STRUCTURE AND EVOLUTION OF CALCIUM-MODULATED PROTEINS*

Author: **Robert H. Kretsinger**
Department of Biology
University of Virginia
Charlottesville, Virginia

Referee: Robert H. Wasserman
Department of Physical Biology
New York State College of Veterinary Medicine
Cornell University
Ithaca, New York

I. INTRODUCTION**

My view of calcium-modulated proteins and processes is summarized by five postulates:[126]

1. All resting eukaryotic cells maintain the concentration of free Ca^{2+} within the cytosol between 10^{-7} and 10^{-8} *M*.
2. The sole function of Ca^{2+} within the cytosol is to transmit information.
3. The target of Ca^{2+}, functioning as a second messenger, is a protein in the cytosol.
4. Calcium-modulated proteins contain EF-hands.
5. Cells initially extruded calcium so they could use phosphate as their basic energy currency; $Ca_3(PO_4)_2$ is insoluble.

The functional implications of this model are discussed by Kretsinger.[128] Here I wish to focus on the structure and evolution of calcium-modulated proteins. I define a calcium-modulated protein by two criteria. First, it is present in the cytosol or is associated with a membrane that faces the cytosol. Second, it must bind calcium with a pK_d ($-\log K_d$) between 5.0 and 6.5 in the presence of 10^{-3} *M* Mg^{2+} normally found in the cytosol. It appears that the pCa ($-\log[Ca^{2+}]$) in a wide variety of resting eukaryotic cells is between 7 and 8. Following a stimulus to a cell there is a transitory rise in the concentration of free Ca^{2+} in the cytosol to about pCa 4.5 (documented by Kretsinger).[128] Calcium functions as a second messenger[199] or coupling factor.[200] In order for

* Manuscript received June 1978.

** Abbreviations: AC, Adenylate cyclase; AMP, Adenosine monophosphate; ATP, Adenosine triphosphate; ATPase, Adenosine triphosphatase; C or N termini, Carboxyl or amino termini of a protein; cAMP, Cyclic adenosine monophosphate; CAF, Calcium-activated factor; CDR, Calcium-dependent regulator or calmodulin; cGMP, Cyclic guanosine monophosphate; CNPDE, Cyclic nucleotide phosphodiesterase; DTNBLC, RLC form of skeletal myosin extractable with 5,5′-dithiobis (2-nitrobenzoic acid); EDTA, Ethylenediamine tetraacetic acid; EDTA LC, RLC of scallop myosin extractable with EDTA; EGTA, Ethylene glycol bis (β-aminoethyl *N,N′*-tetraacetic acid); ELC, Enzymic light chain of myosin; HMM, Heavy meromyosin; ICBP, Intestinal calcium-binding protein; L1 or A1, Traditional designation for the longer of the two ELCs; L4 or A2, Traditional designation for the shorter of the two ELCs; MCBP, Muscle calcium-binding parvalbumin; ϕ, ψ, Dihedral angles representing rotations about nitrogen, α-carbon and α-carbon, carbonyl carbon single bonds; PP, Pyrophosphate ($P_2O_7^{4-}$); RLC, Regulatory light chain of myosin; SF1, Subfragment, or head, of myosin; SR, Sarcoplasmic reticulum; SDS, Sodium dodecyl sulphate; TNC, Calcium-binding component of troponin; TNI, Inhibitory component of troponin; TNT, Tropomyosin-binding component of troponin; V_{max}, Maximum velocity, Δ substrate/Δ time, of a reaction; NRT, Nucleotide replacements per 10^{10} codon years.

a protein to be modulated by Ca^{2+}, it must release its Ca^{2+} during rest ($pK_d < 7.0$) and bind Ca^{2+} during the second messenger pulse ($pK_d > 4.5$).

Based on amino acid sequence data and the crystal structure of carp parvalbumin, one can identify a homolog family consisting of five groups of proteins — calcium-dependent regulator (CDR), calcium-binding component of troponin (TNC), myosin light chains (ELC and RLC), muscle calcium-binding parvalbumin (MCBP), and intestinal calcium-binding protein (ICBP). The general characteristics and functions of these so-called EF-hand proteins will be discussed in Section II and their proposed structures and evolutionary relationships in Section III. Note that none of these homologous calcium-modulated proteins are themselves enzymes, but that at least three of them (CDR, TNC, and LC) function by modifying the functions of enzymes, the ultimate targets of calcium functioning as a second messenger.

