Review Letter–Hypothesis

Primary sequence analysis and folding behavior of EF hands in relation to the mechanism of action of troponin C and calmodulin

Jean Gariépy and Robert S. Hodges

Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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The primary sequence of EF hands encodes for elements of secondary structure which includes the presence of hydrophobic and charged domains in the helical regions of these sites. The hydrophobic and charged surfaces located in the N-terminal region of EF hands offer a potential site of interaction with complimentary surfaces on target proteins. Although the binding of calcium to the EF hands of calmodulin and troponin C may lead to a local exposure of these domains, it is the tertiary structure of these proteins that probably dictates the extent to which these domains are exposed and the selectivity of these proteins for target proteins.

Calcium-induced folding *Calmodulin* *Sequence analysis* *Troponin C*

1. INTRODUCTION

An EF hand can be described as a linear sequence of 30–35 amino acids, where the N- and C-terminal α-helical regions are flanking a 12-residue calcium binding loop [1]. To date, at least 6 families of EF hand containing proteins have been established: parvalbumins, troponin C's, calmodulins, myosin light chains, intestinal calcium binding proteins and the brain specific S-100 proteins. The secondary structure analysis of various EF hand containing proteins has indicated that the C-terminal α-helical region is initiated in the loop region and that a β-turn region separates both helical segments [2]. The β-turn probability is particularly high for the first 4 residues ($+X, +Y$ region) of the loop [3]. These results agree with tl folding pattern of EF hands as observed in tl crystal structures of carp parvalbumin [1] ar bovine ICBP [4]. In the case of calmodulin ar troponin C, their ability to bind calcium associated with their role as modulator protein Calcium binding to their EF hand sites induc changes in their secondary and tertiary structur These changes in turn, result in the formation ar exposure of hydrophobic surfaces that are in clo proximity or represent binding regions to targ proteins such as troponin I and phosphodiesteras This article examines the possible features of E hands that could describe the mechanism of actic of these calcium binding proteins.

Abbreviations: EF hand, second calcium binding site of carp parvalbumin for which the crystal structure is known; ICBP, intestinal calcium binding protein; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide

2. GENERATION OF AN AVERAGE EF HAND SEQUENCE

The primary sequence of 30 different EF site exemplifying the 6 families of EF hand containi

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proteins, were aligned to trace common features to all EF sites (fig.1). Note that all sequences listed represent EF hand sites that bind calcium including 4 sequences containing double amino acid insertion sites [5–10]. A general sequence pattern emerges from our analysis when one tabulates the type and frequency of amino acid at each position of the EF hand (table 1). Note that the amino acids were grouped by order of polarity [11]. We did not include in our tabulation, the inserted residues present in the S-100 and ICBP sequences. The average amino acid sequence deduced from this sequence analysis is listed in fig.2A.

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                 ++++Loop+++++
++N-Helix++++                  ++C-Helix+++

