Diversity of Conformational States and Changes Within the EF-Hand Protein Superfamily

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ABSTRACT The EF-hand motif, which assumes a helix-loop-helix structure normally responsible for Ca2+ binding, is found in a large number of functionally diverse Ca2+ binding proteins collectively known as the EF-hand protein superfamily. In many superfamily members, Ca2+ binding induces a conformational change in the EF-hand motif, leading to the activation or inactivation of target proteins. In calmodulin and troponin C, this is described as a change from the closed conformational state in the absence of Ca2+ to the open conformational state in its presence. It is now clear from structures of other EF-hand proteins that this “closed-to-open” conformational transition is not the sole model for EF-hand protein structural response to Ca2+. More complex modes of conformational change are observed in EF-hand proteins that interact with a covalently attached acyl group (e.g., recoverin) and in those that dimerize (e.g., S100B, calpain). In fact, EF-hand proteins display a multitude of unique conformational states, together constituting a conformational continuum. Using a quantitative 3D approach termed vector geometry mapping (VGM), we discuss this tertiary structural diversity of EF-hand proteins and its correlation with target recognition. Proteins 1999;37:499–507.

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Key words: calcium signaling; EF-hand protein; conformational change

INTRODUCTION

Over 200 proteins serving as both Ca2+ sensors and buffers in eukaryotes are known to contain one or more so-called EF-hand motifs.1 First discovered in the crystal structure of parvalbumin,2 the EF-hand motif is a helix-loop-helix structure with a Ca2+ ion bound to the interhelical loop region. Upon Ca2+ binding, the sensor proteins undergo conformational change and in turn regulate a vast number of target proteins; calmodulin (CaM) alone regulates many intracellular targets such as CaM kinases, myosin light chain kinases and calcineurin (reviewed in Crivici and Ikura3), and NMDA receptor.4 Other EF-hand proteins are more specialized. Troponin C (TnC) serves as a Ca2+ sensor in muscle cells,5 Recoverin, expressed solely in retinal rod cells, acts as a Ca2+ sensor in vision.6 Guanylate cyclase activating proteins (GCAPs) specifically activate retinal guanylate cyclases only at low Ca2+ levels (> 100 nM).7 Some of these EF-hand proteins, such as S100B and recoverin, have been implicated in a number of neurological diseases including Alzheimer’s disease, Down’s syndrome, epilepsy and retinal degradation.8–10

Members of the EF-hand superfamily employ the same helix-loop-helix motif to carry out their diverse biological functions. This motif has been quantitatively characterized in the past by one angle between the two helices.11–13 In this review we compare the structures of 90 EF-hand motifs in 31 proteins by a newly developed method which enables us to define the conformational variation of EF-hand motifs in a more precise manner. Our analysis provides a novel way of characterizing an EF-hand (or any other two-helix structural motif), highlighting trends in conformational changes and uncovering subtle differences between EF-hand motifs that were previously considered similar. This review intends to provide a better understanding of how homologous proteins use different structural characteristics for their specific roles in biological functions.

VECTOR GEOMETRY MAPPING ANALYSIS

The interhelical angle between the two helices has been widely used to describe the conformational state of an EF-hand motif. However, the information provided by this angle alone is insufficient to describe the complete movement of one helix with respect to the other. The Vector Geometry Mapping (VGM) method14 directly compares the position of the exiting (sequentially second) helix of an EF-hand with respect to its entering (sequentially first) helix by orienting the two helices in a coordinate system.

Abbreviations: CaM, calmodulin; TnC, troponin C; VGM, vector geometry mapping; RMSD, root mean square deviation; CNA, calcineurin A; CNB, calcineurin B; ELC, myosin essential light chain; RLC, myosin regulatory light chain; GCAP, guanylate cyclase activating protein; EH domain, Eps15 homology domain; PLC, phospholipase C; skMLCK/smMLCK, skeletal/smooth muscle myosin light chain kinase; CaMKII, Ca2+–CaM dependent protein kinase II; NPF motif, Asn-Pro-Pro-Phe motif.