There are several other proteins that appear to be calcium modulated based on physiological evidence and/or their calcium affinities. For none of them are amino acid sequence studies completed. It will be exciting to know which of these belong to the EF-hand homolog family. There are many calcium-modulated functions whose associated proteins remain to be identified. This is a particularly promising area of research.

II. CHARACTERISTICS AND FUNCTIONS OF PROTEINS IN THE EF-HAND HOMOLOG FAMILY

A. Overview

In the following five sections are outlined the important functional characteristics of the known EF-hand proteins. They are related by their common origin and their similar structures. Further, they are all involved with some aspect of the functioning of calcium as a second messenger. Yet the diversity of structure and function within the homolog family makes it one of the most fascinating and rewarding yet studied. Inevitably I must introduce many abbreviations and new notations. I beg the reader's forbearance and suggest reference to the list of abbreviations, list of amino acid sequences (Tables 2 and 3), and parvalbumin structure description (Figure 1 and Tables 4 and 5).

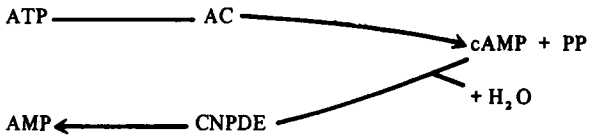
B. Calcium-Dependent Regulator (CDR)

As will be discussed, the various functions and sources of this protein initially gave rise to several designations — phosphodiesterase activator protein, modulator protein, and the most generally used, calcium-dependent regulator (CDR). Calmodulin (modulated by calcium) seems to be gaining general acceptance.

Its multiple functions as well as its broad cellular and species distribution recommend CDR as the prototype of calcium-modulated proteins.[25,36,247a] Waisman et al.[243] identified CDR by activation of bovine heart cyclic nucleotide phosphodiesterase, in the following phyla: Cnidaria, Mollusca, Annelidia, Nemathelminthi, Arthropoda, Echinoderma, Porifera, and Chordata. Within vertebrates, CDR has been identified in many tissues[34] as well as cultured fibroblasts, glioma cells, and lymphoma cells and has been isolated from brain, testes, heart, adrenal medulla, and erythrocytes. The richest sources are testes at 100 mg/kg wet wt tissue[52] and brain at 60 mg/kg.[250] Although CDR is easily EGTA-water extracted from these tissues, apparent lack of, or low yield of, CDR in other tissues may reflect an inadequate extraction procedure.

CDR in the presence of Ca^{2+} activates cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (CNPDE), adenylate cyclase (EC 4.6.1.1) (AC), skeletal muscle myosin ATPase (in vitro), erythrocyte membrane (Ca^{2+}) ATPase, and myosin light chain ki-

nase. Cheung[35] (1969) and Kakiuchi and Yamazaki[113] first described and characterized a "phosphodiesterase activator protein". Since most, if not all, eukaryotic cells employ cAMP (and possibly cGMP) as a second messenger, most cells can be expected to have a CNPDE as well as an AC to control cytosol levels of cAMP:



Most eukaryotic cells appear to have at least one particulate and several soluble forms of CNPDE. At least one of the soluble forms is CDR activated. The significance of the particulate and soluble forms of CNPDE is not clear.[254] Gnegy et al.[83] reported the "release of CDR by cAMP-dependent ATP: protein phosphotransferase from subcellular fractions of rat brain". Ho et al.[102] have prepared CDR-dependent CNPDE 80% pure. From 3.0 kg of bovine hearts they prepared 1.1 mg of CNPDE representing 6.7% of the activity in the original extract. The specific activity of CNPDE is 120 × 10^{-6} mol of cAMP per milligram·minute and can be activated fivefold by CDR and 10^{-5} $M Ca^{2+}$.