                 X  Y  Z-Y -X -Z
IAEFKAAFDMF* DADGGGD*ISVKE LGTVMRML I   RSTnC
KEELDAIIEEV* DEDGSGT*IDFEE FLVMMVRQ II  RSTnC
EEELAECFRIF* DRNADGY*IDAEE LAEIFRAS III RSTnC
DEEIESLMKDG* DKNNDGR*IDFDE FLKMMEGV IV  RSTnC
PEELQEMIDEV* DEDGSGT*VDFDE FLVMMVRC II  BCTnC
EEELSDLFRMF* DKNADGY*IDLEE LKIMLQAT III BCTnC
EDDIEELMKDG* DKNNDGR*IDYDE FLEFMKGV IV  BCTnC
IAEFKEAFSLF* DKDGDGT*ITTKE LGTVMRSL I   BBCalm
EAELQDMINEV* DADGNGT*IDFPE FLTMMARK II  BBCalm
EEEIREAFRVF* DKDGNGY*ISAAE LRHVMTNL III BBCalm
DEEVDEMIREA* NIDGDGQ*VNYEE FVQMMTAK IV  BBCalm
IAEFKEAFSLF* DKDGDGT*ITTKE LGTVMRSL I   TCalm
EAELQDMINEV* DADGDGT*IDFPE FLSLMARK II  TCalm
EEELIEAFKVF* DRDGDGL*ITAAE LRHVMTNL III TCalm
DEEVDEMIREA* DIDGDGH*INYEE FVRMMAK  IV  TCalm
ADAVDKVMKEL* DEDGDGE*VDFQE YVVLVAAL II  S-100a
QEVVDKVMETL* DSDGDGE*CDFQE FMAFVAMI II  S-100b
ADDVKKAFAII* DQDKSGF*IEEDE LKLFLQNF II  Cparv
DGETKTFLKAG* DSDGDGK*IGVDE FTALVKA  III Cparv
TEDVKKVFHIL* DKDKSGF*IEEEE LGFILKGF II  Rparv
VKETKTLMAAG* DKDGDGK*IGADE FSTLVSES III Rparv
PRTLDDLFQEL* DKNGDGE*VSFEE FQVLVKKI II  PICBP
PSTLDELFEEL* DKNGDGE*VSFEE FQVLVKKI II  BICBP
IQEFKEAFTVI* DQNRNGI*IDKED LRDTFAAM I   DTNB
IQEFKEAFNMI* DQNRDGF*IDKED LHDMLASM I   CGRLC
IQEMKEAFSMI* DVDRDGF*VSKDD IKAISEQL I   SRLC

Double insertion sites

METLINVFHAHS GKEGDKYKLSKKE LKELLQTE I   S-100a
VVALIDVFHQYS GREGDKHKLKKSE LKELINNE I   S-100b
PAELKSIFEKYA AKEGDPNQLSKEE LKQLIQAE I   PICBP
PEELKGIFEKYA AKEGDPNQLSKEE LKLLLQTE I   BICBP
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Fig.1. Primary sequence of 30 EF hands that bind calcium. *Abbreviations:* RSTnC, rabbit skeletal troponin C [48]; BBCalm, bovine brain calmodulin [49]; TCalm, *Tetrahymena* calmodulin [44]; S-100a and S-100b, α- and β-subunit chains of bovine brain S-100 proteins [7,8]; Cparv, carp parvalbumin [50]; Rparv, rabbit parvalbumin [51]; PICBP, porcine intestinal calcium-binding protein [6]; BICBP, bovine intestinal calcium-binding protein [5]; DTNB, rabbit skeletal DTNB light chain [52]; CGRLC, chicken gizzard regulatory light chain [53]; SRLC, scallop regulatory light chain [53]; *, insertion site. The italicized portion of the sequence denotes the calcium-binding loop region where X, Y, Z, $-Y$, $-X$, $-Z$ represent the calcium coordinating positions of the loop. The roman numerals indicate which of the native protein EF hand sites are listed.

3. COMPOSITION OF THE CALCIUM BINDING LOOP

The primary sequence of a calcium binding loop (12 residues) reflects more than just a series of ligands and side chains designed to maximize the binding of Ca^{2+}. The segment Asp,*P*,Asx,Gly, Asx,Gly, where *P* is a positive side chain, represents the most conserved sequence of the EF hand site and is predicted to be a strong β-turn forming region [12]. The side chains of aspartic acid and/or asparagine residues represent calcium coordinating ligands at positions $+X$, $+Y$ and $+Z$ (fig.2A). In view of the fact that glutamic acid residues rarely occur in β-turn structures [12] and are only present in this region of the EF hand in the case of distorted EF sites (site I of S-100a,b and ICBPs), one can conclude that other structural requirements besides the type of calcium ligand must be met in order to generate a functional EF hand. This β-turn region is shown in fig.2B as part of a model EF hand.

