmsd, root mean square deviation; SCPs, sarcoplasmic Ca²⁺-binding proteins; skMLCK, skeletal-muscle myosin light-chain kinase; sTnC, skeletal-muscle troponin C; Tnl, troponin I; TR2C, calmodulin tryptic fragment containing Ca²⁺-binding sites 3 and 4; yCaM, yeast calmodulin.

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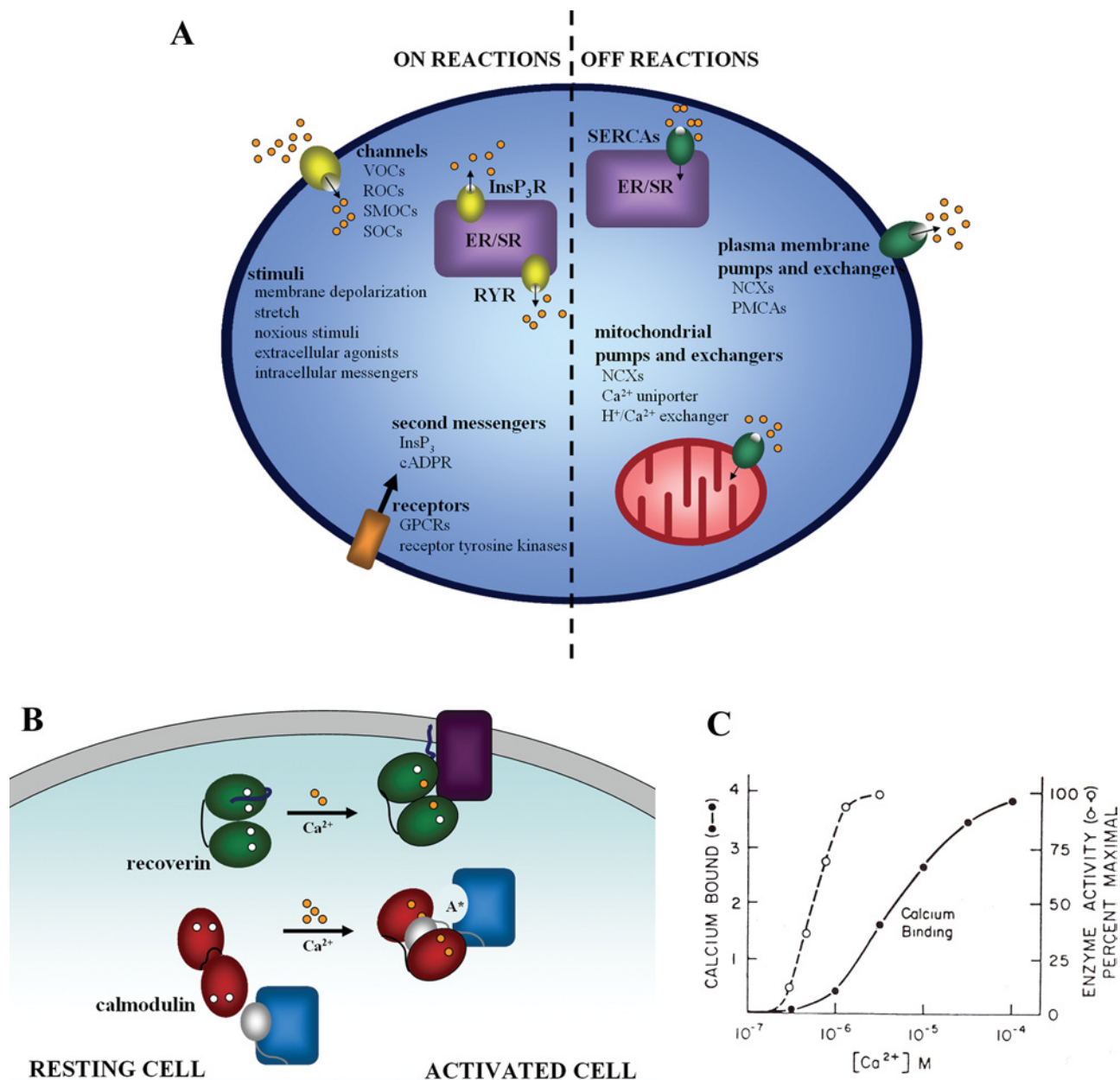


Figure 1 Ca^{2+} signalling and the cell

(A) The 'on reactions', triggered by various stimuli, result in an increase in the intracellular Ca^{2+} concentration. This increase is either due to release from intracellular stores, such as the ER/SR, through channels such as the $\text{Ins}(1,4,5)\text{P}_3$ or ryanodine receptor (InsP_3R and RyR respectively) or via the entry of external Ca^{2+} through various channels, including voltage-operated (VOCs), receptor-operated (ROCs), second-messenger-operated (SMOCs) and store-operated (SOCs) channels. Release of Ca^{2+} from the ER/SR is triggered by second messengers, including $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) and cyclic ADP-ribose (cADPR) generated as a result of signalling cascades initiated by receptors located in the plasma membrane, including G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases. During the 'off reactions', Ca^{2+} is removed from the cytoplasm, since it is sequestered again in the ER/SR by the SR/ER Ca^{2+} -ATPases (SERCAs), stored in the mitochondria via a Ca^{2+} uniporter or NCXs ($\text{Na}^+/\text{Ca}^{2+}$) and $\text{H}^+/\text{Ca}^{2+}$ exchangers, or extruded from the cell through NCXs and plasma membrane Ca^{2+} -ATPases (PMCA). (B) The conformational change that occurs when the sensors bind Ca^{2+} allows these proteins to interact with their protein targets which, in many cases, leads to the activation of the target. In the case of CaM, the Ca^{2+} -bound form binds to the autoinhibitory domain (grey) of its enzyme target (blue), which results in exposure of the active site (A^*). For recoverin, Ca^{2+} binding results in the extrusion of a myristoyl group, drawing this protein to the plasma membrane and its membrane-bound target protein (purple). (C) Comparison of the Ca^{2+} -binding curve of CaM (●) and the enzyme activation curve of its target cyclic-nucleotide phosphodiesterase (○). The dependence of the enzymatic activity on the Ca^{2+} -bound state of CaM is clearly indicated. Also evident is the small change in Ca^{2+} concentration required to activate the enzyme, a consequence of the positive co-operativity through which CaM binds Ca^{2+} . (C) is taken from [174] and reproduced with the permission of the publishers. © 1994 The National Research Council of Canada.

conformational change. However, we will touch only briefly on the latter topic, as the reader is directed to recent reviews by Grabarek [3] and Capozzi et al. [4].

Falke et al. [5] and Linse and Forsen [6] have remarked on the finely tuned metal-binding ability of the EF-hand motif. A

sensitivity to minor changes in amino acid sequence enables this motif to exhibit a range of Ca^{2+} affinities functionally matched to the role of each EF-hand-containing protein (see Supplementary Table 2 at <http://www.BiochemJ.org/bj/405/bj4050199add.htm>). The affinity observed is affected by intramolecular interactions,

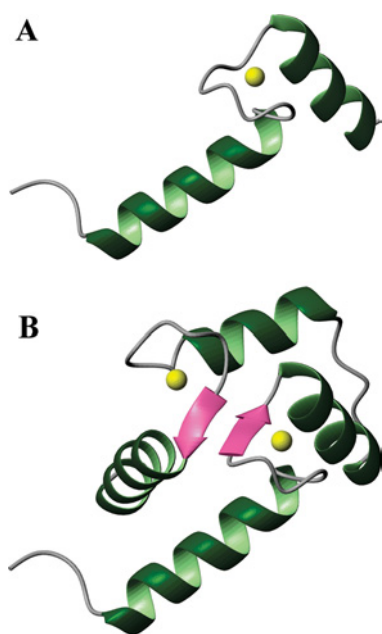


Figure 2 The EF-hand

(A) A single EF-hand from the N-terminal domain of CaM. Extending for 29 consecutive amino acid residues, the EF-hand consists of a nine-residue entering helix, a nine-residue loop and an 11-residue exiting helix. (B) A pair of EF-hands from the N-terminal domain of CaM. An additional secondary-structural element is observed in the pair as a small β -sheet formed between residues in the latter part of the loops, through which passes the pseudo-2-fold axis of symmetry that relates the EF-hand pair, in most cases the functional unit of Ca^{2+} binding (PDB code 1EXR) [175].

since, owing to contacts with other EF-hand motifs, Ca^{2+} can be bound in a co-operative manner as well as by the intermolecular interactions formed with target proteins. Both of these interactions have noteworthy functional consequences, as they enable the targets of the sensor class to be activated by a small change in intracellular Ca^{2+} concentration (Figure 1C). Finally, in many cases the EF-hand motif possesses important selectivity over the chemically similar Mg^{2+} cation which, in the resting cell, can occur at an intracellular concentration of up to four orders of magnitude higher than that of Ca^{2+} . The final section of this review addresses in detail these Ca^{2+} -binding abilities, focusing on both the molecular and thermodynamic causes.

THE EF-HAND: A Ca^{2+} -BINDING UNIT

Named after a Ca^{2+} -binding motif first seen formed by helices E and F in the crystal structure of parvalbumin [7], the EF-hand is composed of a typical helix–loop–helix structural unit: two α -helices bridged by a Ca^{2+} -chelation loop (Figure 2). For most protein Ca^{2+} -binding motifs, including the EF-hand, a turn–loop structure provides the bulk of the ligands for the bound cation. Unlike helices and sheets, which can only provide a few appropriate ligands in a given sequence, owing to geometric or spacing constraints, the turn–loop structure is flexible and can readily supply three ligands from a sequence of five amino acids [8].

Ca^{2+} is a ‘hard’ metal ion and likes ‘hard’ ligands that provide an interaction dominated by ionic forces with little covalency [5]. As such, oxygen is the co-ordinating atom of choice and consequently EF-hands, and in particular EF-loops, are rich in the negatively charged amino acids glutamic acid and aspartic acid (Figure 3A). Of the two amino acids, Ca^{2+} ligands are more commonly provided by the side chain of aspartic acid residues,

a frequency which may reflect the preference of the EF-loop for carboxylate ligands with a less bulky side chain [9]. Consistently, asparagine is present to a greater extent than glutamine. The preferred co-ordination geometry of the Ca^{2+} ion is seven ligands arranged in a pentagonal bipyramidal fashion. This is the geometry of the Ca^{2+} ion in water and, in most protein binding sites, including EF-hands, this geometry is preserved with six or seven of the chelating groups provided by the binding motif [10].

The canonical EF-loop

In the canonical EF-loop, five of Ca^{2+} 's seven co-ordinating groups are provided by the nine-residue loop. The remaining two come from a bidentate carboxylate ligand supplied by the side chain of an acidic amino acid located in the exiting helix, three residues removed from the loop's C-terminus. Though this residue is not structurally part of the loop, it is commonly referred to as the EF-loop's twelfth residue. Of the ligands provided by the loop itself, three or four come from the side-chain carboxy groups of negatively charged amino acids and one from a backbone carbonyl group. Frequently, the co-ordination sphere of the Ca^{2+} ion is completed by a water molecule hydrogen-bonded to one of the side chains of the loop (Figure 3B). The chelating residues of the loop are notated in two ways, the first based on linear position and the second on the tertiary geometry imposed by their alignment on the axes of a pentagonal bipyramid: 1(+X), 3(+Y), 5(+Z), 7(−Y), 9(−X), 12(−Z). The Y- and Z-axis pairs align along the vertices of an approximately planar pentagon, whereas the X-axis pair takes up an axial position perpendicular to the Y/Z plane [10]. In addition to the Ca–O bonds formed through the chelating interactions, the canonical EF-loop also contains an extensive network of hydrogen bonds between both chelating and non-chelating residues. These bonds help to stabilize the otherwise unfavourably close proximity of the negatively charged oxygen atoms in the co-ordination sphere of the bound Ca^{2+} .

Several of the loop's residues contribute individual and identifiable roles to both the stabilization and fold of this structure. Through numerous intraloop hydrogen bonds, the first residue plays an important part in defining a precise stereochemical arrangement for the loop (Figure 3C). The conserved glycine residue found at the sixth position facilitates the unusual main-chain conformation (φ and $\psi \sim 60^\circ$ and 20° respectively) which results in a 90° turn that enables the remaining Ca^{2+} ligands to take up co-ordinating positions. The eighth residue of the loop is a highly conserved hydrophobic residue [11], the main chain NH and CO groups of which face away from the Ca^{2+} -binding site towards the loop of the paired EF-hand. This forms the short anti-parallel β -sheet with the corresponding groups of the paired loop's eighth position. As well as chelating the bound Ca^{2+} ion either directly or indirectly via a bridging water molecule, the hydrogen-bonding pattern formed by the side chain of loop position 9 initiates the exiting helix, a conformation stabilized through additional hydrogen bonds with the side-chain carboxylate group of position 12. Finally, the bidentate side-chain ligands supplied by the twelfth loop position are critical to both the structure and function of the EF-loop. The role played by the ligands of the twelfth loop position is discussed in depth in this review.