Most if not all AC activity is membrane associated. It is normally studied as a particulate suspension or dissolved in 1% Lubrol®. There have been reports of both stimulation, usually at pCa 6, and of inhibition, at about pCa 4, by calcium. However, since none of these preparations were pure, their characteristics neither support nor refute the involvement of CDR. Cheung et al.[34] prepared a detergent-dispersed AC from rat and bovine brain. Brostrom et al.[23] suggested that CDR "controls enzymatic activities responsible for the synthesis of cAMP and for the hydrolysis of cGMP." In 1976, Brostrom et al.[24] demonstrated a 40% stimulation of AC activity of a particulate fraction from homogenates of C-6 glioma cells by addition of CDR and calcium. Rodan and Feinstein[205] found that in platelets Ca^{2+} decreased the V_{max} of AC while stimulating the synthesis of cGMP by guanylate cyclase (EC 4.6.1.2); however, they do not mention the effect of added CDR. Brostrom et al.[23] postulated that the CDR-sensitive CNPDE primarily hydrolyzes cGMP; hence, an increase in free Ca^{2+} in the cytosol would increase cAMP levels via activation of AC and decrease cGMP levels via CNPDE. Alternatively, Cheung et al.[34] suggested a kinetic or compartmental interpretation. AC on the plasma membrane would be first activated by Ca^{2+}-CDR, then as the Ca^{2+} diffuses through the cytosol, the free CNPDE would be activated. A pulse of cAMP would result. Any model of the control of cAMP and cGMP levels must reconcile the apparent contradiction of CDRs stimulating both the forward and the reverse reactions.

A third activity (and possible biological function) of CDR was first reported by Amphlett et al.[5] In a reconstituted actomyosin system from white skeletal muscle of rabbit, CDR from bovine brain is nearly as effective as troponin C (TNC) in relieving the ATPase inhibition by troponin I (TNI). Dedman et al.[51] reported similar results using rat testis CDR. TNC can stimulate CNPDE, even though its affinity for CNPDE is 1/600 that of CDR. CDR probably does not regulate the myosin ATPase of skeletal muscle; however, it might regulate other actomyosin systems. Mahendran and Berl[150] reported the "Isolation of Troponin-like Complex from Bovine Brain Cortex", which is distinct from CDR. It is probable that a TNC or CDR is involved in microfilament activation and possibly in subsequent exocytosis.

A factor that activates the calcium pump (Ca^{2+} and Mg^{2+}) ATPase of erythrocytes

was first described by Bond and Clough.[16] The calcium affinity, heat stability, and molecular weight of the factor suggested its similarity to CDR.[86,148] Jarrett and Penniston[110] have established that the factor contains trimethyllysine (a unique characteristic of CDR) and comigrates with brain and testis CDR on electrophoresis; further, all three stimulate erythrocyte ATPase equally.

The fifth function reported for CDR is activation of myosin light chain kinase from chicken gizzard[49] and from rabbit skeletal muscle.[264] The active kinase is a heterodimer with the enzymic subunit having mol wt 105,000 and the CDR regulatory subunit having mol wt 17,000. Waisman et al.[244] characterized a similar, if not identical, CDR dependent protein kinase from rabbit skeletal muscle. Phosphorylase kinase (Section IVB3), myosin light chains (Section IID), and histones serve as substrates for the protein kinase. They suggested "that this protein kinase mediates the regulatory effects of Ca^{2+} in many physiological processes" and "that the activation of phosphorylase kinase . . . may be . . . a mechanism for the mediation of the Ca^{2+} effect on glycogenolysis during neuronal stimulation of muscle contraction." Schulman and Greengard[213] reported that brain extracts contain a kinase whose phosphorylation of membrane protein is stimulated by an endogenous heat-stable protein plus calcium.

A possible sixth protein regulated by CDR is the brain "modulator binding protein", which elutes from G-200 Sephadex® with the Ca^{2+} form of CDR as a complex of mol wt 160,000.[248] It is not known whether this modulator binding protein is itself an enzyme or whether it functions as a competitor of AC and CNPDE for CDR.[247]

Using indirect immunofluorescence, Welsh et al.[255] followed the localization of CDR throughout the cell cycle of 3T3, PtK_1, and various other vertebrate cells. They observed the following distributions of CDR antibodies: interphase, similar to stress fibers; prophase, random distribution; prometaphase, associated with half-spindles; through anaphase, in the spindle region; late anaphase, in the interzone region; and late telophase, near the midbody and not in the cleavage furrow. They suggest that CDR "may mediate the calcium effects on the mitotic apparatus".

One can anticipate that more CDR-like proteins and associated functions will be identified.