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common to all studied EF-hands (Fig. 1). The coordinate system is arbitrarily defined; in this study, the first EF-hand in sequence of Ca2+-free calmodulin (apo-CaM EF1) is used as a reference. The two helices of apo-CaM EF1 are represented by vectors, with the endpoints determined by averaging the coordinates of the first or last eleven backbone N, Cα and C' atoms on either end of the helix. The +z axis is defined along the vector representing the entering helix of apo-CaM EF1 and the +x axis is defined by the N-terminal end of its exiting helix vector. The intersection of the x and z axes defines the origin. The entering helices of the other EF-hands studied are then superimposed on that of apo-CaM EF1 (root mean square deviation, RMSD = 0.55 ± 0.48 Å). Superposition of multiple NMR structures also yields similar values (0.68 ± 0.52 Å). In principle the basis for superposition could be set to either the entering or exiting helix, although the use of the latter was less successful (3.93 ± 4.66 Å), mainly owing to partial disorder of the exiting helix in some EF-hands (e.g., CaM EF4, myosin regulatory light chain EF4).

Three angles quantify the exiting helix vector’s geometric position relative to the entering helix. θ is the angle between the entering and exiting helices and is 180° less the interhelical angle previously described.15 α, the horizontal plane angle, is measured between the +x axis and the xy projection of the exiting helix vector, counterclockwise about the +z axis. ω is the counterclockwise angle of rotation about the exiting helix vector axis and is a descriptive measure of conformational change. The position of the exiting helix is further described by the coordinate of its N-terminal end. Because all vectors are defined in the same coordinate system, the VGM program (available at http://diana.oci.utoronto.ca/ikura/datasoft.html) can also provide other geometry-based parameters such as helix lengths and interhelical distances.

Members of the EF-hand superfamily display great variability in their structures,16,17 even among homologs of one subfamily (e.g., skeletal and cardiac muscle TnC18). This diversity appears as a non-discrete range of conformations, as illustrated by the VGM approach (Figs. 2 and 3). In most EF-hands, the exiting helix points (N to C termini) in the same general direction in the horizontal plane as those of CaM and TnC in the Ca2+-free and Ca2+-loaded states (Fig. 2). The interhelical distance between inner endpoints (the C-terminal end of the entering helix and the N-terminal end of the exiting helix) is 11.8 ± 1.6 Å (Fig. 3). The average (x,y,z) coordinate of the exiting helix’s N-terminal end is (8.6 ± 3.5, 1.0 ± 4.2, -1.9 ± 2.5) (Fig. 3), compared to (10.9, 0, 0) for the reference EF-hand. The conformational space is defined by ranges of the interhelical angle θ (89% within the range 30° < θ < 90°) and horizontal plane angle ω (80% within the range 80° < ω < 140°). The propensity of most of the EF-hands to assume this geometric arrangement is governed by the sequence of the interhelical Ca2+ binding loop, and the packing of side chains within and between EF-hands.

Twenty-five EF-hands from nine proteins (for which both the apo and Ca2+-bound forms are available) showed variable magnitudes of ∆θ (−8° < ∆θ < 60°), ∆ω (−66° < ∆ω < 19°) and ∆Dv (−66° < ∆Dv < 18°). Many of the EF-hands studied had negative ∆ω values (a decrease in ω), indicating that upon Ca2+ binding, the exiting helices of these EF-hands undergo a clockwise change in orientation with respect to the entering helix. Evidently there are some trends in Ca2+-induced conformational change; below we consider several well-characterized examples that shed light on the diversity in conformation and function.