As the above discussion reflects EF-loop structural information predominantly obtained through X-ray crystal structures, it represents a static view. MD (molecular dynamics) simulations corroborate many of these points, but they have shed light on the transitory nature of the Ca^{2+} ligands. Although EF1 (EF-hand 1) remained essentially as observed in the crystal structure, in the course of simulations the bound Ca^{2+} ion in EF2 of CaM had a tendency to gain an extra water molecule as a ligand, the

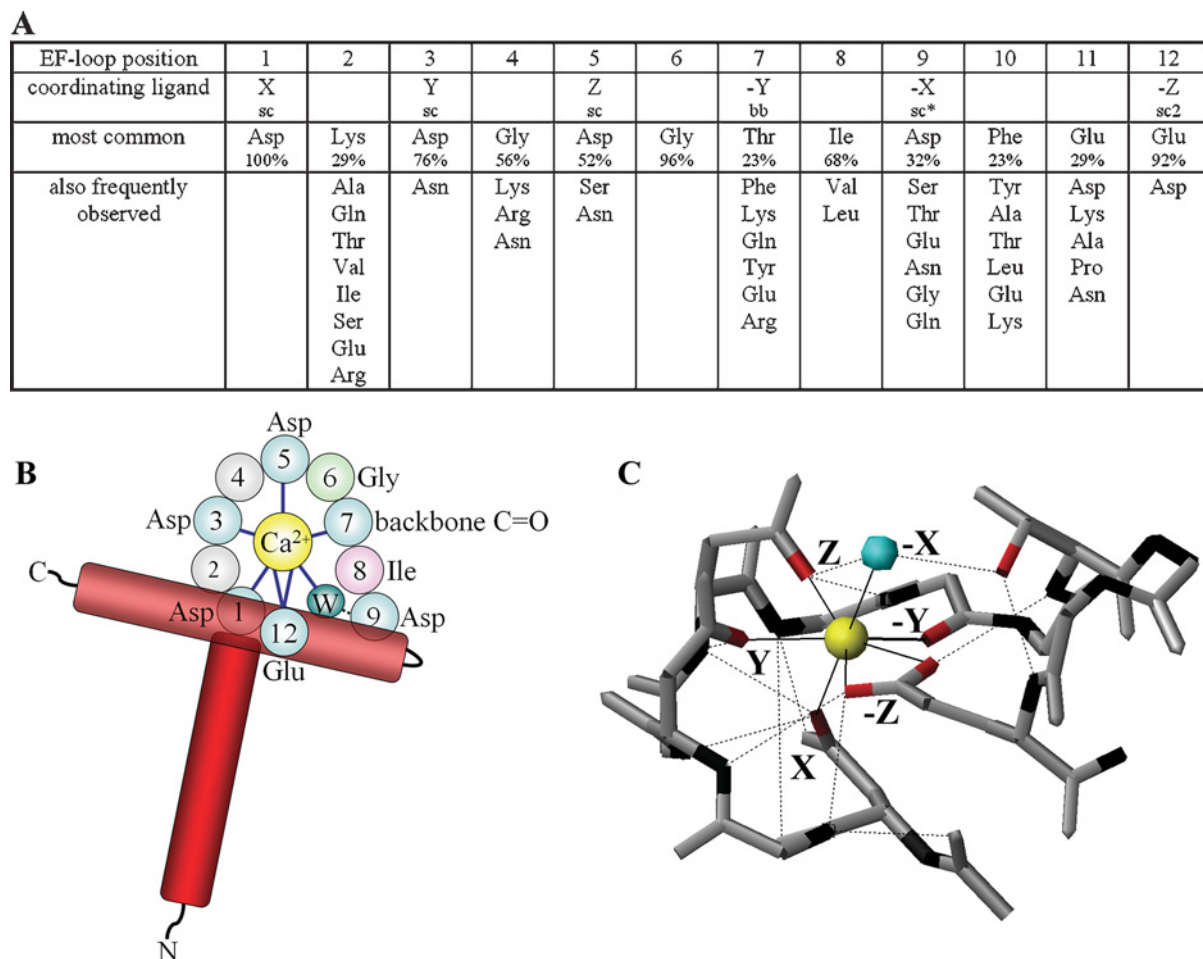


Figure 3 The co-ordination sphere of the canonical EF-loop

(A) The sequence preference of the EF-hand loop. The Ca^{2+} ligands are indicated by both their position in the EF-loop and in the co-ordinating array with whether or not co-ordination occurs via the side chain (sc) or through the backbone (bb) indicated below. The asterisk (*) highlights the ligand typically provided by a water molecule that is hydrogen-bonded to the side chain of the amino acid found at position 9. Also noted in the Figure are the most common amino acids at each position, with their corresponding percentages of occurrence, and those that occur with a frequency greater than 5% in known EF-loops [5]. (B) A schematic diagram of the Ca^{2+} co-ordination sphere with the entering and exiting helices in red, the co-ordinating protein ligands in blue and co-ordinating water molecule (W) in teal (dark blue). Light green corresponds to the conserved glycine residue that provides the bend in the loop. Purple highlights the conserved hydrophobic residue that forms the short β -sheet in the paired EF-hand. Also indicated are the most common amino acids found at the critical positions. (C) Ca^{2+} co-ordination by the canonical EF-hand (EF1 of CaM) illustrating both the pentagonal bipyramidal co-ordination of the Ca^{2+} ion (continuous lines) and the extensive hydrogen bonding pattern found in the loop (broken lines) [10]. The backbone NH groups are indicated in black, the side-chain oxygen atoms in red, the Ca^{2+} ion in yellow and the co-ordinating water in blue (PDB code 1EXR) [175]. An interactive three-dimensional version of (C) is available at <http://www.BiochemJ.org/bj/405/0199/bj4050199add.htm>.

appearance of which coincided with a decreased contribution from the main-chain carbonyl group of the $-Y$ ligand [12]. This ligand exchange results in two water molecules in the co-ordination sphere, a liganding scheme that increases the flexibility of the loop and perhaps the Ca^{2+} off-rate [13]. A second short equilibrium simulation on Ca^{2+} -bound EF3 of the C-terminal domain of CaM found that the co-ordination number of the Ca^{2+} ion fluctuated between seven and eight as the Asp^{95} (+Y ligand) flipped between monodentate and bidentate [14]. As this same phenomenon was observed in EF2 of calbindin $\text{D}_{9\text{k}}$ [15], it appears that the current van der Waals parameters of Ca^{2+} that are used in the simulations, co-ordination numbers of 7 and 8, are in a quasi-equilibrium.

Non-canonical EF-loops

Although the Ca^{2+} chelation scheme outlined above is employed by the majority of EF-hands, the composition and length of functional binding loops vary significantly in the 'EF-handome', a term first coined by Haiech et al. [16] (Figure 4). The different

non-canonical EF-loops can be classified into four groups, three of which demonstrate different ways whereby the pentagonal bipyramidal co-ordination is achieved. The first group contains EF-hands that, although they are the same length as the canonical sequence (12 residues), do not use canonical ligands to bind the Ca^{2+} ion (Figure 3C). This group contains two types of deviation. In approx. 10% of known EF-hands, an aspartic acid residue instead of a glutamic acid residue occupies loop position 12. These 'Asp12' EF-loops tend to be smaller and more compact than the canonical loop [17,18] a characteristic that has effects on Ca^{2+} co-ordination and Mg^{2+} selectivity (discussed later). As seen in the crystal structure of EF3 from CIB (calcium- and integrin-binding protein), the side chain of Asp12 is too short for both oxygen atoms to co-ordinate Ca^{2+} , and although one does, the second is replaced by a bridging water molecule [19]. The second deviation in this group are EF-loops that bind Ca^{2+} through an increased use of main-chain carbonyl groups such as EF4 of AtCBL2 [*Arabidopsis thaliana* (thale cress) calcineurin B-like protein] [20]. This deviation is most likely a response to the

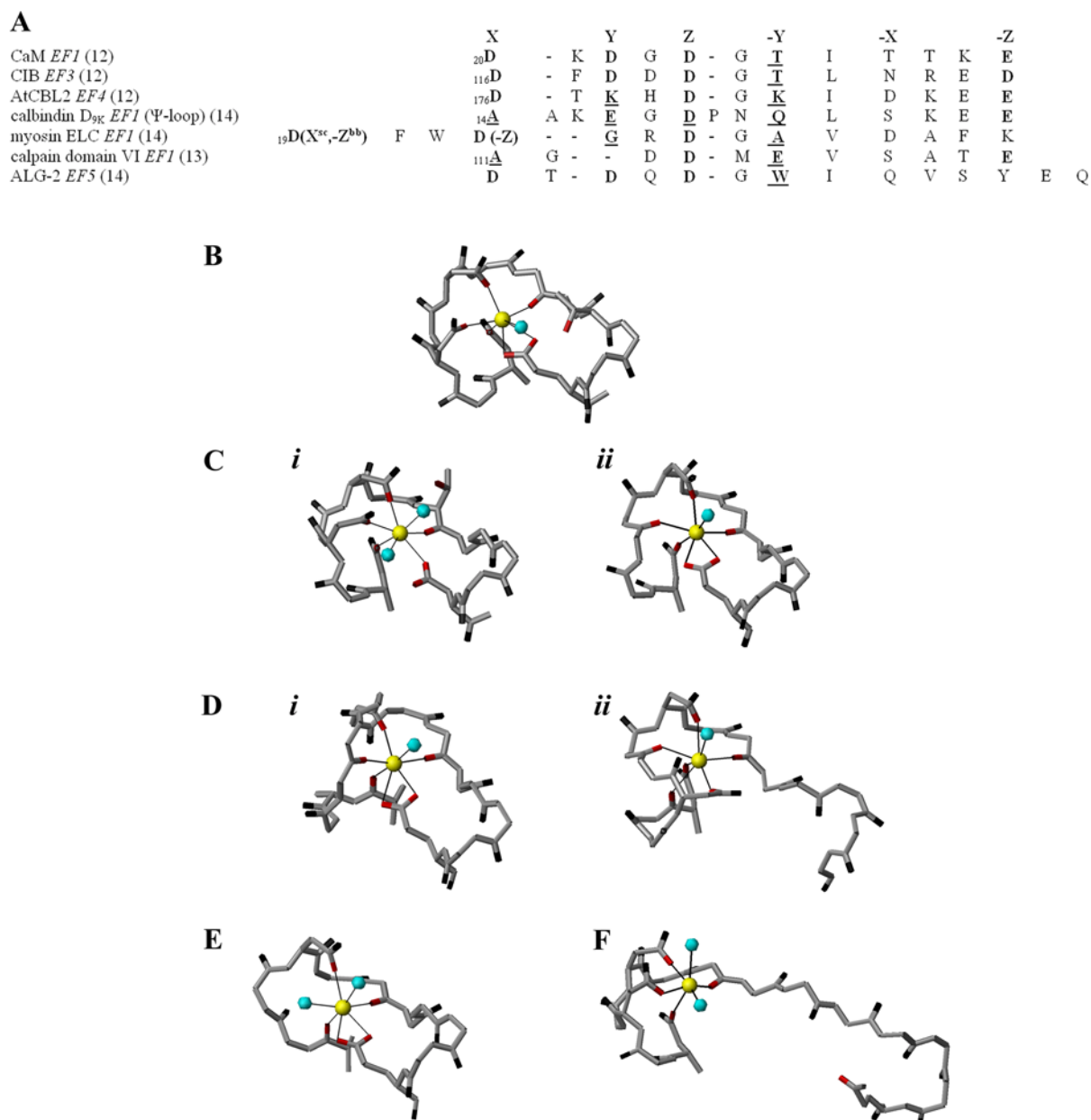


Figure 4 Non-canonical EF-loops

(A) Sequences of representative non-canonical EF-loops. The Ca^{2+} ligands at the co-ordinating positions are indicated in bold with those ligands provided by the carbonyl group of the backbone underlined. Indicated in parentheses is the length of the loop. Again a water molecule directly co-ordinates the bound Ca^{2+} ion at the $-X$ position. (B) The loop of CaM EF1 (PDB code 1EXR) indicating the canonical arrangement. (C) EF-loops that have the canonical length, but do not chelate the Ca^{2+} ion with the canonical ligands: *i*, CIB EF3 which, due to the short length of Asp¹², has a water ligand as the second co-ordinating group at the $-Z$ position (PDB code 1X05); *ii*, AtCBL2 EF4 has a lysine residue at the $+Y$ position and as such chelates the Ca^{2+} ion through the backbone carbonyl group (PDB code 1UHN). (D) EF-loops with insertions: *i*, the Ψ -hand of calbindin D_{9k} overcomes a two-residue insertion by turning inside out and using the backbone carbonyl groups for co-ordination (PDB code 3ICB); *ii*, scallop myosin ELC EF1 uses backbone carbonyl groups and co-ordination from ligands provided by the entering helix to overcome an unusual separation of aspartic acid residues and a substitution of a lysine residue for the C-terminal glutamic acid ligand (PDB code 1WDC). (E) EF1 of calpain domain VI is shorter than the canonical sequence and has an additional ligand provided by a backbone carbonyl group as well as an additional water molecule (PDB code 1DVI). (F) EF5 of ALG-2 binds the Ca^{2+} ion through octahedral co-ordination since, because of an insertion, the too-distant C-terminal ligand potentially provided by a glutamine residue is replaced by a single water molecule (1HQV). In (B)–(F) the Ca^{2+} ion is represented by a yellow sphere, water molecules by blue spheres, the chelating groups as red sticks and the side chain and backbone carbonyl groups as black sticks. For clarity, position 9 of the loop, which is hydrogen-bonded to the co-ordinating water molecule, is not shown [175].

substitution of otherwise disabling amino acids, since, through a shift in the entering helix, the main-chain carbonyl oxygen atom substitutes for the missing side-chain carboxy group.

The second group of non-canonical EF-loops are those with insertions (Figure 4D). An entire subfamily, the S100s and related proteins including calbindin D_{9k} , contain a non-canonical EF-loop

in EF1 termed the ‘pseudo-EF-loop’ forming an EF-hand variant known as the Ψ -hand (‘pseudo-EF-hand’). To accommodate a two-residue insertion in the first part of the loop, this segment is turned inside-out with the dipoles of the main-chain carbonyl groups providing the chelating $+X$, $+Y$ and $+Z$ ligands. Interestingly, the presence of these unusual liganding groups

does not prevent the formation of several favourable hydrogen bonds and, at least in the case of calbindin D_{9K} , high-affinity Ca^{2+} binding still occurs. Main-chain co-ordinating groups are also used to overcome a three-residue insertion between the +X and +Y positions and enable pentagonal bipyramidal geometry in EF1 of AtCBL2 [20] and a one-residue insertion between these same positions in EF1 of the extracellular glycoprotein BM40 (also known as SPARC and osteonectin) [21]. Interestingly, the loops of EF1 and the canonical EF2 of BM40 can be superimposed with an rmsd (root mean square deviation) of only 0.25 Å (1 Å = 0.1 nm), demonstrating that the one-residue insertion of EF1 results only in a very localized effect. The final non-canonical EF-hand that falls into this subgroup is the single functional EF-hand found in the ELC (essential light chain) of scallop (*Argopecten irradians*) myosin. The EF-loop of its EF1 is unusual in a number of ways: (i) although there is a significant number of aspartic acid residues present, owing to their unusual separation, main-chain carbonyl groups provide a number of ligands; (ii) a substitution of lysine for the C-terminal glutamic acid ligand is compensated for by an insertion of two residues after the first ligand, enabling Ca^{2+} co-ordination entirely by the N-terminal part of the loop; and (iii) instead of coming from a glutamic acid residue found in the exiting helix, the final two ligands are provided by two side chains of the entering helix [22].

Members of the third group of non-canonical EF-hands are rarer. At present there is only one example of a Ca^{2+} chelation loop shorter than the canonical sequence: the eleven-residue loop of EF1 in most members of the penta-EF-hand subfamily (Figure 4E). In these EF-loops, as is the case for several of the loops mentioned above, the lack of prototypic side chains in the X and Y positions is compensated for by both an alternative loop conformation that allows for the oxygen of a main-chain carbonyl group to act as a ligand and by co-ordination through an additional water molecule [23].