C. Troponin

The identification and characterization of troponin[64] as the molecule that imparts calcium sensitivity to skeletal muscle was the key event in joining calcium physiology and biochemistry. As found in skeletal muscle, troponin (review by Collins[42]) is a trimer consisting of a calcium-binding component (TNC), a tropomyosin-binding component (TNT), and an inhibitory component (TNI).

The TNI-TNC dimer is dissociated from the trimer by treatment with salt. Further, the dimer does not dissociate in 6 *M* urea. Even though the amino acid sequences do not reveal a homology between TNI and TNT, the TNC-TNI_2 trimer is very stable and imparts calcium sensitivity to a reconstituted tropomysin-actomyosin system.

Actin enhances the (Mg^{2+}) ATPase activity of myosin, as either thick filaments, individual molecules, heavy meromyosin (HMM), or as subfragments (SF1). Tropomyosin combined with actin does not prevent this enhancement. When calcium-free troponin combines with the actin-tropomyosin complex, the previous enhancement is abolished. However, when calcium binds to troponin the tropomyosin shifts deeper into the groove of the actin helix.[96,106,180] Supposedly, this shift uncovers the site on actin that interacts with myosin.

Troponin is widely distributed and appears quite conserved evolutionarily. Lehman and Szent-Györgyi[142] recombined the three troponin components, one each from rabbit, chicken, lobster, and *Limulus* and generated functionally active troponin when

assayed in the rabbit actomyosin system. This is particularly interesting since lobster TNC binds only one equivalent of Ca^{2+} in m*M* Mg^{2+},[140,203] while skeletal TNC binds four and cardiac three.

As discussed in the next section, myosin ATPase can be calcium regulated via thick filament, thin filament, or dual control. Any actomyosin system containing troponin appears to be under thin filament or dual control (see Hitchcock[100] and Korn[122] for reviews of cell motility); however, scallop contains troponin-like proteins.[84] It is difficult to prove that a particular tissue lacks troponin, but this does appear to be the case for smooth muscle.[220]

TNC-like proteins have been reported in various nonmuscle tissues, e.g., bovine adrenal medulla[133] and platelets.[173] From the initial reports it is difficult to determine whether these proteins more closely resemble CDR or TNC. I suggest that CDR be characterized as containing ε-*N*-trimethyllysine (residue 332 domain numbering, Table 3) and stimulating the CDR-dependent form of CNPDE at low molar ratios. Further, TNC appears to function bound to TNT and to TNI.

D. Myosin Light Chains (ELC and RLC)

Myosin is a hexameric ATPase comprised of two (nearly) identical, catalytic heavy chains about 200,000 mol wt and two pairs of light chains 20,000 and 30,000 mol wt locusts[260] and ranging from 18,000 to 21,000 mol wt in vertebrates. There are three general classes of vertebrate myosin — skeletal, cardiac, and smooth — each having light chains and possibly heavy chains different from one another. The myosins of nonmuscle cells — platelets[2] and myoblasts[215] — most closely resemble smooth muscle myosin.

It now appears that all light chains can be considered to be either regulatory (RLC) or essential for enzymatic activity (ELC). The myosin from scallop striated muscle requires about 10^{-5} *M* Ca^{2+} for ATPase activity. If only one of its two identical RLCs (called EDTA-LC) is removed by washing with EDTA, its ATPase activity is expressed even in the absence of Ca^{2+}; the scallop myosin is desensitized. Such desensitized myosin can be resensitized by the addition of certain light chains from a variety of vertebrate and invertebrate sources. This functional test defines the RLC.[114] The ELCs are recognized by two characteristics: their removal by alkali results in a loss of myosin ATPase activity, and they do not resensitize scallop myosin.

The RLCs and ELCs are both homologous to CDR, TNC, MCBP, and ICBP (Table 3). They both contain four EF-hand domains. Their amino acid sequences are, in general, consistent with the characteristics expected for the four EF-hand model described for TNC and CDR. However, three reservations are discussed in section III.C: the ELCs do not bind calcium; RLC binds only one Ca^{2+} ion when associated with the heavy chain and probably one[255a] when isolated from it. Bagshaw and Reed[265] found that "the effective rate of Ca^{2+} binding to the DTNB light chain" in the myosin complex "is several orders of magnitude too slow to participate in the activation of muscle contraction, which is complete within 100 ms". The nonbinding loops, like that in cardiac TNC, differ from those of parvalbumin. Second, the RLCs have six to nine amino acid deletions in domain two. Stafford and Szent-Györgyi[226] have completed extensive hydrodynamic studies, which indicate that the RLC monomer free in solution is roughly 100 × 6.5 × 6.5 Å. Finally, they argue that this shape obtains when the RLC is attached to the heavy chain. I summarize the characteristics of the ELCs, then RLCs and conclude by conceding that no simple model is consistent with all of these characteristics.