**OPEN, CLOSED AND SEMI-OPEN CONFORMATIONS**

EF-hands occur in interacting pairs to form stable structural domains and typically undergo a conformational change upon binding Ca2+ (Fig. 4). As first proposed for TnC19 and later for CaM,15,19,20 these domains exhibit a “closed conformation” (in which the helices of the EF-hand are closer to anti-parallel) in the Ca2+ free state, and an “open conformation” (in which the helices are more perpendicular) when bound to Ca2+ (Fig. 2a). The large conformational change is characterized by a large opening of the helices (26° < ∆θ < 60°) and a swing clockwise about the entering helix (−34° < ∆ω < 0°). As a result, two large hydrophobic surfaces are exposed for target interaction. The exiting helices of all EF-hands but CaM EF2 also undergo large clockwise twist motion (−59° < ∆Dv < −40°) for CaM, −2° for EF2; −35° and −12° for TnC). In addition, calcineurin B (CNB), a CaM subfamily member which associates with calcineurin A (CNA) to form protein phosphatase calcineurin, displays the open conformation.
Fig. 2. VGM plots for EF-hand proteins. Axes are as defined in Figure 1. Entering helix is shown in white; except where noted, exiting helices are green for Ca$^{2+}$-free structures, cyan for Ca$^{2+}$-bound structures. Numbering of EF-hands is in order as they occur in sequence. a: CaM (PDB codes 1DMO, 3CLN) and TnC (5TNC, 1AVS). Vectors representing the closed conformation are in red and orange (CaM and TnC respectively), open conformation in dark blue and light blue. b: Ca$^{2+}$-bound CNB (1TCO). c: Myosin, regulatory domain (1WDC). ELC is in light gray and pink (N- and C-terminal domains), RLC in dark gray and pink. d: Myristoylated recoverin (1IKU, 1JSA). e: Calbindin D$_{28}$ (obtained from W.J. Chazin), calcytin (refined structures obtained from W.J. Chazin) and S100B (refined apo structure and 1QLK obtained from D.J. Weber). Apo and Ca$^{2+}$-bound EF2 of calcytin and S100B are labeled as C2 and S2 respectively. f: Calpain domain VI (1AJ5, 1DVI; obtained from M. Cygler). g: Spectrin (obtained from A. Pastore). h: Ca$^{2+}$-bound oncomodulin (cyan, 1RRO) and parvalbumin (blue, 3PAT). i: Ca$^{2+}$-bound BM-40 (1BM0). j: Ca$^{2+}$-bound sarcoplasmic calcium binding protein (2SCP). k: Ca$^{2+}$-bound EH domain (1EH2; obtained from M. Overduin). l: Ca$^{2+}$-free phospholipase C-$\delta$ 1 (21SD). Figures 2–6 were created using Molscript.
in the Ca\textsuperscript{2+}-bound state (Fig. 2b). Details of a Ca\textsuperscript{2+}-induced conformational change for CNB are unknown, since the Ca\textsuperscript{2+}-free structure has not yet been determined.

A third conformational state has been identified in essential and regulatory light chains (ELC and RLC) of myosin.\textsuperscript{21} The C-terminal domain EF-hands (EF3 and EF4) assume a unique semi-open conformation, in which the two helices of each EF-hand appear closer together than in the open conformation, but further apart than in the closed conformation. Interaction between the light chains and the heavy chain of myosin serve to stabilize the conformation. The EF-hands of the semi-open domains (which do not bind Ca\textsuperscript{2+}) show much less structural congruence in comparison to the EF-hands of the closed and open conformations, implying a kind of "micro"-diversity within the semi-open conformation (Fig. 2c). The exiting helices of ELC EF3 and EF4 and RLC EF3 point in very different directions, but are all positioned halfway between those of the closed and open EF-hands. Conversely, RLC EF4 appears better aligned with the group of open EF-hands. Quantitatively, the C-terminal EF-hands of the light chains are represented by a large range of \(\phi\), which encompasses the \(\phi\) ranges of the closed and open EF-hands, while the range for \(\theta\) overlaps that for the open EF-hands (data not shown). Indicatively, the N-terminal domain (EF1 and EF2) of the RLC is considered to be of open conformation and that of the ELC is considered closed,\textsuperscript{21,22} which is supported by VGM analysis. However, ELC EF2 is unusual in spatial orientation when compared to CaM and TnC's closed conformation, and also when compared to other EF-hand conformations. This is reflected in the lowest \(\phi\) value of all hands (\(-161^\circ\)). ELC EF2 is the only EF-hand of the light chains that makes no contact with the heavy chain and consequently, of the four light chain domains, the N-terminal domain of the ELC is the most weakly anchored to the heavy chain.\textsuperscript{22}

**EF-HAND-MYRISTOYL INTERACTION**

In addition to Ca\textsuperscript{2+}-dependent protein-protein interactions, the EF-hand appears to recognize non-peptide enti-
or other acyl group at the N-terminus.23 (More recently, it was reported that CaM also binds an N-terminal myristoylated portion of CAP-22/NAP-22.) In Ca\(^{2+}\)-free recoverin, the covalently attached myristoyl group is sequestered in a hydrophobic cavity formed by the helices of EF1 and EF2 and the entering helix of EF3 (not shown).