Of course, one way in which a non-canonical EF-loop can try to bind Ca^{2+} is to abandon the pentagonal bipyramidal co-ordination scheme. An octahedral coordination scheme is seen in EF5 of the apoptosis-linked protein ALG-2 (apoptosis-linked gene-2) (Figure 4F). The loop of this EF-hand in all known members of the penta-EF-hand subfamily contains a two-residue insertion in the C-terminal part of the loop that inactivates the site in all members except ALG-2 [24]. In this protein the Ca^{2+} is co-ordinated only by the N-terminal part of the loop and has an octahedral geometry, as the potential glutamine ligand is too distant and it is replaced by a single water molecule [25]. Octahedral co-ordination is also seen in EF1 of the conventional myosin ELC from the acellular true slime mould *Physarum polycephalum* [26]. The unusual conformation of this EF-loop prevents the loop's C-terminal ligands from participating in the co-ordination scheme.

The EF-hand pair

The EF-hand motif almost always occurs in pairs, creating an 11 Å distance between the two bound Ca^{2+} ions [27] (Figure 2B). Stacked against one another in a face-to-face manner, this pair forms a four-helix bundle with the amphipathic helices packed together to make a hydrophobic core. The structural integrity of the two EF-hand motifs is further stabilized through the short antiparallel β -sheet formed between the pairs' EF-loops [3]. Even though the two EF-hands in the domain are related by an approximate 2-fold axis of symmetry that passes through the eighth position of the loop [10], they are not identical. In fact, an EF-hand's position in a pair is strictly defined and designated as either 'odd' or 'even'. This asymmetry translates into asymmetric dynamic and Ca^{2+} -binding properties well documented in CaM [28,29] and calbindin D_{9K} [30,31].

Two pieces of evidence point to the pervasiveness of this pairing. Firstly, it is maintained even if one of the EF-hands is non-functional, a good example of which is seen in the Ca^{2+} -bound forms of the SCPs (sarcolemmal Ca^{2+} -binding proteins) of the invertebrates *Nereis diversicolor* (a ragworm) and *Branchiostoma* (formerly *Amphioxus*; the lancelet). For *Nereis* SCP, although EF2 is non-functional, owing to an extensive insertion, it is still paired with a functional EF1 and the two-fold symmetry is approximately preserved [18]. In the case of *Branchiostoma* SCP, the non-functional EF4 is again paired with a Ca^{2+} -bound EF3, but in this case the antiparallel β -sheet between the two EF-hands is lost [17].

The second piece of evidence is provided by peptide studies on individual EF-hands. The hydrophobic packing of the EF-hand motif provides a strong driving force for peptide dimerization, the extent of which has been demonstrated in experiments using isolated EF-hands from several EF-hand containing proteins, including CaM [32], TnC (troponin C) [33,34], calbindin D_{9K} [35] and *Nereis* SCP [36]. These peptides are observed to form homodimers in the presence of Ca^{2+} , which includes a short β -sheet formed between the loops. Tellingly, when a homodimer is mixed with its natural partner, a heterodimer is preferentially formed at the expense of the homodimer. This suggests a highly specific packing of the core [11], a suggestion corroborated by the 200-fold increased affinity of the heterodimer compared with the homodimer for Ca^{2+} [37,38]. Interestingly, in addition to dimer formation, there is a cation-induced transition from a random-coil structure to a helix-loop-helix conformation, a transition which may have important implications for protein folding [39]. Finally, the interdependent folding of a pair of Ca^{2+} -binding sites is supported by calorimetric studies showing that the Ca^{2+} -saturated forms of TnC and CaM unfold as a 'co-operative block' [40].

Even though the structure of the EF-hand pair is highly conserved throughout this protein family, diversity is introduced through both structural additions to the N-terminus of the Ca^{2+} -binding domain and by modifications to both the length and composition of the linker connecting the two EF-hands. An additional and significant level of diversity is also introduced through the topic of the next subsection – the organization of the EF-hand domains in a protein.

EF-hand domain organization

The smallest member of this family is calbindin D_{9K} , a 9 kDa protein which contains a single EF-hand pair (Figure 5). Although the other members of the S100 subfamily to which this protein belongs also contain a single pair of EF-hands, these members occur as part of either homo- or hetero-dimers, giving each of the S100 proteins four EF-hands. Most of the proteins of the EF-hand family contain two pairs, each similar to calbindin D_{9K} , totalling four EF-hand motifs. Three different arrangements of the two domains have been observed, depending on the conformation and length of the linker between the domains of paired EF-hands. In the first, two independent globular domains are connected by a flexible linker. As seen for sTnC (skeletal-muscle TnC) [41], and extensively for CaM [42], the plasticity of the linker in this arrangement allows the two EF-hand domains to take up a variety of orientations with respect to each other, enabling an extensive array of target interaction arrangements. In the second conformation, which is seen in the structure of recoverin and other members of the NCS (neuronal Ca^{2+} -sensor) subfamily, a U-shaped linker between the domains places the four EF-hands in a compact tandem array on one face of the protein [43,44]. Here, the relative disposition of the domains is fixed by intra- and inter-domain

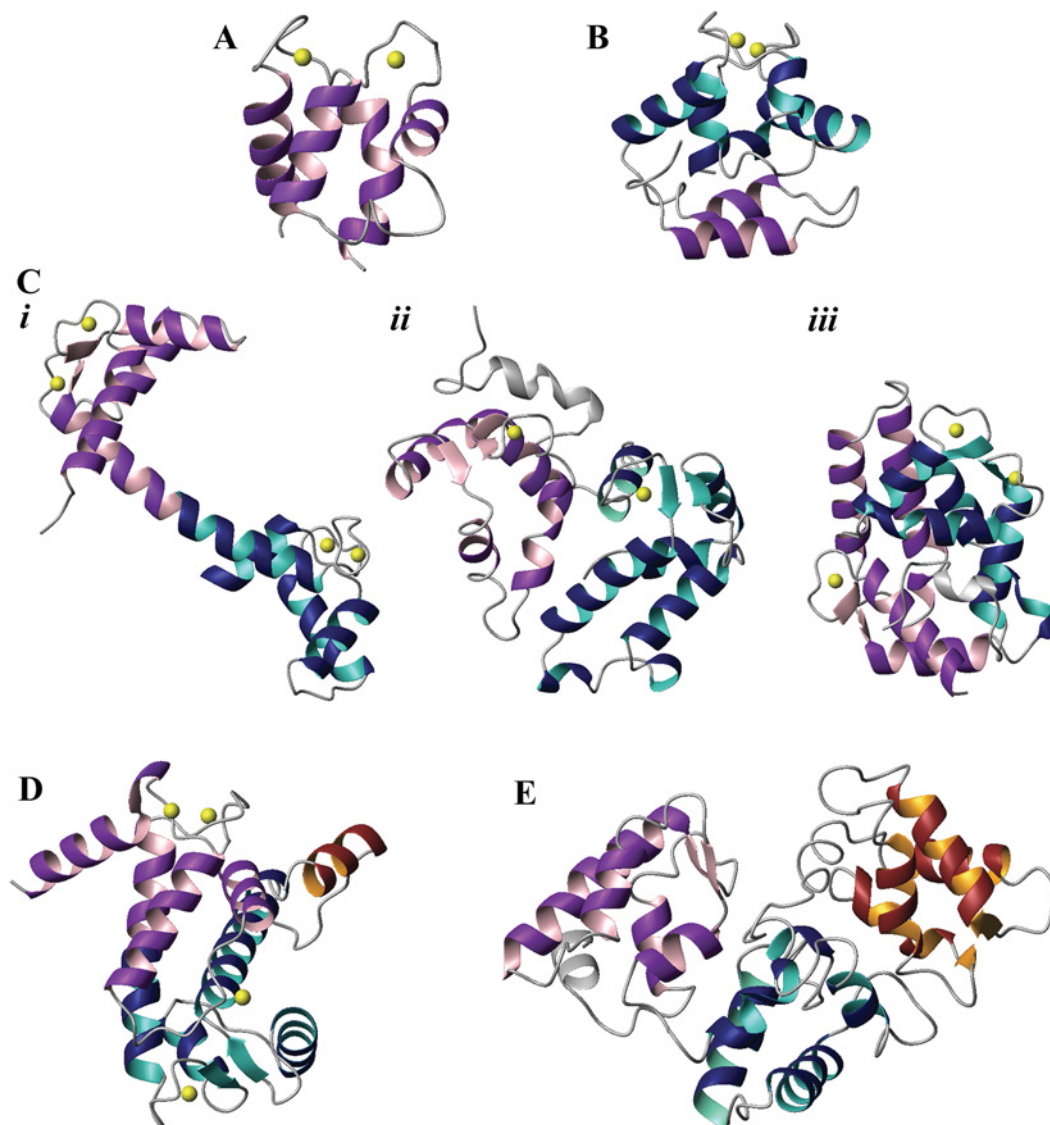


Figure 5 EF-hand domain organization

(A) Calbindin D_{9K} is the smallest member of this family and contains a single pair of EF-hands (PDB code 3ICB). (B) Parvalbumin is a three-EF-hand protein with the non-functional EF-hand serving to stabilize the Ca^{2+} -binding pair (PDB code 4CPV). (C) EF-hand proteins with four EF-hands: *i*, the two independent domains of CaM are connected by a flexible linker (PDB code 1EXR); *ii*, in recoverin the two domains are placed in tandem on the same face of the protein (PDB code 1JSA); *iii*, in *Nereis* SCP the EF-hands form a compact globular fold with the pairs of EF-hands on opposite sides of the molecule (PDB code 2SCP). (D) Domain VI of calpain is a member of the PEF (penta-EF-hand) subfamily and has five Ca^{2+} -binding motifs, although the fifth EF-hand does not remain unpaired, since it promotes dimerization to create a ten-EF-hand structural unit (PDB code 1DVI). (E) Calbindin D_{28K} contains three paired EF-hand domains totaling six EF-hands (PDB code 2G9B). As this is an NMR structure, no direct information on the Ca^{2+} ions is obtained. In (A)–(E) the first EF-hand pair is coloured purple/pink, the second is blue/turquoise and the third is brown/orange. Helices that are not part of the EF-hands are coloured grey [175].

contacts. The final observed arrangement for the four EF-hands is the compact globular fold seen in the structure of the invertebrate SCPs [17,18]. In this conformation, the two pairs of EF-hands reside on opposite faces of the compact molecule. In fact, the highly apolar surfaces of the two domains exhibit such an affinity and specificity for each other that they preclude recognition and binding to the hydrophobic surface of a target protein [45]. Finally, there are those members of the EF-hand superfamily which contain six EF-hands, including the neuroprotective protein calbindin D_{28K} and its homologue calretinin, as well as a subfamily of ER regulatory proteins, including reticulocalbindin (see Supplementary Table 1 and references cited therein). For calbindin D_{28K} the three pairs of EF-hands form a compact and ellipsoid-type conformation similar to that of the NCS subfamily

[46]. In these larger EF-hand-containing proteins, rarely are all the EF-hands active in binding Ca^{2+} .

Because the EF-hand motif has a strong tendency to be part of a pair, examples of EF-hand proteins that contain an odd number of EF-hands are rare. A single, functional EF-hand is found in the C-terminal domain of the CaV1.2 subunit of the voltage-gated Ca^{2+} channel. Despite the non-canonical sequence found in its EF-loop, this motif is believed to bind bivalent cations and may function in Ca^{2+} homeostasis [47]. For the parvalbumins, a three-EF-hand protein subfamily, the unpaired EF1 lacks several characteristic features of the EF-hand motif. Functionally, this site does not bind Ca^{2+} , but serves to stabilize the Ca^{2+} -binding EF2/3 pair by behaving like an endogenous peptide bound to the C-terminal domain in a manner reminiscent of Ca-CaM-peptide

interactions [48]. Interestingly, substitution of a canonical loop into this inactivated EF-hand does not restore Ca^{2+} binding and causes an overall destabilization of the structure [49]. Finally, an entire subfamily contains five EF-hands. However, the function of this fifth hand is to promote self-association to create a ten-EF-hand structural unit.

This dimerization property of the EF-hand is particularly interesting, as it suggests that, in addition to being a Ca^{2+} -binding unit, it can also serve as a dimerization motif. In structures of penta-EF-hand subfamily members, EF5 of the first monomer is packed against the equivalent section of the second, burying one-fifth of the total surface of each monomer and forming a tightly associated four-helix bundle [23,25,50,51] (Figure 6A). Pairing between fifth EF-hands is also used to mediate heterodimer formation between domains IV and VI of calpain as well as ALG-2 and peflin [52]. Interestingly, the structure of the EF5/5' pair mimics that of the traditional EF-hand pair, including the antiparallel β -sheet.

In the previously discussed proteins, the EF-hand is the major structural unit. This is not always the case, as this motif also occurs as one domain of a multidomain protein (see Supplementary Table 1). In many instances, such as that seen for domains IV and VI of calpain (Figure 6B), the EF-hand pair mediates domain-domain interactions. Similarly, the EF-hand domain in both the *P. polycephalum* protein CBP40 [53] and Cb1 [54] is near the centre of the protein, where it creates a large interface (~ 1500 and ~ 800 Å respectively) that serves to position the other domains.

The topics discussed in this subsection, the Ca^{2+} ion co-ordination scheme, the structural unit of the EF-hand pair, as well as the organization of this pair in EF-hand containing proteins, highlights the adaptability of the EF-hand Ca^{2+} -binding motif. Owing to the plasticity of the loop, site-specific mutations, insertions and deletions introduced over time are accommodated in many cases through non-canonical Ca^{2+} co-ordination mechanisms. Variation in the amino acid composition of the EF-hand helices leads to different helix packing interactions between the EF-hands as well as between a given EF-hand domain and the rest of the protein. Finally, an additional level of complexity to this motif is provided by the diversity of the response to Ca^{2+} binding, a topic that is discussed in the next section.