ELC L1 (or A1) of rabbit has 41 additional residues at the N terminus relative to L4 (or A2), and five of the first eight common residues differ.[72] The remainder of the

two molecules, comprising all four EF-hand domains, have identical sequences even though they are coded by different genes. The 41 N terminal residues of L1 have 9 Lys, 10 Pro, and 13 Ala residues. The unusual N terminus is somehow responsible for its reduced mobility in SDS polyacrylamide gel electrophoresis. Because of this, an apparent molecular weight of 25,000 to 27,000 is often cited instead of its true molecular weight of 21,000. In rabbit skeletal muscle, the molar ratios per myosin hexamer are 1.3 (L1) and 0.7 (L4); they cannot occur in a 1:1 ratio in each hexamer.[72] This ratio is particularly significant because an N terminal analysis of the chymotrypsin digest of rabbit skeletal heavy chain reveals two different peptides in the ratio of about 1.8:1.[152] This ratio is similar to that of the ELCs and supports the idea that isoenzymes of myosin may exist in vivo. Recent work by Wagner and Weeds[246] and Winstanley et al.[261] indicates that the ELCs do not have a significant effect on myosin ATPase activity in the absence of actin but do affect actin-activated ATPase activity and may be involved in actin binding.

Regulatory light chains (RLC) are defined by their ability to resensitize scallop myosin, that has been desensitized by the loss of one EDTA-LC. The intact molluscan myosin hexamer binds two Ca^{2+} with high affinity.[230] If one of the two identical molluscan RLCs is extracted with EDTA, there is a complete loss of calcium regulation of actin-activated ATPase activity as well as a loss of one of the two calcium-binding sites. The extracted light chain does not bind calcium, but can be recombined with the desensitized myosin to restore full calcium regulation and calcium binding.[114] Kendrick-Jones and Jakes[116] found that prolonged incubation of isolated scallop RLC with trypsin produces a fragment that has 14 residues removed from the C terminus. This fragment will not recombine with myosin heavy chains. RLC bound to myosin heavy chain is rapidly degraded by chymotrypsin in the presence of 1.0 m*M* EDTA but remains intact in 1.0 m*M* Ca^{2+}.

Kendrick-Jones and Jakes[116] suggested that the RLCs lie parallel to each myosin head in an extended form in which the length of the RLC is about the same as that of the myosin head. The C terminus of RLC is bound near the hinge region. The N terminus is near the actin-binding site and blocks myosin interaction with actin in the relaxed state. Ca^{2+} binding by the RLCs is suggested to switch on the myosin by inducing a conformational change in the RLCs that results in a tighter binding of the RLCs to the myosin heads in such a way as to allow the myosin heads to interact with actin. Removal of one of the two RLCs may result in the remaining RLC being bound to both myosin heads in a position that does not block the actin-binding site on the myosin even in the absence of Ca^{2+}. The major feature of this model is that it does not require cooperativity between the RLCs or the myosin heads to account for Ca^{2+} regulation. They also considered a cooperative model in which RLCs bind near the hinge region of the myosin head and regulate either the orientation of the myosin heads or the "actin searching movement of the myosin head".[116] Szent-Györgyi et al.[230] observed that, although the actin-heavy meromyosin ATPase activity from molluscan muscle is calcium sensitive, isolated SF1 heads with intact RLCs are not. Calcium binding is also "normal".

Lehman et al.[141] showed that certain muscles are heavy chain regulated in the complete thick and thin filament system. That is, the myosin, ATPase is inactive unless calcium is present. The protein that imparts this calcium sensitivity is troponin in the case of thin filament and a light chain in the case of thick filament regulated myosin. However, it soon became apparent that a third class existed — muscles with dual control. Scallop myosin is thick filament controlled; however, many of the RLCs that can resensitize scallop myosin come from muscles that contain troponin and are supposedly under thin filament control. Skeletal and cardiac RLCs do restore calcium sensitivity

to desensitized scallop myosin in the presence of actin, but they do not restore the capacity to bind a second equivalent of Ca^{2+} ions[114] as does scallop RLC.