The *average* sequence pattern in this part of the EF hand also shows the presence of conserved glycine residues which probably play an important role in the construction of the β-turn segment (position 4) and represent an essential residue (position 6) that allows the peptide backbone to undergo a large change in direction in this part of the EF loop [1,13] and permits the proper folding of ligands around the metal [14]. Noticeable exceptions again include the distorted site I of S-100a,b and ICBPs, and the defunct sites II and III of rabbit skeletal muscle alkali light chains [15,16].

Position 8 and to a lesser extent, position 10 of the EF loop region are conserved hydrophobic sites that offer a natural extension to the hydrophobic region of the C-terminal α-helical region (fig.2C). Their presence in association with the C- and N-terminal hydrophobic surfaces may aid toward the dehydration of the Ca^{2+} inner-sphere complex and suggest a global involvement of the EF site in a metal dehydration process. This explanation appears more plausible than the dehydration mechanism proposed in [17] which only involves the

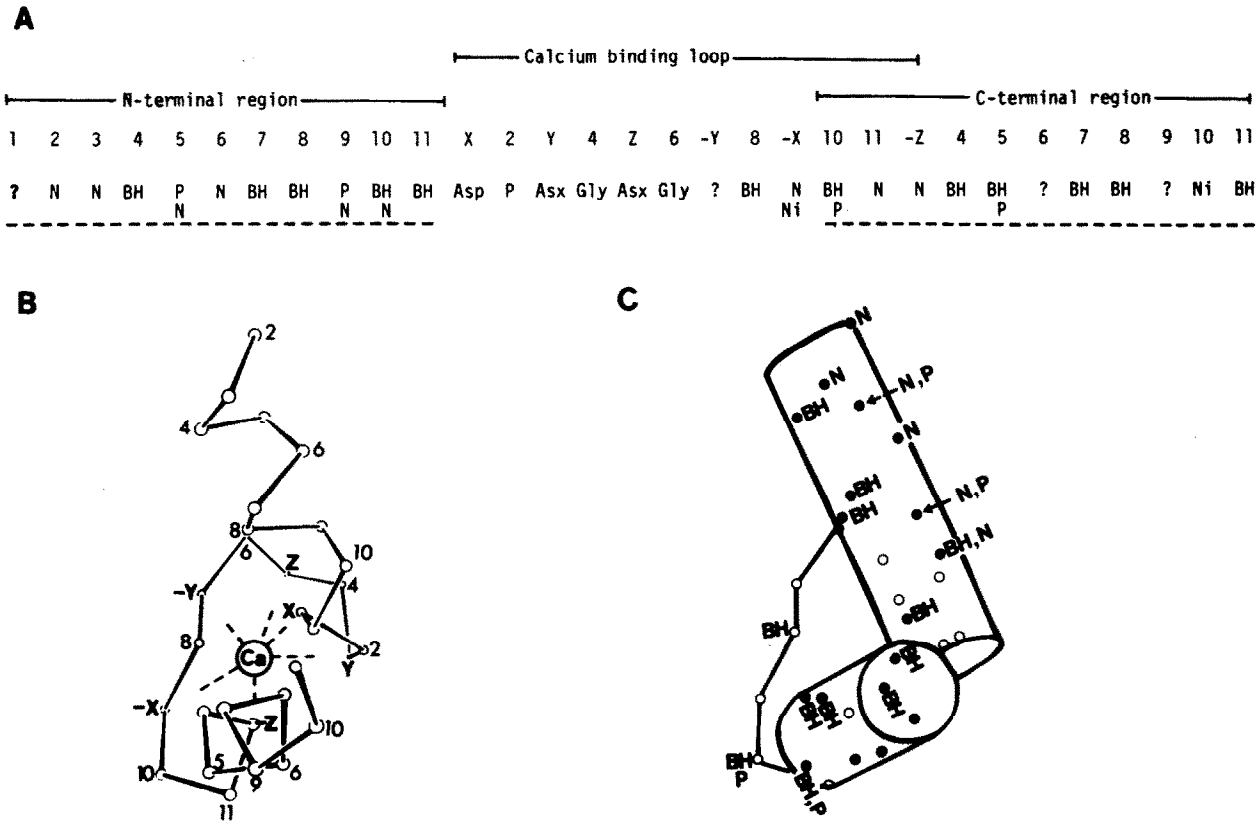

Fig.2. Primary sequence and folding pattern of EF hands. (A) Average sequence of an EF hand. The dotted lines denote regions of pseudo sequence homology. The coordinating residues in the sequence occupy positions *X, Y, Z, −Y, −X* and *−Z*. (B) Representation of a model EF hand along its α-carbon backbone (adapted from [18]). (C) Location of hydrophobic and charged domains in the helical regions of EF hands: (○) residues located in the calcium binding loop; (●) residues present in the helical regions. *Abbreviations:* BH, bulky hydrophobic residue; N, negatively charged residue; P, positively charged residue; Ni, non-ionic residue; ?, weakly conserved residue; Asp, aspartic acid; Asx, aspartic acid or asparagine; Gly, glycine.

residues at positions 7 (least conserved site of the loop) and 10 of the EF loop.

The omnipresence of a glutamic acid at position $-Z$ (table 1) may well be linked to its frequent occurrence in α-helices ($P\alpha$, 1.53; strong α-helix former) [12] as compared to aspartic acid ($P\alpha$, 0.98; α-helix indifferent). In addition, the side chain length at this position ($-Z$) may represent a critical factor in the ability of calcium to induce part of the C-terminal helix.

4. COMPOSITION OF THE C- AND N-TERMINAL REGIONS