CONSEQUENCES OF Ca^{2+} BINDING

Ca^{2+} binding and induced structure formation

Traditionally, the role of Ca^{2+} binding has been examined through the lens of signal transduction, which focuses on the Ca^{2+} -induced conformational changes and their effects on target interactions. Although this consequence of Ca^{2+} binding is important and will be discussed in the next paragraph, Ca^{2+} can also play a *de novo* structural role and, in many cases, the binding of this cation is key to the structural integrity of the protein. EF-hands that serve a structural function have a Ca^{2+} affinity high enough for Ca^{2+} to be bound even in the resting cell. Proteins such as the sarcoplasmic CaBPs (the invertebrate SCPs [55,56]), the C-terminal domain of CaVP (calcium vector protein) [57] and the prokaryotic protein calerythrin [58] all exhibit an unfolded, molten-globule-like state in the absence of Ca^{2+} *in vitro*. Another example is the bilobal TnC, in which the two EF-hands of the C-terminal domain have an approx. 100-fold higher affinity for Ca^{2+} than have the N-terminal EF-hands – an affinity high enough to ensure Ca^{2+} saturation in the resting cell. Like the aforementioned proteins, the C-terminal domain of TnC is unfolded in the absence of Ca^{2+} [59]. When compared with the N-terminal domain, this lack of structure is thought to be due to the absence of stabilizing hydrophobic interactions that overcome the destabilizing electrostatic interac-

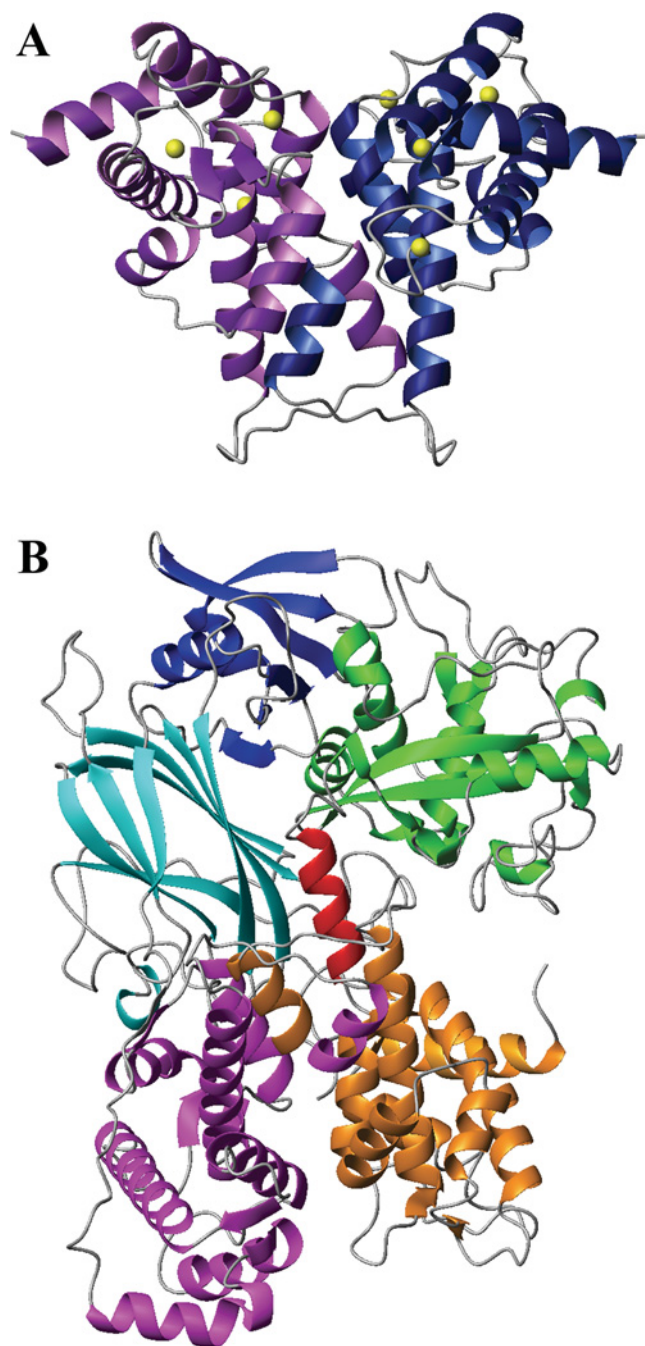


Figure 6 Intermolecular organization of EF-hand proteins

(A) The homodimer of calpain domain VI formed through interactions between EF5/5' (PDB code 1DVJ). One monomer is in purple; the other is in blue. (B) The structure of apo-calpain, a multidomain protein with the different domains highlighted by different colours (PDB code 1DF0). Domains IV and VI (orange and magenta respectively) are members of the PEF subfamily and interact via their unpaired EF5 [175].

tions, the main source of which are the carboxy ligands of the EF-hand motif [60]. Interestingly, a structural role for the EF-hand has also been suggested for several multidomain signalling proteins, since for both phospholipase $\text{C}\delta$ [61] and EH (Eps15-homology) domains [62], Ca^{2+} binding has no effect on the function of the protein. In contrast with these proteins, in which Ca^{2+} -binding induces structure formation, most EF-hand-containing proteins are structured in the apo state, and the binding of this cation leads

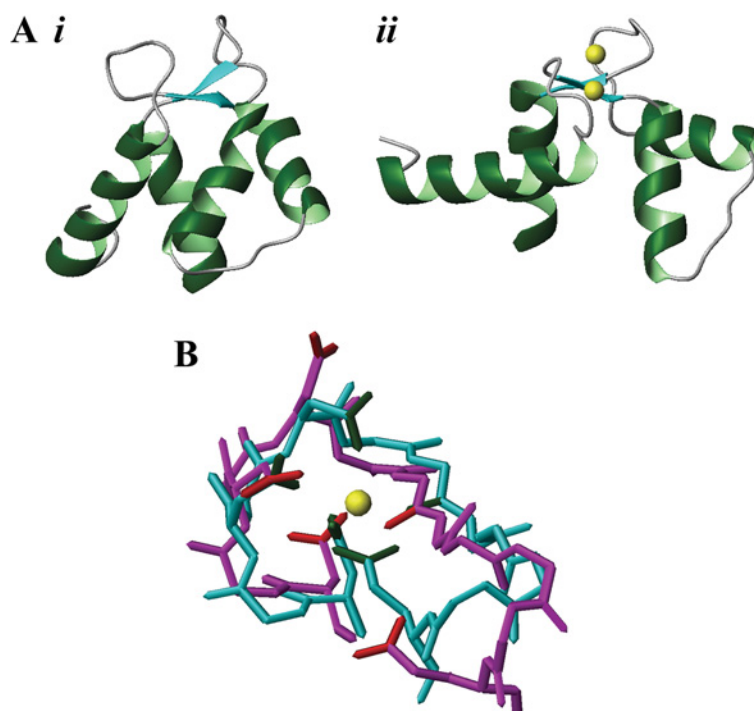


Figure 7 Ca^{2+} -induced conformational changes

(A) The closed-to-open transition as seen in the N-terminal domain of CaM. The helices move from a more antiparallel arrangement in the apo form (i) to being nearly perpendicular in the Ca^{2+} -bound state (ii). Hydrophobic residues that were buried in the apo form are now exposed to the solvent. (B) Changes in the loop structure upon Ca^{2+} binding. The apo loop from EF1 of CaM is indicated in magenta, with the Ca^{2+} ligands in red. The Ca^{2+} -bound loop is in turquoise, with the ligands in green. The bound Ca^{2+} ion is represented by the yellow sphere, and the water ligand is omitted for clarity. In the apo structure, most of the N-terminal ligands of the loop are in place to bind the Ca^{2+} ion. The major difference between the structures is seen in the placement of Glu12, which must move several angstroms to chelate the Ca^{2+} ion [PDB code for (A)–(B) 1EXR] [175].

to a conformational change through which this protein transduces the message of an increased Ca^{2+} concentration.

Ca^{2+} binding and conformational change

Initially this Ca^{2+} -induced conformational transformation was considered in terms of a closed-to-open domain transition quantified through the change in interhelical angle of vectors defined by the helices that bind Ca^{2+} . For the members of the CaM subfamily and several other EF-hand-containing proteins, these helices, antiparallel in the apo structure with interhelical angles of 130–140°, open upon Ca^{2+} binding to become roughly perpendicular with an angle that approximates to 90° [63,64] (Figure 7A). Through this change in angle there is a loss of interhelical contacts between the entering and exiting helices of the EF-hand as the top where Ca^{2+} binds is ‘pinched’ together and the bottom moved apart – a conformational change that exposes once-buried hydrophobic side chains and creates an extensive target-protein interaction site. In CaM these surfaces are approx. 10 Å × 12.5 Å in each domain [65].

At a molecular level this conformational change appears to be a consequence of Ca^{2+} chelation by the EF-loop. Initially the Ca^{2+} ion is bound by ligands in the N-terminal part of the loop (1, 3, 5 and 7), as this region is quite flexible and ‘waves around’ to catch the ion [14]. At this point the C-terminal ligands, predominantly those provided by the twelfth position, are too far away to chelate the bound Ca^{2+} ion directly (Figure 7B). In order for the loop to complete the ion’s co-ordination sphere (an energetically favourable process), the exiting helix must be repositioned by ~2Å. It is the movement of this helix that causes the conformational change in the EF-hand.

The repositioning of Glu12 is stabilized not only through the Ca–O ‘bonds’ that form, but also through two new hydrogen bonds between this side chain and the backbone at positions 2 and 9. The importance of these hydrogen bonds to the Ca^{2+} -induced conformational change has been seen in structural studies in which this glutamic acid residue is replaced with a glutamine residue [28,66,67]. The inability of the glutamine residue’s side chain to simultaneously form both of these hydrogen bonds leads to a conformational exchange between two conformations similar to those of the closed and open states. This conformational exchange suggests that, unlike in the wild-type protein, the equilibrium here is not being pushed towards the open structure and that one additional hydrogen bond (~12 kJ/mol) is required to shift the balance [66].

This two-step binding pathway suggests an explanation for the observed asymmetry in the EF-hand pair mentioned above. As the linker between EF1 and EF2 provides a strong covalent coupling between helices II and III of the pair, rotation of helix II will cause pulling on helix III, forcing it to follow and re-orient. By contrast, as there is no covalent link between helices I and IV, a rotation of helix IV by itself can occur more readily. The consequence of this smaller conformational cost of Ca^{2+} binding to EF2 compared with EF1 is seen in several EF-hand proteins, including CaM, TnC and CIB, where the second EF-hand of the pairs is filled first, since the binding affinity is higher.

The closed-state-to-open-state conformational change seen in the CaM subfamily is not observed in all EF-hand subfamilies. For the S100s, Ca^{2+} binding induces a ‘change in hand’-type motion, as the interhelical angle between the entering and exiting helices of EF2 is modified from a negative angle to a positive one similar to that seen in CaM [68]. For recoverin and other

members of the NCS subfamily, Ca^{2+} binding results in domain rotation. Through this rotation a myristoyl group that had been buried within protein core was exposed, leading these proteins to translocate to the plasma membrane [64]. In sorcin and potentially all other members of the penta-EF-hand subfamily, though Ca^{2+} binding seems to have little structural effect on the EF-hand domain itself, due to altered interhelical interactions, a large conformational change is thought to occur as the contacts between the C-terminal EF-hand domain and the N-terminal domain are weakened. This results in the exposure of a large hydrophobic protein target binding site [69,70]. Interestingly, for the aforementioned proteins, and as in the CaM subfamily, mutational studies indicate that it is still chelation by the ligands provided by the 12-loop position that trigger the Ca^{2+} -induced conformational change. Finally, the Ca^{2+} -buffer proteins calbindin D_{9k} and the parvalbumins experience minimal structural changes upon Ca^{2+} binding.

The diversity of EF-hand conformations has been attributed to the torsional flexibility of a region of the loop termed the 'EF β -scaffold' [3]. Encompassing positions 6–8 the EF β -scaffold forms a well-defined hinge around which the rotation of a few bonds enables the Ca^{2+} -induced conformational change. Amazingly, orientations of the exiting helix differing by as much as 30° may be compatible with Glu12 binding in the Ca^{2+} -co-ordinating position. Significantly for the latter proteins mentioned in the previous paragraph, through rotation of these bonds the EF β -scaffold is able to close the loop on its own without requiring a large change in the interhelical angle.

The above paragraphs describe fixed conformations for the Ca^{2+} -bound and -unbound EF-hand pair which, owing to protein dynamics, probably does not reflect the reality of the situation. From the anisotropic and anharmonic disorder seen in the 1.0 Å crystal structure of Ca-CaM [71] and NMR relaxation data from the structures of both apo and Ca-CaM [29,65,72–74], a model was proposed whereby CaM samples quasi-continuous substates rather than cycling between two well-defined conformations that depend on the Ca^{2+} -bound status. Both apo- and Ca-CaM sample very large and partially intersecting volumes of conformational space, explaining the open-type conformation sometimes seen for apo-CaM and the closed-type conformation sometimes seen for Ca-CaM. Similar to the classical Monod–Wyman–Changeux model, the binding of Ca^{2+} to the unbound form stabilizes certain substates at the expense of others, as it results in structural changes in the EF-hands which decrease the frequency with which the closed substates are sampled. This suggests that, at least for CaM, the Ca^{2+} -bound and apo forms seem to be less distinct structurally, which helps to explain the plasticity of this protein in the presence of its target proteins. The dynamics that enable the sampling of many substates is attributed to the flexibility in the backbone angles of the loop residues, predominantly those of position 8.

The Ca^{2+} -induced conformational changes discussed in this section have energetic consequences on the ability of each EF-hand to bind this cation. These energetic consequences will be discussed in the next section.

DETERMINANTS OF Ca^{2+} AFFINITY

When attempting to understand the Ca^{2+} -binding ability of the various EF-hands, four factors must be taken into account (Figure 8). The first is the intrinsic Ca^{2+} affinity of each EF-hand (ΔG_{intr}), which reflects both the ligands present in the loop as well as the contributions of non-co-ordinating interactions. The second is the ability of a given EF-hand to bind Mg^{2+} , a cation chemically similar to Ca^{2+} that can pose significant selectivity problems owing to its high cytosolic concentration. The third factor is

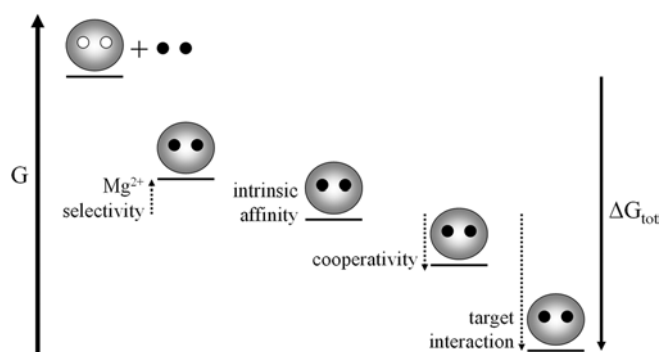


Figure 8 Schematic representation of the major determinants of observed Ca^{2+} -affinity (ΔG_{tot})

This affinity reflects not only the EF-hand's intrinsic ability to bind Ca^{2+} , but also favourable (co-operativity, target interactions) and unfavourable (competition from Mg^{2+}) energetic couplings.

co-operativity, a phenomenon that enables an EF-hand pair to bind Ca^{2+} as a unit, despite any differences in intrinsic affinity, thereby creating a rapid response to this second messenger. The final factor for those EF-hand proteins that act as sensors is the target protein, whose presence can have a significant impact on the affinity. Taken together, the observed Ca^{2+} affinity of an EF-hand can be described as:

$$\Delta G_{\text{obs}} = \Delta G_{\text{intr}} + \Delta \Delta G_{\text{sel}} + \Delta \Delta G_{\text{co-op}} + \Delta \Delta G_{\text{interact}} \quad (1)$$

where the effects of Mg^{2+} selectivity ($_{\text{sel}}$), co-operativity ($_{\text{co-op}}$) and target interaction ($_{\text{interact}}$) are seen as an energetic coupling ($\Delta \Delta G$) that reflects the difference in affinity [ΔG_{tot} (total free-energy change) of binding] in the presence and absence of these factors (e.g. $\Delta \Delta G_{\text{sel}} = \Delta G_{\text{tot, presence of Mg}} - \Delta G_{\text{tot, absence of Mg}}$).