Bremel[19] used the competitive actin-binding assay of Lehman et al.[141] to demonstrate that a myosin-linked regulatory system is present in smooth muscle of higher vertebrates. This result is particularly significant in view of the similarities of nonmuscle myosin to that of smooth muscle. The RLCs from chicken gizzard will hybridize with the scallop myosin and restore not only Ca^{2+} sensitivity but also binding of a second Ca^{2+} ion.

The similarities between the RLCs of smooth and skeletal muscle — ability to resensitize scallop myofibrils, similar sequence, and identical phosphorylation site (Section III.F) — suggest that the RLC of vertebrate striated muscle may have a similar regulatory function. However, the low Ca^{2+}-binding affinity of native skeletal myosin (see Section III.D), the requirement of complexed actin for restoring Ca^{2+} sensitivity to desensitized scallop myosin, and the failure to Ca^{2+} binding to desensitized scallop myofibrils indicate that this regulatory role may have been lost during the evolution of the skeletal and cardiac systems.

Calcium regulation of arthropod actomyosin ATPase is associated with the thick and the thin filaments. The fast muscles of crustacea were thought to be an exception in that they appeared to exhibit only thin-filament control.[142] Recently, Lehman[139] demonstrated that the fast muscles from crustacea exhibit myosin regulation when ATPase activity is assayed at ionic strengths approaching those in vivo (0.1 *M* KCl). Similarly, studies on rabbit skeletal myofibrils indicate myosin-linked regulation that can be abolished only at low ionic strength. Purified rabbit skeletal myosin shows no Ca^{2+}-dependent ATPase activity regardless of the ionic strength used. Thus, a myosin-linked regulation may exist in vertebrate skeletal muscle that is lost during purification.

Margossian et al.[153] prepared SF1 fractions of skeletal myosin in the presence of Mg^{2+} or EDTA. Mg^{2+} SF1 fractions contain RLCs whereas EDTA SF1 fractions do not. No differences in content of ELC L1 or of ELC L4 were apparent. They then studied the effect of Ca^{2+} on actin binding by the SF1 fractions; Ca^{2+} had no effect on EDTA SF1 interactions but significantly decreased the binding by Mg SF1. The midpoint for the binding transition was pCa 5. Skeletal RLCs can be added back to the EDTA SF1 fractions, and a partial calcium response is obtained. These results indicate that the RLC of skeletal myosin, in response to an increase in Ca^{2+}, may regulate interaction of the myosin heads with actin. The same results were obtained with HMM preparations. Possible regulatory roles related to phosphorylation of RLCs are discussed in Section III.F.

As indicated in the preceding three paragraphs, there is inferential evidence that the RLCs might impart calcium sensitivity to skeletal muscle. This suggestion was reinforced by Morimoto and Harrington[167] who found that calcium ($pCa^{2+} \simeq 5.4$ with m*M* Mg^{2+}) induces a reversible conformational change in myosin evidenced by an increased sedimentation coefficient and reduced relative viscosity. Huxley[106] and Haselgrove[96] obtained X-ray diffraction patterns of toad semitendinosus muscles that had been stretched so far as to preclude any overlap of thick and thin filaments. One interpretation of the altered diffraction pattern following addition of calcium is that the myosin heads move away from the thick filament. However, there has been no direct demonstration of calcium regulation of skeletal myosin other than via its binding to troponin. On the contrary, Sutoh and Harrington[229] chemically cross-linked myosin and concluded from their "results that neither the presence of Ca ions nor the shift from resting to rigor state (at rest length) results in release of myosin heads from the thick filament surface." Mendelson and Cheung[161] studied fluorescence depolarization