Another striking feature from this analysis is the internal sequence homology between the N- and C-terminal α-helical regions (fig.2A; dotted line regions). Kretsinger [13,18] pointed out the presence of regularly spaced hydrophobes in the helical regions of EF hands. The internal sequence homology is best emphasized in the case of site III of rabbit skeletal troponin C where the homologous segments EELAECFR and EELAEIFR are present on opposite sides of the calcium binding loop (fig.1). From the average sequence, we notice that these pseudo-homologous regions are composed of an ordered array of charged and hydrophobic residues. Considering the α-helical nature of these 2 regions, one realizes that the spacing of the hydrophobes will place these side chains on the

same side of the helix, creating hydrophobic surfaces on both sides of the calcium binding loop. A similar observation can be made for charged residues in the N-terminal region of the EF hand. Fig.2C depicts the position of hydrophobic and charged surfaces on a model EF hand domain. Kanehisa and Tsong [19] conducted a study on the secondary structure of 47 globular proteins which indicated that helices are generally found nearer to the surface of proteins and tend to have hydrophobic and hydrophilic surfaces on opposite sides of their helices as a result of an alternating sequence of hydrophobic and charged residues.

Our group has demonstrated using synthetic peptide analogs of site III of rabbit skeletal troponin C that the formation of a calcium–peptide complex is concomitant with the induction of helices in parts of these regions and that the presence of α-helices prior to calcium binding (presence of trifluoroethanol) enhances the affinity of the site for calcium [20–22]. The removal of these helical regions however leads to a large decrease in the ability of the resulting EF hand site to interact with calcium.

In summary, the primary sequence of a protein represents a degenerate code, in relation to its ability to code for elements of secondary structure. This statement implies that a moderately substituted sequence as in the case of EF hands, can still retain its basic structural properties and folding pattern. In addition, although all EF hands possess potential hydrophobic and charged domains, their formation and exposure remains a function of calcium concentration and of the tertiary structure of the protein.