Determinants of intrinsic Ca^{2+} affinity

The EF-hand presents an intriguing mystery, as the Ca^{2+} dissociation constants found in this protein family range from 10^{-9} to 10^{-4} M, with no major correlation between affinity and the nature or position of the ligands (Supplementary Table 2). However, if one considers thermodynamics and the following well-known equations, a few conclusions can be made:

$$\Delta G = -RT \cdot \ln K_a \quad (2)$$

Where R is the gas constant and T is the temperature.

$$\Delta G = \Delta H - T \Delta S \quad (3)$$

Where where ΔH is the change in the enthalpy of the system, T is the temperature in degrees kelvin and ΔS is the change in entropy. The intrinsic Ca^{2+} affinity is determined by the difference in the Gibbs' free energy (ΔG) between the unbound and bound states. Clearly, the larger and more negative the ΔG , the higher the affinity. At a constant temperature, phenomena that increase ΔS or decrease ΔH between the unbound and bound states should lead to higher affinity (Figure 9).

Entropic contributions to affinity

When Ca^{2+} binds to an EF-hand, water that was 'frozen' in its co-ordination sphere is 'released' to the bulk solvent. An effect

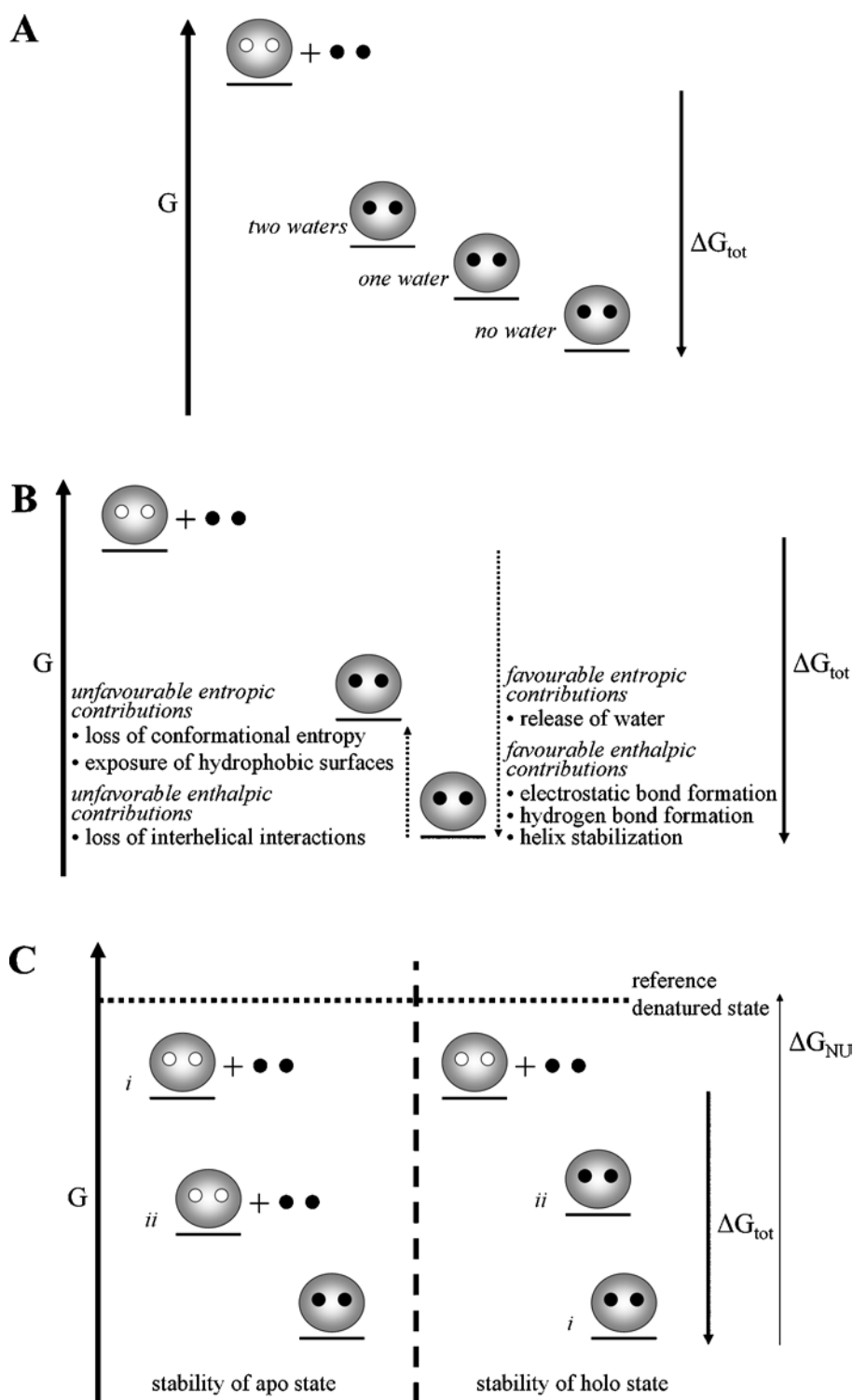


Figure 9 Determinants of the intrinsic Ca^{2+} -binding ability of the EF-hands

(A) The effect on affinity (ΔG_{tot}) of water molecules remaining in the co-ordination sphere. As the increase in solvent entropy is thought to be the major contributor to the free energy of EF-hand Ca^{2+} -binding, those EF-hands in which two water molecules remain will bind Ca^{2+} with a lower affinity than those in which all of the Ca^{2+} ligands are provided by the protein. (B) The intrinsic Ca^{2+} affinity of an EF-hand (ΔG_{tot}) is dependent upon a balance between favourable and unfavourable enthalpic and entropic factors. For each EF-hand the characteristics of these factors and the balance between them are unique. (C) The relative stability of both the apo and the Ca^{2+} -bound states also affect an EF-hand's intrinsic Ca^{2+} affinity. The more stable the apo state or unstable the Ca^{2+} -bound state compared with a reference denatured state (*ii* versus *i*), the lower the Ca^{2+} affinity of a given EF-hand.

that is fairly constant per bound Ca^{2+} ion, this gain in solvent entropy is so large that it is thought to dominate the favourable increase in ΔG that occurs when Ca^{2+} binds to an EF-hand [6]. The

magnitude of this entropic change is dependent on the number of water ligands exchanged for protein ligands: the more water ligands that remain, the lower the affinity (Figure 9A).

This has significant implications for the identity of the ninth and twelfth loop positions. Owing to its length, the co-ordinating side chain of a glutamic acid or glutamine residue in the ninth position has the ability to chelate the bound ion directly, and not through the bridging water molecule which is needed if a shorter amino acid is found in this position [75]. The consequence of this is apparent when the EF-hands of parvalbumin as well as EF3 of the parvalbumin subfamily member oncomodulin are compared with that of EF2 of oncomodulin. Unlike the glutamic acid residue found here in the aforementioned EF-loops, EF2 of oncomodulin has an aspartic acid residue in the ninth position and a water molecule in its co-ordination sphere. The resulting loss in increased solvent entropy due to the more incomplete loop chelation is thought to contribute to the lower affinity of this site compared with that of EF3 of oncomodulin and those found in parvalbumin [76]. The same reasoning applies in many cases when an aspartic acid residue is found instead of a glutamic acid residue in position 12 of the EF-loop. As seen from the crystal structure of CIB, the side chain of the aspartic acid residue is too short for both oxygen atoms to co-ordinate the bound Ca^{2+} ion, and the co-ordination sphere is completed by a water molecule [19]. The effect of this extra water molecule is reflected in the fivefold decrease in affinity of EF3 (Asp12) compared to EF4 (Glu12) of CIB [77].

The magnitude of the favourable entropy term in eqn (3) can be decreased if unfavourable factors contribute to the entropy of Ca^{2+} binding to EF-hands. For some EF-hands the loss of EF-loop conformational entropy is higher than for others. Both crystallographic B-factors and NMR relaxation data indicate that a glycine triplet in the *N*-terminal part of the EF1 loop of sTnC is more disordered, and consequently more stable, in terms of conformational entropic free energy, than EF2 [78,79]. The cost of ordering this flexible loop is reflected in its tenfold lower affinity compared with EF2 [80]. This entropic cost of loop ordering is drastically decreased in 'preformed' EF-hands, such as the Ψ -hand of calbindin $\text{D}_{9\text{k}}$, and it contributes to the high Ca^{2+} affinity of this site [81]. It is likely that many EF-hands use such conformational entropy costs due to loop flexibility in the apo form to fine-tune their Ca^{2+} affinity [82]. A second unfavourable entropic factor is the Ca^{2+} -induced exposure of hydrophobic surfaces, as experienced by the Ca^{2+} sensors, a cost (ΔG) measured for the *N*-terminal domain of sTnC to be ~ 8.4 kJ/mol [83]. This cost of exposing hydrophobic surfaces is reflected in a reduced Ca^{2+} affinity with respect to the theoretical maximum attained if the unfavourable effects of the Ca^{2+} -induced conformational change did not counteract the favourable effects of releasing the water molecules [63] (Figure 9B). For the Ca^{2+} sensors, this costly conformational change would not occur if Ca^{2+} could be accommodated in the closed state. It is likely that the co-ordination geometry required to bind the Ca^{2+} ion imposes geometric constraints on the EF-hand, the strain in the closed conformation being relieved in the open state. Clearly, for all the members of the EF-hand family, whether or not an EF-hand opens in response to Ca^{2+} , is dependent on the balance between conformational strain and exposed hydrophobic surfaces.

Enthalpic contributions to affinity

Metal ion binding to proteins is usually entropically driven ($\Delta H > 0$) because of the high dehydration enthalpies of bivalent cations [84]. However, the overall enthalpy of bivalent-cation binding can be exothermic ($\Delta H < 0$) if it is coupled to an enthalpically favourable process such as those mentioned below [77,85–87].

A major contributor to the favourable enthalpy changes upon Ca^{2+} binding is the formation of constructive electrostatic interactions – the electrostatic 'bonds' that form between the ligands of the loop and the bound Ca^{2+} ion. From this one would expect the EF-hand affinity to reflect the number of carboxylate ligands. This is not the case, however, for as much as there is electrostatic attraction between these groups and the bound Ca^{2+} ion, there is also electrostatic repulsion, not only in the apo, but also in the Ca^{2+} -bound, form. An early explanation relating Ca^{2+} affinity and the identity of the ligands is known as the 'acid-pair hypothesis' [88]. This hypothesis proposes that Ca^{2+} -binding ability is dependent on the number and arrangement of the charged ligands in the EF-loop, specifically that four acid residues paired along the *X*- and *Z*-axes would be the most stable arrangement of anionic charges as in this configuration dentate–dentate repulsion is more reduced than in any other. The acid-pair hypothesis goes further and predicts that the introduction of a fifth carboxylate chelating residue will decrease Ca^{2+} affinity owing to an increased level of electrostatic repulsion [89]. Subsequent studies provided evidence against this hypothesis with respect to both of its proposals. Mutational studies on the *N*-terminal domain of CaM found that Ca^{2+} affinity increased with pairing along any of the three axes [90]. That an acid pair along the *Y*-axis increases affinity is surprising, since the ligand at the *Y* position is provided through the backbone carbonyl group; however, this affinity increase probably reflects a higher Ca^{2+} association rate in this mutant. Additionally, this study showed that, whereas the formation of a third pair created too much electrostatic repulsion, the addition of a fifth ligand leads to an increased affinity, a finding also seen with oncomodulin [91].

A more recent hypothesis relating the nature of the liganding groups and Ca^{2+} affinity is termed the 'charge-ligand-balanced model' [92]. Supported by work on isolated EF-loops of CaM grafted on to a protein scaffold, this model proposes that both the number of negatively charged chelating groups and the presence of non-co-ordinating residues that balance the dentate–dentate repulsion of adjacent amino acids are major determinants of the intrinsic Ca^{2+} -binding affinities of the EF-loop. The importance of non-ligand residues has also been seen in peptide-binding studies with sTnC [93].

Additional loop-based interactions that are thought to contribute positively to the enthalpic change of Ca^{2+} binding include hydrogen-bond formation and further electrostatic interactions. The significance of hydrogen bonds in Ca^{2+} affinity has been seen in both site-directed-mutagenesis studies of EF2 of sTnC [94] as well as X-ray analysis of this protein's crystal structure [41]. These studies revealed the importance of Asp1 of the EF-loop for both Ca^{2+} co-ordination and maintenance and stability of the loop structure through the extensive hydrogen-bonding network between its carboxylate oxygen atoms and various other residues within the same loop. The precise steric dimensions of the aspartic acid side chain are pivotal, as neither mutant loops containing a glutamic acid nor an asparagine residue in this position bind Ca^{2+} with high affinity. Additional electrostatic interactions are thought to take advantage of either the excess formal positive or negative charge that is common in EF-loops. These excess charges are thought to be involved in electrostatic interactions with both buried and surface charges of the protein, even though some are up to 20 Å from the bound metal ion [95]. Kinetically, surface charges in the vicinity of the EF-hand site are thought to influence the Ca^{2+} on-rate, helping to tune EF-hand affinity [96].