Fig. 5. N-terminal domain of recoverin (1IKU). In the Ca\(^{2+}\)-free state, the myristoyl group (shown as a space-filling model) is sequestered in a hydrophobic pocket formed by EF1, EF2 and the entering helix of EF3 (not shown).

Proteins of the S100 subfamily contain two EF-hands of varying Ca\(^{2+}\) binding affinity and form homodimers or heterodimers with other S100 proteins (Fig. 6a), with the exception of calbindin D\(_{9k}\). Ca\(^{2+}\)-sensor proteins calcyclin,\(^{32–34}\) and S100B\(^{35–40}\) are dimeric in both the presence and absence of Ca\(^{2+}\). Psoriasin is a dimer in the Ca\(^{2+}\)-bound form, although it may only bind Ca\(^{2+}\) at EF2 (Brodersen et al.\(^{41}\)). p11 is also a dimer, but cannot bind Ca\(^{2+}\) due to mutations in the Ca\(^{2+}\)-binding loops of the EF-hands.\(^{42}\) In all cases, the dimeric interface involves the entering helix of EF1 and the exiting helix of EF2. This interface varies from \(\approx 2,400\) Å\(^{2}\) for Ca\(^{2+}\)-bound human S100B and psoriasin, to \(\approx 3,200\) Å\(^{2}\) for apo calcyclin,\(^{43}\) and the S100 dimer is probably a tight association (\(K_d < 10^{-9}\) M).\(^{44}\)

The EF-hand exiting helices of the dimeric S100 proteins are represented by vectors in the same region of the plot (\(80^\circ < \phi < 110^\circ, 50^\circ < \theta < 80^\circ\)) (Fig. 2e). The most notable exceptions are the exiting helices of apo-calcyclin EF2 and apo-S100B EF2 which are sloped in an opposite direction (Fig. 3). Consequently the \(\phi\) values are negative (\(-53.1^\circ\) and \(-117.8^\circ\) for rabbit calcyclin and rat S100B respectively) and the \(\theta\) values more accurately represent the angle formed by the other helical endpoints (i.e., the N-terminal end of the entering helix and the C-terminal end of the exiting helix). Upon binding Ca\(^{2+}\), these EF-hands are reoriented (\(\Delta \phi \approx 200^\circ\)) into the aforementioned common region, undergoing the largest conformational change observed among all EF-hands studied.

With the exiting helix of EF2 fully involved in the dimer interface, the conformational change observed in EF2 of both calcyclin and rat S100B are in fact due to Ca\(^{2+}\)-induced movement of the entering helix with respect to the rest of the molecule.\(^{48}\) This results in an exposure of a relatively large hydrophobic surface which is likely required for target interaction. The recently determined structures of p11 and its complex with an annexin II peptide have shown that without binding Ca\(^{2+}\), this protein may mimic a Ca\(^{2+}\)-bound S100 protein structure that is “complex-ready”.\(^{42}\)

Calbindin D\(_{9k}\) is the most evolutionarily distant member of the S100 subfamily and is the only known monomeric
S100 protein. This may be because the two helices essential for dimer formation in S100 proteins are considerably shorter. Calbindin D\(_{9k}\) undergoes very little conformational change upon Ca\(^{2+}\) binding (\(\Delta\phi = 1.8^\circ\) and 16.0\(^\circ\), \(\Delta\theta = -8.4^\circ\) and \(-7.0^\circ\) for EF1 and EF2, respectively), consistent with its buffering function. However, a similarity in locality of the largest structural change (the entering helix of EF2) may indicate that calbindin D\(_{9k}\) has evolved from an ancestral sensor protein.

The small subunit domain VI of calpain is unique, containing five EF-hands, the fifth of which is essential for dimer formation (Fig. 6b) and Ca\(^{2+}\) activation of calpain.\(^{57,48}\) The dimer interaction surface is much more extensive (4,269 Å\(^2\)) in comparison to the S100 proteins, and the dissociation constant likely much smaller (\(K_d \sim 10^{-12}\) to \(10^{-15}\) M for the calpain subunit heterodimer),\(^{49}\) implying that the calpain dimer is more stable than the S100 dimer. EF1, EF2, and EF3 bind Ca\(^{2+}\), but little conformational change occurs (Fig. 2f). The exiting helices of EF2, EF3, and EF5 are positioned in the same cluster in both Ca\(^{2+}\)-free and bound states. The position of EF4 is unusual (Fig. 3), as evident in low \(\epsilon\) values (15\(^\circ\) and 22\(^\circ\) for Ca\(^{2+}\)-free and bound, respectively), and in part allows for stabilizing interaction between two pairs of EF-hands (EF1-EF2 and EF3-EF4).\(^{47-48}\) The position of EF1 may result from an atypically long linker between EF1 and EF2, and its relatively larger conformational change appears in concert with a movement of the EF1-EF2 linker closer to EF3 and EF4 (Blanchard et al.\(^{47}\)).