5. IMPORTANCE OF THE EF HAND STRUCTURE IN THE FUNCTION OF CALCIUM BINDING PROTEINS

It has become clear that the mechanism of action of calmodulin correlates with the calcium-induced exposure of hydrophobic patches on its surface [23–26]. The presence in solution of various aromatic ligands such as antipsychotic drugs [27] and W compounds [28] effectively inhibit the interaction of this modulator protein with target enzymes. The localization of a phenothiazine binding site in the N-terminal region of site III of rabbit skeletal troponin C [29] and probably bovine brain calmodulin [30] indicates that target proteins may recognize a helical arrangement of hydrophobic and negatively charged side chains [29,31]. It was demonstrated using a W-7-coupled Sepharose column or a phenothiazine–Sepharose conjugate, that hydrophobic regions on rabbit skeletal troponin C and bovine brain S-100 proteins are also exposed in the presence of calcium [32,33]. It should be noted that carp parvalbumin and chicken ICBP failed to interact with the phenothiazine-bound matrix, in the presence of calcium [33].

Finally, spectroscopic studies on rabbit skeletal troponin C and its fragments [34–40] have indicated that the binding of calcium to the calcium

Table 1

Tabulation of amino acid frequencies at each position of the EF hand

	N-terminal region										Calcium binding region												C-terminal region										
	Residue position										Residue position												Residue position										
	1	2	3	4	5	6	7	8	9	10	11		+X	2	+Y	4	+Z	6	-Y	8	-X	10	11	-Z		1	2	-Z	4	5	6	7	8	9	10	11
Met	1	0	0	1	0	0	5	5	0	4	0	Met	0	0	0	0	0	0	0	0	0	0	0	0	Met	0	0	0	0	1	0	8	13	0	2	2
Cys	0	0	0	0	0	0	1	0	0	0	0	Cys	0	0	0	0	0	0	0	1	0	0	0	0	Cys	0	0	0	0	0	0	0	0	0	0	1
Ile	6	0	0	3	3	0	3	6	0	3	4	Ile	0	2	0	0	0	0	1	19	0	0	0	0	Ile	0	0	0	1	0	1	3	2	0	0	3
Leu	0	0	0	13	0	0	6	1	0	2	5	Leu	0	0	0	0	0	0	0	1	4	0	1	0	0	Leu	1	0	0	15	6	2	10	6	0	0	7
Val	2	1	1	6	0	0	5	0	0	3	4	Val	0	1	0	0	0	0	0	6	0	2	0	0	Val	2	0	0	0	3	5	5	6	2	0	2
Phe	0	0	0	5	0	0	1	18	0	0	7	Phe	0	0	0	0	0	0	0	4	0	0	9	0	0	Phe	9	0	0	13	0	1	1	3	2	0	2
Tyr	0	0	0	0	0	0	0	0	0	0	3	Tyr	0	0	0	0	0	0	0	4	0	0	3	0	0	Tyr	3	0	0	1	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0	0	0	0	Trp	0	0	0	0	0	0	0	0	0	0	0	0	Trp	0	0	0	0	0	0	0	0	0	0	0
Ala	2	6	2	0	1	2	9	0	2	3	2	Ala	2	3	0	2	0	0	0	0	0	4	2	0	Ala	4	2	0	0	1	3	0	0	7	7	0
Gly	0	1	0	0	0	1	0	0	0	0	4	Gly	2	0	0	21	1	26	0	0	2	0	1	0	Gly	0	1	0	0	4	0	0	0	0	3	0
Pro	5	0	0	0	0	0	0	0	0	0	0	Pro	0	0	0	0	0	2	0	0	0	0	2	0	Pro	0	2	0	0	0	0	0	0	0	0	0
Ser	0	1	0	0	1	2	0	0	3	0	0	Ser	0	2	0	0	4	0	0	0	8	0	1	0	Ser	0	1	0	0	1	1	0	1	1	3	2
Thr	1	0	3	2	0	2	0	0	1	1	0	Thr	0	0	0	0	0	0	6	0	3	2	0	0	Thr	2	0	0	0	1	5	1	0	3	2	1
Asn	0	0	0	0	0	1	0	0	3	0	0	Asn	1	0	8	2	4	0	2	0	2	0	0	0	Asn	0	0	0	0	0	0	0	0	1	4	0
Gln	1	3	0	0	3	0	0	0	1	1	0	Gln	0	3	0	0	0	0	1	0	0	0	2	0	Gln	0	2	0	0	2	2	0	0	5	1	1
Asp	4	3	3	0	7	5	0	0	2	2	0	Asp	25	0	18	0	21	0	1	0	12	0	7	3	Asp	0	7	3	0	0	2	0	0	0	0	0
Glu	7	13	21	0	2	13	0	0	5	9	0	Glu	0	3	4	0	0	0	4	0	2	2	11	27	Glu	2	11	27	0	0	4	0	0	2	1	4
His	0	0	0	0	0	0	0	0	3	0	1	His	0	0	0	0	0	0	2	0	0	0	0	0	His	0	0	0	0	1	2	0	0	0	0	0
Arg	0	1	0	0	1	0	0	0	5	0	0	Arg	0	3	0	3	0	0	2	0	0	0	0	0	Arg	0	0	0	0	3	1	0	0	4	4	0
Lys	1	1	0	0	12	4	0	0	5	2	0	Lys	0	13	0	2	0	2	2	0	1	7	4	0	Lys	7	4	0	0	7	1	0	0	5	3	3