As evidenced by the variation in Ca^{2+} dissociation constants for EF-hands with very similar loop sequences, helical effects on the binding enthalpy also need to be considered. First, as seen through additive peptide studies, helices play a major role in

promoting high-affinity Ca^{2+} binding to the EF-hands [37,97,98]. Secondly, as the electrostatic force is inversely proportional to the polarity of the binding site, the degree of hydrophobicity of the flanking helices and the loop itself can shape the local electrostatic field through contributions to the dielectric environment [99]. The C-terminal EF-hands of cTnC (cardiac-muscle TnC) have an approx. 20-fold higher affinity for Ca^{2+} than the corresponding EF-hands in CaM, an affinity attributed, through CaM–TnC chimaera studies, to their increased hydrophobicity [100]. Of course, an increased hydrophobicity of the flanking helices would help to stabilize the protein core, which itself would increase Ca^{2+} affinity, especially in those EF-hands that do not undergo extensive conformational changes [101]. Thirdly, favourable helix–dipole interactions will lead to a more negative enthalpic change [102]. The significance of these charge–charge interactions is thought to partially explain the presence of high- and low-affinity sites in sTnC. The high affinity of EF3 is in part attributable to the lysine residue at position 2, since, in the crystal structure of sTnC [41], the side chain of Lys¹⁰⁷ is exposed to the solvent and directed back towards the entering helix. A similar occurrence is found with the position-2 lysine residue of EF4, suggesting that this residue also acts to stabilize the entering helix by neutralizing the partial negative charge at the C-terminal end of the helix [103]. In the lower-affinity EF2, this position in the loop is occupied by a glutamic acid residue, which would have a destabilizing effect on the incoming helix [93]. Stabilization of the exiting helix in a similar manner is also thought to occur [95].

Finally, interhelical interactions also play a role in the binding enthalpy. However, at least for those proteins that undergo a large Ca^{2+} -induced conformational change, this role is unfavourable. The interhelical interactions are much more favourable in the Ca^{2+} -free form, as the adjacent helices within a given EF-hand are nearly antiparallel. This orientation increases the number of energetically favourable interactions in two ways, as it provides both a greater contact area among all four helices and favourable electrostatic interactions due to the orientations of the dipoles of the helices. As calbindin D_{9k} does not undergo significant conformational changes, such as greatly altered helix packing and the exposure of hydrophobic residues, its Ca^{2+} binding is dominated by the favourable effects of relieved electrostatic repulsion in the loop and increased solvent entropy, and occurs with relatively high affinity.

Contributions of stability towards affinity

A final factor that affects an EF-hand's affinity for Ca^{2+} is the relative stability of each state (Figure 9C). The diversity of Ca^{2+} affinity in this family reflects differences in the apo and Ca^{2+} -bound forms of the EF-hands, as the total free-energy change that occurs upon Ca^{2+} binding, ΔG_{tot} , is proportional to the energy of the Ca^{2+} state compared with that of the apo state plus two solvated Ca^{2+} ions and is influenced by the stability of each conformation [101]. Stability can be seen in the energy change that occurs as the folded protein transitions to the denatured state, the free energy of denaturation (ΔG_{NU}). Each protein will have a unique denatured state at a unique energy level that can then be used as a reference to compare the stabilities of the apo and Ca^{2+} -bound proteins. The importance of this reference state is seen in the strong correlation between the energy of denaturation of the Ca^{2+} -bound state [$\Delta G_{\text{NU}(\text{Ca})}$] and the Ca^{2+} affinity. It appears that the more stable the Ca^{2+} -bound state, the larger the ΔG between the two states tends to be. The stability of the apo state is also important as a more unstable apo state leads to higher-affinity binding due to a larger difference in free energy of the apo and Ca^{2+} -bound forms. The significance

of the apo-state stability on the Ca^{2+} affinity can be seen in the following examples.

Even though the two lobes of CaM share 46 % sequence identity and superposition of the Ca^{2+} -bound structures leads to an rmsd of only 0.75 Å, there are significant energetic differences between them, differences that manifest themselves as dissimilar Ca^{2+} affinities [104]. The higher affinity of the two C-terminal EF-hands is thought to be partially due to the greater instability of their apo state when compared with those of the N-terminal domain [65,105]. Studies on the isolated N-domain suggest that residues of the interdomain linker are the cause of this increased apo-state stability [106,107]. As seen in the crystal structure, these residues form favourable contacts with the helices of the N-domain, contacts that must be unfavourably broken for these EF-hands to bind Ca^{2+} . In this way, residues of the interdomain linker increase the stability of the apo state at the expense of the Ca^{2+} -bound state, the consequence of which is a decreased Ca^{2+} affinity. In fact, through favourable effects on the stability of the apo state, both interdomain linkers as well as C-terminal domain tails are thought to help tune the Ca^{2+} affinity of an EF-hand. The correlation between affinity and domain stability was also seen in sTnC mutants with truncations of the N-terminal helix [108].

Electrostatic repulsion can also play a role in apo-state destabilization. At first glance the increased Ca^{2+} affinity of a pentacarboxylate EF-loop variant of oncomodulin would seem to negate the acid-pair hypothesis mentioned above [91]. However, upon structural examination of this variant, co-ordination by four carboxylate ligands is seen to be maintained as this EF-loop experiences ligand exchange [76]. Although the fifth carboxylate ligand does not directly increase the Ca^{2+} affinity, thermodynamic data indicate that it works indirectly by increasing electrostatic repulsion in the apo state, thereby decreasing its stability. As the apo form of this protein is destabilized, the ΔG between the bound and unbound states increases resulting in a greater Ca^{2+} affinity.

In fact, suboptimal stability has evolved into the helix packing of the Ca^{2+} -free forms of CaM and sTnC. The N-terminal domain helices of both of these proteins contain buried polar groups, the extent of which is unexpectedly high and energetically unfavourable, due either to high desolvation costs or a reduction in conformational entropy associated with an inability to solvate a large number of the side-chain configurations [109]. Their presence destabilizes the apo state and, if they are substituted with hydrophobic residues, Ca^{2+} affinity decreases.

Finally, for those EF-hand proteins in which Ca^{2+} binding induces structure from a molten-globule apo state, the inherent instability of this state, as well as the many hydrogen bonds, van der Waals interactions and electrostatic attractions associated with structured proteins, leads to high-affinity binding of this metal ion. The four-EF-hand phytoprotein aequorin illustrates this point. Aequorin is homologous with the invertebrate SCPs and the prokaryotic calerythrin, but, unlike these proteins, it maintains an apo state that is very similar in structure to the Ca^{2+} -bound one [110]. The folded conformation of apo-aequorin is thought to be due to a bound chromophoric coelenterazine group in the centre of the protein, the hydrophobic nature of which is likely to bring significant structure to the apo form. However, the increased stability this structure brings to the unbound form of aequorin results in a 10^2 – 10^3 -fold decrease in Ca^{2+} affinity compared with the SCPs and calerythrin.

EF-hands and Mg^{2+} selectivity

In the cell, the EF-hand selectively binds Ca^{2+} in the presence of 10^3 – 10^5 -fold higher concentrations of K^+ and Na^+ . Explained through the electrostatic repulsion model [111], the negative

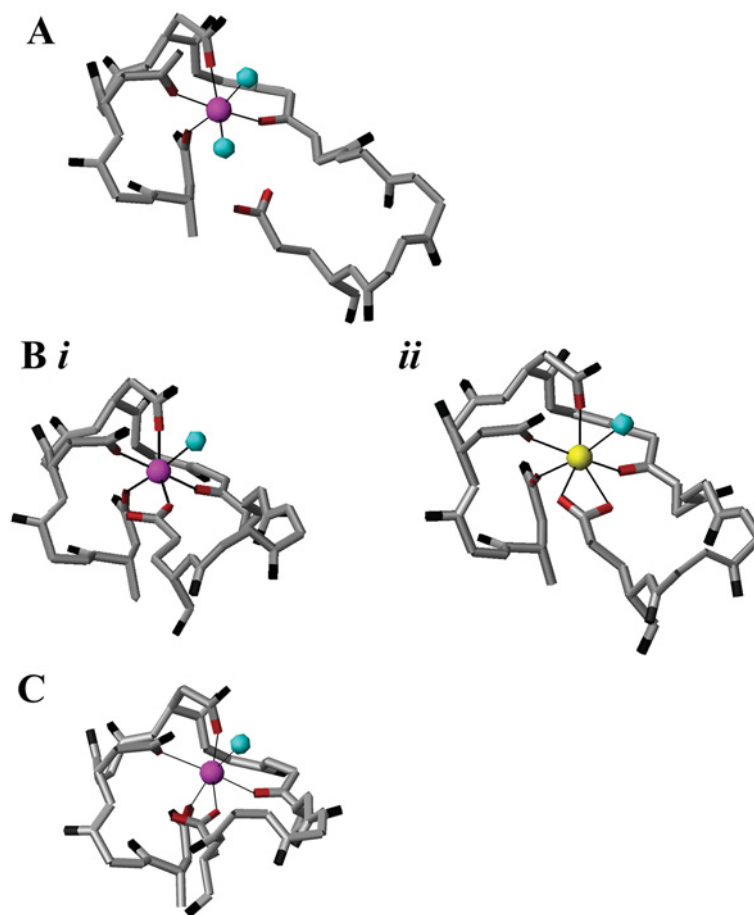


Figure 10 Mg^{2+} binding to EF-loops

(A) The Ca^{2+} -specific EF-loop of calbindin D_{9k} EF2. In this EF-loop, Mg^{2+} is only bound to the N-terminal ligands of the loop. The potential ligand provided by Glu12 is too far from the bound cation to be used; instead a water molecule is found here (PDB code 3ICB). (B) The Ca^{2+}/Mg^{2+} loop of parvalbumin EF3. With Mg^{2+} bound, the side chain of Glu12 is rotated 120° to provide only one ligand (i), contrasting with the bidentate ligand used for Ca^{2+} co-ordination (ii) (PDB codes 4PAL and 4CPV respectively). (C) The Ca^{2+}/Mg^{2+} loop of scallop myosin RLC. The side chain at position 12 of this loop is provided by an aspartic acid residue, creating a smaller binding site that more greatly favours binding of Mg^{2+} (PDB code 1WDC). In (A)–(C) the Mg^{2+} ion is represented by a magenta sphere, the water ligand as a blue sphere, the protein ligands as red sticks and the backbone and side chain carbonyl groups are black. For clarity, position 9 of the loop, which is hydrogen-bonded to the co-ordinating water molecule, is not shown [175].

charge of the EF-hand co-ordinating array is adjusted so that these univalent cations lack sufficient positive charge to stabilize the repulsion between the co-ordinating oxygen atoms. Owing to its greater charge, Ca^{2+} and other similar-sized bivalent and trivalent cations are able to overcome this O–O repulsion. In the test tube, this motif has been shown to bind spherical metal cations from groups Ia, IIa and IIIa, including the lanthanides [112], a property that has been exploited in several *in vitro* experiments. As most of the cations found in these groups have extremely low concentrations *in vivo*, competition between Ca^{2+} and these ions is not a significant factor for EF-hands. The high concentration of free Mg^{2+} in the cell (0.5–2.0 mM) does, however, pose a problem, as competition from this cation decreases the observed affinity of many EF-hands [113].

Ca^{2+} -specific and Ca^{2+}/Mg^{2+} -binding EF-hands

The similar properties of Ca^{2+} and Mg^{2+} lead to a complicated case of cation selectivity, as CaBPs must be able to discriminate against a 10^2 – 10^4 -fold excess of Mg^{2+} . The selectivity of Ca^{2+} -specific sites is thought to be provided by two distinct properties of these metal ions, namely (i) the stringent requirement of Mg^{2+}

for six ligands arranged in an octahedral geometry and (ii) Mg^{2+} 's smaller ionic radius, resulting in a higher energetic cost of dehydration [114]. By controlling ligand flexibility, EF-hands can exclude Mg^{2+} by preventing the required sixfold co-ordination or the preferred formation of a smaller binding site. Despite these selectivity mechanisms, owing to the excess Mg^{2+} concentration, many Ca^{2+} -specific EF-hands bind Mg^{2+} in the resting cell. NMR studies indicate that the EF-hands of CaM bind Mg^{2+} with dissociation constants in the millimolar range [115], implying that the protein is almost 50% saturated with this cation at resting Ca^{2+} levels [116]. Although Mg^{2+} binds the same sites as Ca^{2+} , in the Ca^{2+} -specific sites Mg^{2+} appears to bind only the ligands of the N-terminal part of the loop (1, 3, 5 and 7) (Figure 10A). By not engaging the C-terminal residues of the loop, including the critical twelfth residue, Mg^{2+} binding has no effect on the overall conformation of the EF-hand, and these sites remain 'off'. This inconsequential binding is supported by the fact that Mg^{2+} causes only localized conformational rigidity in the EF-loop, and the structure of Mg-CaM is nearly the same as apo-CaM [115]. A similar effect is seen with Mg^{2+} binding to the Ca^{2+} -specific EF-hands of calbindin D_{9k} [117].

In contrast with the Ca^{2+} -specific EF-hands, $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites have an Mg^{2+} -affinity severalfold higher and, at least in the resting cell, functionally bind the cation. This second class of site binds Mg^{2+} in almost the same manner as it binds Ca^{2+} , including the use of a ligand provided by the twelfth loop residue. In fact, this side chain is thought to play a key role in adapting the number of co-ordination ligands of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ EF-loop for the different cations. Seen in the Mg^{2+} -bound structures of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites of parvalbumin [118] and a molecular-dynamics simulation of the C-terminal domain of sTnC [119], through a 120° rotation of the $\text{C}_\alpha\text{-C}_\beta$ bond of Glu12, this amino acid provides a bidentate ligand for Ca^{2+} and a monodentate ligand for Mg^{2+} (Figure 10B). This bond rotation has also been observed in the Asp¹² EF-loop of molluscan myosin RLC (regulatory light chain) [22,120] (Figure 10C). In $\text{Ca}^{2+}/\text{Mg}^{2+}$ EF-hands, the flexibility of this side chain is critical, since, if it is sterically blocked into a bidentate configuration, these EF-hands become Ca^{2+} -specific [121]. As rotation of a side chain is limited by the local environment, it is difficult, if not impossible, to predict from the primary structure whether a given EF-hand is a Ca^{2+} - or a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -specific site. More commonly for Asp12 loops, rotation of the $\text{C}_\alpha\text{-C}_\beta$ bond at this position does not occur, and this residue provides a single ligand for both Ca^{2+} and Mg^{2+} [19,122]. As this ligand arrangement favours Mg^{2+} binding at the expense of Ca^{2+} , it leads to a reduction in cation selectivity and explains the tendency of Asp12 loops to be $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites. Finally, unlike Ca^{2+} -specific EF-hands, Mg^{2+} binding to $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites induces conformational changes that typically lead to structure formation from a molten globule. It is thought that, as for Ca^{2+} binding, the ligand provided by the twelfth position is probably key to this structural change.

Determinants of Mg^{2+} affinity

While enthalpic factors such as the presence of a Z-axis acid-pair are thought to play a role in Mg^{2+} affinity [123,124], entropic factors are more significant. As is the case for Ca^{2+} , increased solvent entropy is thought to be one of the driving forces of EF-hand Mg^{2+} binding, and the number of water molecules replaced by protein ligands has significant implications for the affinity for this cation. In fact, this entropic contribution is thought to be a significant reason behind the weaker Mg^{2+} affinity of Ca^{2+} -specific EF-hands when evaluated against that of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites [125]. As Mg^{2+} binding by the Ca^{2+} -specific EF-loops does not involve co-ordination by ligands provided by the twelfth loop position when compared with $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites that do chelate through this position, an extra water molecule is included in the co-ordination sphere [117]. This extra water molecule diminishes the favourability of the solvent entropy increase and leads to lower Mg^{2+} affinity.