It has been recently shown that neurocalcin, a member of the recoverin subfamily, dimerizes both in crystal and in solution\(^{50}\) (Fig. 6c). The dimeric interface involves only four residues of EF3, plus two residues from the linker between the two domains and one residue from the N-terminal helix. Dimerization is likely induced by Ca\(^{2+}\) binding (JBA, unpublished). The hydrophobic surface of dimer interaction (2,309 Å\(^2\)) is comparable to that of the S100 proteins. Given neurocalcin’s sequence similarity to GCAP, it has been proposed that GCAP may also be dimeric.\(^{28}\)

Clearly, proteins from different subfamilies use different mechanisms for dimerization. Although there is still much to be revealed about these proteins and their siblings, this diversity in dimerization is likely to enable EF-hand proteins to effectively regulate multiple target proteins.

A CONFORMATIONAL CONTINUUM

As discussed thus far, there are several structural means by which an EF-hand protein can operate. With each new structure added to the ever-growing superfamily, it becomes more apparent that their structures and Ca\(^{2+}\)-induced conformational changes are as diverse as their functions.\(^{11}\)

This “continuum” of conformational states is exemplified by several EF-hand-containing proteins that are the sole structure-solved members of their subfamilies, or are larger proteins containing other non-EF hand domains. One of the latter is spectrin, whose subunit \(\alpha\) contains two Ca\(^{2+}\) binding EF-hands that undergo moderate Ca\(^{2+}\)-induced conformational change\(^{51}\) (Fig. 2g). The movement of EF1’s exiting helix away from the entering helix is marked by a significant decrease in \(\phi\) (\(\Delta\phi = -46^\circ\)), but little change in interhelical angle (\(\Delta\theta = -1.5^\circ\)). EF2 undergoes a large conformational change that may initiate Ca\(^{2+}\) binding to EF1 (Lundberg et al.\(^{50}\)). It is interesting to note that \(\alpha\)-spectrin forms a heterodimer with \(\beta\)-spectrin, but the dimerization does not require the EF-hands of the \(\alpha\) subunit.\(^{53}\)
EF-HAND CONFORMATIONAL DIVERSITY

The recently determined structure of Cbl, a regulator of T-cell receptor signalling, contains a pair of EF-hands in its N-terminal domain, the second of which binds calcium.54 The exiting helix of the first EF-hand is sloped in an opposite direction (α = −80.9°), much like that of apo-calcyclin or apo-S100B EF2. Although the helices of the second EF-hand are positioned similarly to those of Ca2+-bound Calcyclin or apo-S100B EF2, although the helices of EF2 are almost parallel to the entering helix (4° for both) (Fig. 2h). Such “closed” helices were not observed in other proteins. The helices of EF2 and EF3, however, are almost as far apart as in the open conformation. Similarly, the Ca2+-bound EF-hand’s exiting helices of BM-40 (Fig. 2i) and of the C-terminal domain of sarcoplasmic calcium binding protein (SCP) (Fig. 2j) are spatially in the same region as the open conformation of Ca2+-bound CaM and Ca2+-bound TnC. The EF-hand’s exiting helices of N-terminal domain SCP point sharply away from the entering helices, as evidenced by the highest 0 values observed (121° and 122° respectively). This may be due to movement of the helices to avoid an energetically unfavorable positioning within the domain, since EF2 does not bind Ca2+ (Vijay-Kumar and Cook55). The Eph15 homology (EH) domain is found in some twenty proteins, and consists of a pair of EF-hands. In the second EH domain of the Eph15 protein, only the second EF-hand binds Ca2+ (de Beer et al.56). In the Ca2+-loaded state, the helices of both EF-hands are close together, much like the closed conformation (α = 58° and 40°, respectively) (Fig. 2k), although EF2 points further away from its entering helix. Ca2+-free phospholipase C-51 (PLC-51), a multi-domain protein, contains four EF-hands (the first of which contains a disordered polypeptide) that do not display a similar conformation like the four EF-hands of apo-CaM (Fig. 2l). However, structurally equivalent residues in PLC-51 to those involved in a CaM-peptide complex interact with the C-terminal, C2 domain of PLC-51 to form a smaller interface.57