specific sites of troponin C (regulatory sites) results in changes in the tertiary structure of the protein and the exposure of hydrophobic site(s), but has little effect on the secondary structure of the protein. These regulatory sites probably possess some partly formed helices [22,37,40] having their hydrophobic surfaces buried and it is the binding of calcium to these Ca^{2+} binding sites that locally exposes the hydrophobic and charged domains. Similar conclusions can be drawn for calmodulin [23–26,41,42]. The extent of exposure of these hydrophobic sites varies, as exemplified by the reduced exposure of the hydrophobic domain(s) of *Tetrahymena* calmodulin in comparison to bovine brain calmodulin [43]. One should note that none of the 11 substitutions observed [44] when comparing the sequence of these two calmodulins results in the loss of hydrophobic residues. However, these two calmodulins are equally able to activate enzymes such as adenylate cyclase, NAD and myosin light chain kinases, but differ in their ability to modulate phosphodiesterase and guanylate cyclase [45]. A similar conclusion can be made when one compares the ability of troponin C to substitute for calmodulin in activating phosphodiesterase [46]. Thus, the action of calcium on these EF hand containing proteins is not limited to the induction of α-helical regions and the formation of hydrophobic and charged surfaces but lies also in its ability to properly expose these sites.

6. CONCLUSIONS ON THE MODE OF ACTION OF EF HAND CONTAINING PROTEINS

In conclusion, we propose that

1. The primary sequence of EF hands codes for elements of secondary structure such as C- and N-terminal helical regions flanking a β-turn segment. The calcium binding loop spans over the β-turn and the beginning of the C-terminal helix and is composed of an arrangement of properly positioned calcium-binding ligands. The calcium-binding affinity of an EF hand however remains largely a function of the tertiary structure it adopts as part of a protein.

2. EF hands possess homologous hydrophobic domains flanking both sides of their calcium-binding loop. In the presence of calcium, parts of the C- and N-terminal regions of an isolated EF hand adopt an α-helical arrangement thus optimizing the geometry of their hydrophobic and charged domains.

3. In the case of EF hand containing proteins, these N- and C-terminal α-helical regions may be preformed in the absence of calcium. These regions are differentially exposed in the presence of calcium so that proteins such as parvalbumin and ICBP do not expose these sites upon calcium binding while troponin C and S-100 protein only partly expose these domains in comparison to calmodulin.

4. The degree of exposure of these hydrophobic and charged domains may explain the selective affinity of *Tetrahymena* calmodulin for guanylate cyclase, bovine brain calmodulin for brain phosphodiesterase, and troponin C for troponin I.

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