Roles of Mg^{2+} binding

Mg^{2+} binding to EF-hands is physiologically important, and three roles are proposed. As previously mentioned, Ca^{2+} binding to several EF-hands serves a structural purpose, bringing form to an otherwise poorly defined molten globule apo state. These EF-hands, such as those in the invertebrate SCPs and the C-terminal domain of TnC, tend to be $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites, guaranteeing occupancy by either cation and structure formation at any Ca^{2+} concentration [126]. As these EF-hands also tend to have affinities that ensure Ca^{2+} saturation at resting intracellular Ca^{2+} concentrations, their ability to bind Mg^{2+} is not as significant as it is for Asp12 structural EF-hands. Owing to the weakened Ca^{2+} affinity

provided by an aspartic acid residue in this position, the ability of these EF-hands to bind Mg^{2+} in $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites is critical, as this cation maintains protein structure in the absence, and sometimes in the presence, of Ca^{2+} [77,87,127]. The ability of many proteins to bind Mg^{2+} in the absence of free Ca^{2+} makes their apo form physiologically irrelevant. However, as the Mg^{2+} -bound conformations tend to differ in subtle ways from the Ca^{2+} -bound form, these proteins, which include CIB, CaBP1 and GCAP-1 (guanylate cyclase activating protein-1), are still able to respond conformationally to increased Ca^{2+} concentration upon cell stimulation.

Since Mg^{2+} is a potent competitor for the EF-hand binding sites, the second role of Mg^{2+} is to modulate the affinity of EF-hands for Ca^{2+} . Whether or not the bound Mg^{2+} ion is fully co-ordinated by the twelfth position of the EF-loop, the kinetic on-rate of Ca^{2+} binding in the presence of Mg^{2+} is slowed, as it is limited by the Mg^{2+} off-rate. This, in turn, slows the Ca^{2+} response. If, due to Mg^{2+} competition, the Ca^{2+} affinity of CaM is reduced by only a factor of 2.6–3.3 over the entire range of cytosolic Ca^{2+} concentrations, no more than 10% of CaM N-terminal domains will be fully Ca^{2+} saturated [128]. Of course, owing to a more complete loop co-ordination, the effect of Mg^{2+} competition on Ca^{2+} affinity is higher for the $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites than for the Ca^{2+} -specific sites. The consequence of this is readily apparent in the two classes of sites found in sTnC in which the Ca^{2+} on-rate for the $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites found in the C-domain is > 100-fold lower than that of the N-domain Ca^{2+} -specific sites – a decrease partially attributed to the dissociation rate of Mg^{2+} [129,130].

Finally, EF-hand Mg^{2+} binding in some proteins appears to have a function distinct from that of Ca^{2+} . First, it is the Mg^{2+} -bound form of DREAM (downstream regulatory element antagonist modulator) that binds sequence-specific DNA targets; the Ca^{2+} -bound form disrupts this interaction [131]. Secondly, it has been suggested that the single EF-hand found in the cytoplasmic C-terminal domain of the cardiac CaV1.2 channel is specific for Mg^{2+} [132]. As most Mg^{2+} in the cell is complexed with ATP, the free Mg^{2+} concentration in cardiac myocytes increases by three or four orders of magnitude in response to falling ATP levels during stress [133]. It has been proposed that this increase is sensed by the CaV1.2 channel, since Mg^{2+} now binds the single EF-hand found in the cytoplasmic C-terminal domain. The consequence of this binding is an inhibition of the ability of the channel to conduct Ca^{2+} and, consequently, an adjustment of the contractile function of the heart under conditions of stress [47].

EF-hands and co-operativity

The third factor that influences an EF-hand's ability to bind Ca^{2+} is co-operativity of ligand binding. Through interactions with their partner, many EF-hands bind Ca^{2+} with positive co-operativity, since binding of Ca^{2+} to the first site enhances the affinity of the second site for this ion. The effect of this phenomenon on the EF-hand pair is a greater sensitivity to an increased Ca^{2+} concentration when compared with that of an isolated, independent EF-hand, since it enables a small change in the Ca^{2+} concentration to control the physiological response (Figure 11A). Electrostatically, Ca^{2+} binding to an EF-hand pair would be expected to be an example of negative co-operativity, since the presence of the first cation not only neutralizes charged groups, but, owing to the close proximity of the binding sites, would repel the incoming Ca^{2+} . As positive co-operativity is observed, the favourable structural effects of the first Ca^{2+} binding must outweigh the unfavourable electrostatic factors.

Positive co-operativity is an affinity enhancement and can be defined in terms of free energy where $\Delta\Delta G_{\text{co-op}}$ reflects the

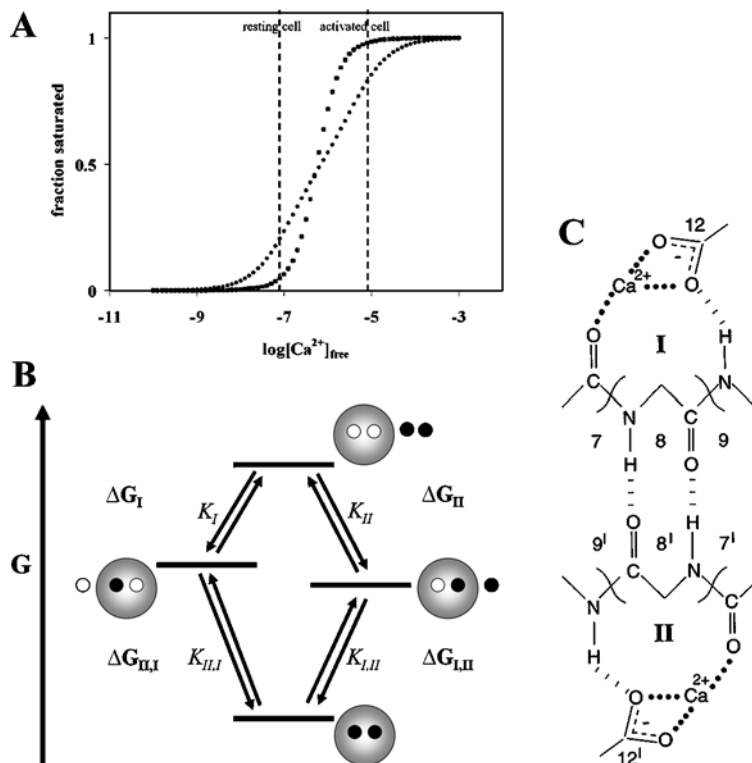


Figure 11 Positive co-operativity of Ca²⁺ binding

(A) Comparison curves for sequential (circles) and co-operative (squares) Ca²⁺ binding. The curves were created from the Ca²⁺ binding constants for CIB (sequential) [77] and the C-terminal domain of CaM (co-operative) [104]. Also indicated is the free Ca²⁺-concentration in the resting and activated cell. (B) The different pathways of Ca²⁺-binding to an EF-hand pair, with the corresponding ΔG and affinity constants specified. (C) The short β-sheet that links residues 7 and 8 of both EF-loops. Indicated are the hydrogen-bond and dipole interactions that are thought to enable the Ca²⁺-binding sites to communicate and enable positive co-operativity. (C) is taken from [27] and is reproduced with the approval of the authors.

difference in affinity of one site in the presence and absence of a Ca²⁺ ion in the other [104]:

$$\Delta\Delta G_{\text{co-op}} = \Delta G_{(\text{II,I})} - \Delta G_{(\text{II})} = \Delta G_{(\text{I,II})} - \Delta G_{(\text{I})} \quad (4)$$

Of course, as most proteins are asymmetrical $\Delta G_{(\text{II,I})} \neq \Delta G_{(\text{I,II})}$, $\Delta G_{(\text{I})} \neq \Delta G_{(\text{II})}$, and $\Delta G_{\text{I,II}} - \Delta G_{\text{II}} \neq \Delta G_{\text{II,I}} - \Delta G_{\text{I}}$ [81] (Figure 11B). The $\Delta\Delta G_{\text{co-op}}$ is related to the observed cation affinity through the microscopic binding constants in the following equation (K_{I} and K_{II} are the intrinsic affinity constants):

$$\Delta\Delta G_{\text{co-op}} = -RT \cdot \ln(K_{\text{II,I}}/K_{\text{II}}) = -RT \cdot \ln(K_{\text{I,II}}/K_{\text{I}}) \quad (5)$$

$\Delta\Delta G_{\text{co-op}}$ is the free energy of inter-site interactions, a coupling term that in many publications is simply defined as $\Delta\Delta G$. If the affinity for the second ion is greater than the first, this term is negative, indicating positive co-operativity; however, some degree of co-operativity could still take place if this value is greater than zero [6]. As $\Delta\Delta G_{\text{co-op}}$ is an energetic term, it is more useful than the traditional Hill coefficient for examining the effects of positive co-operativity on Ca²⁺ binding. For calbindin D_{9K} the free energy of co-operativity is -5 kJ/mol at moderate salt concentrations (0.15 M) [134], a value that results from many contributions and tends to be small because of the cancellation among large terms. For example, compensation for the electrostatic repulsion between the two Ca²⁺ ions implies that the sum of the favourable structural effects associated with ion binding is larger (more negative) than the net free energy of co-operativity [135].

The mechanisms of co-operativity are varied and hard to determine, since this requires characterization of the various metal-bound states along the different binding pathways – a requirement difficult to achieve owing to the co-operative nature of Ca²⁺ binding. However, the intermediate states of a number of EF-hand proteins have been characterized and conclusions have been drawn regarding the conformational effects and molecular mechanisms of positive co-operativity.

Conformational effects: changes in structure and dynamics

The intermediate states of calbindin D_{9K} and a tryptic fragment comprising the C-terminal domain of CaM (TR2C) have been captured through a series of techniques, including site-directed mutagenesis [13,28,136], binding experiments with a Ca²⁺ analogue (Cd²⁺) [67,137,138] and NMR spectroscopy, in which, by characterization of chemical exchange as a function of Ca²⁺ concentration, the weakly populated (Ca²⁺)₁ state of the wild-type protein was observed [29]. From these studies it appears that the conformational effects of co-operativity are two-fold: structural and dynamic. The structural effects are dominated by enthalpic factors, as for both calbindin D_{9K} and TR2C the binding of the first Ca²⁺ ion leads to the greatest reorganization of structure, be it changes in the protein core or preformation of the partner binding loop [29,67,137]. Upon binding of the second Ca²⁺ ion, backbone chemical shifts of residues involved in this binding are the only significant changes observed. For calbindin D_{9K}, the result of a singular Ca²⁺-binding event, no matter which binding

pathway is employed, is an intermediate state more similar to the $(Ca^{2+})_2$ than the apo form. For the C-terminal domain of CaM, the $(Ca^{2+})_1$ state does depend on the binding pathway, since, unlike the predominantly open conformation of $(Ca^{2+})_1$ TR2C with Ca^{2+} bound to EF4, the $(Ca^{2+})_1$ TR2C with Ca^{2+} in EF3 is in a closed conformation and not much communication between the EF-hands occurs [28,66,136]. As the TR2C intermediate states were captured through an E12Q mutation that significantly reduced the affinity for Ca^{2+} of the mutated EF-hand, the asymmetry in binding reflects, first, the importance of EF4's Glu12 residue for domain opening and, secondly, an intrinsic preference for binding Ca^{2+} to EF4 before EF3. Unlike in calbindin D_{9K} , where $K_I \approx K_{II}$, in CaM $K_{IV} > K_{III}$.

As would be expected, the dynamic effects of positive cooperativity are dominated by entropic factors [139]. NMR studies on both calbindin D_{9K} [81] and CaM TR2C [73] propose that changes in the flexibility of the EF-loops and internal motions of the domain core lead to positive co-operativity in binding. In the apo state, the domain structure of these proteins is quite dynamic, a situation that allows the sampling of a large number of conformations, most of which exhibit low Ca^{2+} affinities. Upon binding of the first Ca^{2+} ion, however, the backbone dynamics decrease to essentially the same level measured for the fully occupied conformation, since both the flexibility of the loops and the internal structural fluctuations decrease [15,31,135]. In effect, both EF-loops are trapped in the Ca^{2+} -occupied conformations. As the binding of the first Ca^{2+} ion pays both the structural costs of the reorganization and the entropic costs of the loss in flexibility and internal dynamics [30,81,140], the second ion binds with higher affinity, since these energetic deductions from its overall free energy of binding do not apply.

Such a mechanism, based significantly on changes in dynamics rather than structure, differs from the classical view of cooperativity in allosteric enzymes [141]. This mechanism of positive cooperativity has also been seen in peptide studies [142] and is suggested for *Nereis* SCP, which undergoes extensive and almost complete changes in structure upon binding the first Ca^{2+} ion [56].

Molecular mechanisms of co-operativity

To date, two molecular mechanisms have been identified that help to explain the observed positive co-operativity. The first involves the short antiparallel β -sheet formed between two EF-hands, a direct interaction that links residues 7 and 8 of the binding loops as well as the two Ca^{2+} ions (Figure 11C). It has been suggested that the two Ca^{2+} -binding sites communicate through changes in the hydrogen bonds that create this sheet [15]. For most EF-hand pairs, the two hydrogen bonds of the β -sheet are present in both the apo and Ca^{2+} -bound states. However, in the apo form these bonds display significant fluctuations, which contrasts with their rigidity in the Ca^{2+} -bound state. This increased stability is thought to be due to a strengthened internal electric field created by a cyclic network of two antiparallel electric 'chains' formed by the donor-acceptor interactions of the β -sheet hydrogen bonds, the metal ion in each site and the side-chain carboxylate group of both sites' Glu12 [27,143]. Following the co-ordination of one Ca^{2+} ion, the cation's positive charge increases the induced dipole of the chain's elements, stabilizing the chain and increasing the strength of the internal electric field. Owing to its antiparallel nature, the paired chain is also stabilized and the side chain of the apo site's Glu12 'pulled' into place so that it can now co-ordinate the second Ca^{2+} ion. In this way the binding of one ion stabilizes the proper conformation of at least two ligands of the other

EF-loop: the oxygen of the carbonyl group (from position 7) and one of the carboxy oxygen atoms of Glu12.