TARGET INTERACTIONS

The Ca2+-induced conformational change described above enables calcium sensor proteins to interact with target proteins in a Ca2+-dependent manner, thereby activating or inhibiting the activity of the targets. From the structures of the EF-hand protein-target complexes that have been solved to date, it appears that hydrophobic residues on the surface play a key role in interaction with the target protein. In Ca2+-loaded CaM and TnC, each of the N- and C-terminal domains augments an exposed hydrophobic surface. In Ca2+-loaded recoverin, many of the exposed hydrophobic residues in the N-terminal domain (which are buried to form the myristoyl binding pocket in the Ca2+-free state) are homologous to those involved in CaM-target binding,26 while the C-terminal domain is prevented from target binding by the K-helix. In both myosin light chain-heavy chain interactions, the overall architecture is similar to that of the CaM-target interaction, despite the ELR and RLC formation of the “semi-open” conformation.21 In dimeric proteins, a target-interacting surface might reside on each monomer, or it may be cooperatively created by the two monomers. A unique mechanism of target binding has been proposed for the EH domain, in which the target is believed to interact with a small hydrophobic patch on the surface of the EF-hand protein.56

Affinities with which these proteins bind their targets vary greatly in accordance with the structural properties of the interaction (Table I). Clearly, a complex with a larger buried surface shared between the protein and its target is more tightly bound. CNA (the catalytic domain) and CNB

<table>
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<td>-</td>
<td>10⁻⁴</td>
<td>de Beer et al.56</td>
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1*Calculated using software described in Jones and Thornton.43
2The domains of the light chains that interact with the heavy chain do not bind Ca2+.3M. Rebe, personal communication.
4p11 does not bind Ca4."
or within the EF-hand. Diversity is also evident in Ca\textsuperscript{2+} the numerous inter- and intra-residue contacts formed by the entering helix. This propensity may be in part due to rather narrow range of positions on the plane horizontal to form an acute angle with the entering helix, and favors a thereby large interacting surfaces (3,594–3,714 Å\textsuperscript{2}). Other Ca\textsuperscript{2+}-free protein complexes bind with less affinity and, as might be postulated for lack of solved structures, with a smaller interface.

The conformational state of these proteins greatly influences, if not determines, their target binding affinity. Further structural and thermodynamic studies may eventually allow for the determination of a quantitative relationship between conformation, binding affinity, and specificity for target proteins.

**CONCLUSIONS**

EF-hand superfamily members clearly exhibit considerable diversity in conformation that appears to be continuous rather than discrete. This is not surprising since it has previously been suggested that proteins in all classes of protein structure exhibit a “continuum of fold motifs”. Despite the conformational diversity in EF-hand proteins, there are clear propensities toward certain helix orientations. In particular, the exiting helix of the EF-hand tends to form an acute angle with the entering helix, and favors a rather narrow range of positions on the plane horizontal to the entering helix. This propensity may be in part due to the numerous inter- and intra-residue contacts formed by or within the EF-hand. Diversity is also evident in Ca\textsuperscript{2+}-induced conformational change displayed by EF-hand proteins. CaM and TnC exhibit the largest changes, calpain and calbindin D\textsubscript{9k} the smallest, and all others displaying conformational changes of varying magnitude both among the EF-hands in the molecule, and among EF-hands of other proteins. Although limited structural information on protein-target complexes is available, there is appreciable correlation between EF-hand conformation and target binding affinity, with multiple domains providing larger binding interfaces and thereby favoring stronger target binding. In light of the conformational diversity evident in known protein structures, EF-hand superfamily members appear to use a wide variety of molecular surfaces to interact—usually in a Ca\textsuperscript{2+}-dependent manner—within various proteins, fatty acid alkyl chains and even with DNA. Clearly, the movement of the “thumb” and “index finger” helices within EF-hands can be fine-tuned in order to accomplish various functions.

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**NOTE ADDED IN PROOF**


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