The second molecular mechanism of positive co-operativity entails interhelical contacts. The importance of these interactions was seen in studies of CaM and cTnC chimaeras, where this phenomenon was fully retained in domain swap mutants but significantly impaired in all variants in which the EF-hands were swapped [144]. The importance of interhelical contacts is also seen in the cross-domain communication and resulting positive co-operativity of Ca^{2+} binding to γ CaM (yeast CaM). Despite an unpaired EF-hand in the C-terminal domain, Ca^{2+} binds strongly and with positive co-operativity to all three EF-hands, owing to the close contacts with the helices of the high-affinity sites in the N-terminal domain [145]. The close contacts between the domains are due to the globular conformation of γ CaM, which differs markedly from the elongated vertebrate CaM in which the two domains are independent in the absence of a target protein. The notion that co-ordinated conformational changes between domains is facilitated by close interhelical contacts can also explain the positive co-operativity observed between all functional EF-hands of *Nereis* SCP which, again, include an unpaired EF-hand [146], as well as between the unpaired EF1 and EF3 of ALG-2 [24]. It has been proposed that the strength of interhelical interactions is one of the main determinants of co-operativity in the EF-hand proteins [3].

In both of these molecular mechanisms of positive co-operativity, the -Z ligand (Glu12) plays an essential role, since the chelation of the bound Ca^{2+} ion by this residue in one EF-hand leads to structural changes that are transduced to the second EF-hand. The importance of this residue, in particular the side chain's ability to act as a bidentate ligand, is readily seen in substitution mutagenesis studies, where its replacement with a glutamine residue is detrimental to both high-affinity and co-operative Ca^{2+} binding [147]. Interestingly, the fact that Mg^{2+} binding does not occur with positive co-operativity and does not involve both ligands of Glu12 adds weight to this argument [117].

Ca^{2+} binding without co-operativity

Even though an EF-hand pair's ability to bind Ca^{2+} through positive co-operativity is almost a central dogma of this field, there are actually several examples of EF-hand-containing proteins that do not appear to bind Ca^{2+} in this way. The binding sites in these proteins can be divided into two classes. The sites in the first class are classified as 'sequential independent', since no co-operativity is observed between them, and the Ca^{2+} concentration at which they become occupied is determined by their intrinsic affinity. The EF-hands of CaVP fall into this category. Ca^{2+} binding to this protein progressively brings structure to a domain that in the apo state is a molten globule; the binding of the first Ca^{2+} organizes only its EF-hand, since the rest of the sequence remains highly fluctuating [57,148]. The binding of the second cation is required to attain the standard paired EF-hand conformation. Interestingly, this lack of co-operativity between the binding sites may be due in part to the presence of an Asn12 instead of a Glu12 residue in the second EF-hand that prevents the critical movement of this side chain. The second example of sequential independent sites are those found in the C-terminal domain of the human protein CIB [77]. As co-operative and sequential binding are not mutually exclusive [149], the observed sequential binding here could be the result of the ~ 5 -fold difference in Ca^{2+} affinities of EF3 and EF4, and co-operativity between the binding events might still occur if the affinity of EF3 is increased as the result of EF4 being occupied. If the observed Ca^{2+} affinity of EF4 reflected the conformational costs of binding the first Ca^{2+} ion to the EF-hand

pair, the presence of a structure-inducing Mg^{2+} ion in EF3 should increase the affinity of EF4, since Ca^{2+} binding to this EF-hand no longer pays the energetic costs of conformational change. However, the influence of Mg^{2+} on the ability of these EF-hands to bind Ca^{2+} suggests that this is not the case, since EF4's affinity for Ca^{2+} is not affected by the presence of this other cation (A. P. Yamniuk, J. L. Gifford, S. Linse and H. J. Vogel, unpublished work). This same type of relationship between the EF-hands was also seen in GCAP-1 [127].

The second class of EF-hands that appear to bind Ca^{2+} without co-operativity are termed 'sequential co-operative sites'. In this class, seen in sTnC and recoverin, the sequential binding observed is structurally linked, since one site is required to be filled before the other. For the EF-hand pair in sTnC's N-terminal domain, the binding of Ca^{2+} to EF2 is required for EF1 to strongly bind Ca^{2+} [80]. Although the binding of each Ca^{2+} ion induces changes throughout the whole domain, these changes are only minor for the binding of the first Ca^{2+} , as this event 'sets the stage' for the major change in structure that accompanies binding of the second Ca^{2+} ion [78,151]. In addition to intrinsic differences in the binding ability and dynamics of the EF-loops (site II contains more negatively charged ligands and is more closely preformed than site I [79,82]), the ~ 10 -fold weaker Ca^{2+} -affinity of EF1 compared with EF2 likely reflects this EF-hand's role in facilitating the conformational change [80]. A similar phenomenon is observed for recoverin. Structural comparison of the apo and Ca^{2+} -loaded forms of this NCS protein revealed that the loop regions of both EF2 and EF3 are equally solvent accessible, thus making them equally attractive for Ca^{2+} ions. However, as the helices of EF2 in apo-recoverin help to house a myristoyl group, Ca^{2+} -binding to this EF-hand is energetically unfavourable [152]. As EF3 lacks this deterrent, Ca^{2+} binds favourably to this EF-hand, resulting in significant conformational changes in the protein. Through these changes the helices of EF2 are repositioned, enabling favourable Ca^{2+} binding to this EF-hand and the resulting extrusion of the buried myristoyl group [153]. In a way, and as with sTnC, this represents an extreme case of positive co-operativity, since the presence of Ca^{2+} in EF3 is required for Ca^{2+} to bind EF2 (a very low-affinity site now binds Ca^{2+} with a K_d of $\sim 10^{-5}$ M) [154]. Experimentally, positive co-operativity has been reported for the myristoylated form of this protein, but the Ca^{2+} -binding process to the unmyristoylated form has been classified as sequential [155]. Instead of reflecting differing consequences of Ca^{2+} binding, this inconsistency is likely the result of an increased Ca^{2+} affinity of EF3 in the absence of the acyl chain relative to that of EF2. As Ca^{2+} now binds this site ~ 10 -fold stronger than EF2 ($\sim 10^{-7}$ versus 10^{-6} M), the binding events appear to be sequential and are not the co-operative phenomenon that occurs when the two binding constants are of similar magnitude (both $\sim 10^{-5}$ M). This increase in affinity for EF3 likely reflects the loss of the energetic costs of exposing the myristoyl group due to the Ca^{2+} -induced conformational changes and, for the reasons mentioned above, this process should still be thought of as co-operative. A similar effect is also seen in the related proteins frequenin and neurocalcin [43].

Effects of protein–protein interactions

The fourth and final factor that affects the observed Ca^{2+} affinity of several EF-hands is the presence or absence of intermolecular interactions. For several EF-hand domains that are part of multidomain structures, the other domains are required for high-affinity Ca^{2+} binding. In isolation, the Ca^{2+} -specific EF-hand in the ELC does not bind Ca^{2+} , since this binding site requires a co-operative stabilizing interaction with both the RLC and the

heavy chain of the intact myosin hexamer [156]. For BM40, high-affinity Ca^{2+} -binding requires the paired EF-hand domain to be embedded in the larger EC domain [157]. In this manner BM40 contrasts with the small cytosolic EF-hand proteins in which the EF-hand pair itself is a stable unit capable of high-affinity Ca^{2+} binding.

An increase in Ca^{2+} -affinity is seen to result from the interaction between Ca^{2+} sensors and their targets. This effect has been well-documented through the use of peptide models to represent target-binding domains of the CaM subfamily members CaM [158–162], sTnC [125], calmodulin-like skin protein [163], the Ca^{2+} regulatory subunit of the phosphatase calcineurin, calcineurin B [164] and CaVP [57]. It is thought that there is a transfer of the free energy of target binding to that of Ca^{2+} binding, since the presence of different target peptides has been seen to increase the affinity of these proteins for Ca^{2+} by up to two orders of magnitude [165]. This free energy coupling ($\Delta\Delta G_{interact}$) provides a quantitative measure of the enhancement in Ca^{2+} affinity in the presence of the peptide, or, equivalently, the affinity enhancement for the peptide when Ca^{2+} is bound. In fact, $\Delta\Delta G_{interact}$ is high enough to restore the Ca^{2+} -binding abilities of E12Q loop mutants [166]. However, in these cases the affinity of target binding is weakened, which likely reflects the energetic cost of generating the appropriate Ca^{2+} -binding structure in the mutant loop. Interestingly, different target peptides affect the sensors differently, indicating another method whereby EF-hand proteins can tune their Ca^{2+} -affinity.

A phenomenon that seems to be the result of intermolecular interactions, the increase in Ca^{2+} affinity is probably due to three consequences of target binding. First, the amphipathic nature of the peptides allows them to shield the exposed hydrophobic patches that result from Ca^{2+} -binding sensor proteins such as CaM [167]. As the exposure of these patches is entropically unfavourable and takes away from the energy of Ca^{2+} -binding (Figure 9B), the fact that the presence of the peptide covers them causes the Ca^{2+} affinity to increase. Secondly, owing to intermolecular interactions, there is a decrease in the Ca^{2+} dissociation rate, which leads to an increase in affinity [158]. This decrease in the dissociation rate may reflect a steric hindrance to Ca^{2+} dissociation and functionally serve to extend the lifetime of the activated protein complex long after the Ca^{2+} transient has subsided. The third mechanism whereby target binding increases Ca^{2+} affinity is through stabilization of the Ca^{2+} -bound state. As mentioned above, in the absence of the target, even with Ca^{2+} bound, there is conformational exchange between open and closed structures. However, the presence of the target shifts this equilibrium towards the open conformation, i.e. the closed conformation is now more rarely sampled, if ever [165]. This stabilization of the Ca^{2+} -bound state itself would lead to a marked decrease in the Ca^{2+} dissociation rate.

The presence of the protein target also has effects on the positive co-operativity. For the interaction between CaVP and CaVPT [168], myristoylated recoverin and rhodopsin kinase [169], and CaM with mastoparan or caldesmon [170], the presence of the target increases the observed co-operativity. That this occurs is not surprising, since, for CaM especially, the presence of the peptide decreases the entropic costs of subsequent Ca^{2+} binding as, once bound to the C-terminal domain, it will cover any exposed hydrophobic surfaces in the N-terminal domain that result from Ca^{2+} binding here, thus increasing the observed affinity. In addition, the more compact structure that forms as CaM collapses around the target enables communication between the helices of the two lobes (perhaps as in yCaM) [171].

This effect of target binding on the observed Ca^{2+} affinity has significant functional consequences. The first is that, for CaBP–target interactions that are constitutively bound together in the

cell such as CaVP and its target [57], the unbound Ca^{2+} affinities of these proteins are not physiologically relevant. The second is that, because of this increase in Ca^{2+} affinity, there is a decrease in the effect of Mg^{2+} competition.

There is evidence, however, that the extent of the *in vitro* effect of the target peptide on Ca^{2+} affinity may not be quite as large *in vivo*. For the interaction between skMLCK (skeletal-muscle myosin light-chain kinase) and CaM, the peptide enhances affinity 220-fold, whereas the intact protein only enhances affinity 13-fold. These affinities result in only partial Ca^{2+} occupancy of the CaM-skMLCK complex at resting cellular Ca^{2+} levels, a state required for effective Ca^{2+} -CaM-mediated activation *in vivo*. By contrast, the much higher affinities observed for the skMLCK-peptide complex would predict significant Ca^{2+} occupancy and activation, even in the absence of a Ca^{2+} signal. This difference in effect likely stems, at least in part, from the energetic cost of removing an autoinhibitory domain from the intact protein, an effect also seen for the full-length Ca^{2+} -ATPase molecule [165].

Finally, target proteins can have a negative effect on the observed Ca^{2+} affinity. In the complex of the Ca^{2+} -activated K^{+} -channel bound to Ca-CaM, Ca^{2+} binds only to the N-terminal domain EF-hands, since the higher-affinity sites in the C-terminal domain have their affinities reduced so that Ca^{2+} can no longer bind. In the structure formed upon target interactions, the EF-hands in the C-terminal domain are found in the apo state, since a severe disruption in the Ca^{2+} -co-ordination spheres of this domain causes Glu12 of both EF-loops to be removed from their Ca^{2+} -binding positions [172]. This prevents Ca^{2+} from binding to these EF-hands. The evolutionary pressures of target-protein interaction may also be responsible for the different combinations of functional EF-hands in subfamilies such as the NCS subfamily, as adaptation to different target proteins leads to the inactivation of some EF-hands [173].

In this final part of this review we have tried to link thermodynamic studies on Ca^{2+} affinity to the molecular structures talked about in the above two sections. This final part has also stressed the importance of the many contributing factors to general EF-hand affinity. The Ca^{2+} -binding abilities of each specific EF-hand is determined by an individual combination of these factors. The consequence of this is that it is nearly impossible from the primary sequence alone to predict the Ca^{2+} affinity of a given EF-hand containing protein.

CONCLUDING REMARKS

An extensive amount of research has been devoted to understanding the structure, function and Ca^{2+} -binding abilities of the 'EF-handome', the group of helix-loop-helix EF-hand proteins. Amazingly, most of this has been accomplished by independent curiosity-driven research groups, which contrasts with the current trend of large research consortia dominating the fields of structural and functional genomics. Despite this, several areas have yet to be fully explored, two of which are relevant to the topics of the present review. The first pertains to the Ca^{2+} -binding abilities of the proteins of this superfamily. As most research in this field to date has focused on the model members CaM, TnC and calbindin $\text{D}_{9\text{K}}$, relatively little is known about the mechanisms of co-operativity or dynamic conformational effects of Ca^{2+} -binding of the other EF-hand proteins. The second area is that of the effect of the target proteins on an EF-hand's ability to bind Ca^{2+} , an effect that can have significant implications for the saturation state of the EF-hand protein in the resting cell. As the effects of intact target interactions are only known for some complexes of CaM, this topic should be examined for other members of this family,

so we gain a deeper appreciation of the role of Ca^{2+} activation in the living cell.

We thank Dr Aaron Yamniuk and Mr Leonard Nguyen (both of the Department of Biological Sciences of this University) for their insightful discussions, as well as Dr Simon Megy (also of the Department of Biological Sciences of this University) for his help with the preparation of the Figures. Research in the authors' laboratories in this area is generously supported through operating grants from the Canadian Institutes of Health Research. J. L. G. is supported by a Studentship from the Natural Sciences and Engineering Research Council of Canada and the Alberta Ingenuity Fund. The Alberta Heritage Foundation for Medical Research provides Scientist Awards to M. P. W. and H. J. V. M. P. W. is recipient of a Canada Research Chair (Tier 1) in Vascular Smooth Muscle Research.

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Received 19 February 2007/24 April 2007; accepted 25 April 2007

Published on the Internet 27 June 2007, doi:10.1042/BJ20070255