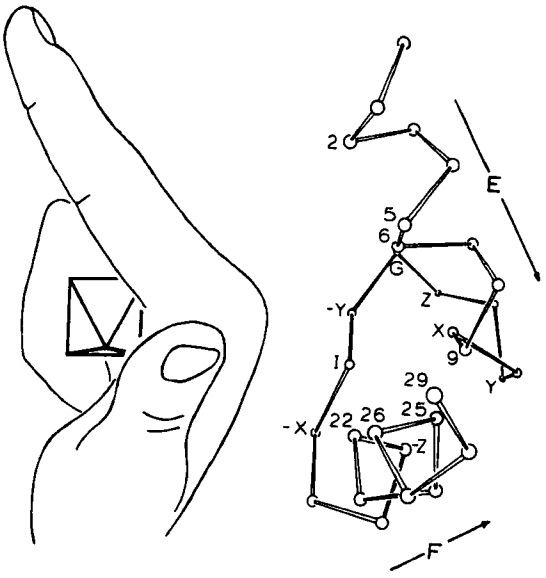


FIGURE 1. The EF hand. The α-carbons that contribute hydrophobic side chains to the core of the protein are numbered as in Tables 2 and 3. The vertices of the octahedron, which represents the oxygen ligands about the Ca^{2+} ion, are indicated by X, Y, and Z. The highly conserved Gly and Ilu are labelled.

and inferred that "calcium does not directly move the crossbridges toward thin filaments on activation of (skeletal) muscle."

We do not yet understand the Ca^{2+} binding of RLC, the structures of ELC or RLC, the effects of phosphorylations in skeletal and cardiac muscle, nor the detailed functions of the myosin light chains in various types of muscle.

E. Parvalbumin (MCBP)

The crystal structure of isotype 3 (or B), pI 4.25, from carp has been determined and refined at 1.9 Å resolution by Moews and Kretsinger.[163] It contains six α-helical regions, A through F. The loops between helices C and D and between E and F each bind a calcium ion. Helix E, loop EF, and helix F resemble the extended forefinger, clenched middle finger, and extended thumb of a right hand (Figure 1). This basic structural and homology domain is called the EF-hand. It is related to the homologous CD-hand by an approximate twofold axis passing through the monomeric protein. The AB-hand, which lies over the tightly packed EF-hand, CD-hand pair, does not bind calcium but apparently functions to cover and contribute to the hydrophobic core. The amino acid sequence and chemical data indicate that all known parvalbumins are isostructural with that of carp 4.25. Details of its crystal structure, evolution, and calcium binding are presented in Section III. In order to consider its function, which remains unknown, I will first discuss its structure in solution and its cellular distributions.

Various spectroscopic and chemical studies show that the solution structure of the two Ca^{2+} form of muscle calcium-binding parvalbumin (MCBP-Ca_2) is very similar to that in the crystal. As is the case for most proteins, the parvalbumin structure is dynamic with all atoms vibrating or oscillating about their average positions, as seen in

the crystal structure. Cave et al.[32] extended the early ^{13}C NMR spectroscopic results of Opella et al.[178] and concluded that the ten phenyl rings show no $\pi \cdot \pi$ interaction and that most of them are packed in a herring-bone arrangement as had been seen in the crystal structure. They also deduced that most of the rings flip about their C_β-C_γ bonds over 1000 Hz, even though the rings reside the great majority of the time in their "crystal positions". The packing of the core of MCBP demands compensatory movements of various side chains associated with each flip in order to avoid unacceptable van der Waals contacts. The structure is dynamic.

Various calcium-binding constants have been published for parvalbumin (Section III.D). In the absence of Mg^{2+}, the $pK_d(Ca^{2+})$ is ~8.4 for both[194] or one[186] of the sites, while in the presence of physiological levels of Mg^{2+} (~2.0 m*M*), the pK_d (Ca^{2+}) is ~6.5. Potter et al.[194] reported the $pK_d(Mg^{2+})$ to be 4.0. Grandjean et al.[90] inferred from ^{23}Na NMR spectroscopy $pK_d(Na^+) \simeq 2.0$ with a greater K^+ affinity. These results, which should be regarded as tentative, suggest that in the resting cell (pCa > 7.0) parvalbumin has either Mg^{2+} or K^+ bound at the calcium sites.

Kretsinger[125] presented a structural model for the cooperative release of Ca^{2+} by MCBP-Ca_2. This model appears to be wrong since no or only slight[184] cooperativity (Hill coefficient 1.05)[186] is observed by Chelex® or equilibrium dialysis studies. More conclusive, several spectroscopic studies have been interpreted in terms of three states: MCBP-Ca_2 ↔ MCMP-Ca_1 ↔ MCBP-Ca_0. Opella et al.,[178] using ^{13}C natural abundance NMR spectroscopy, identified the resonances of the guanido carbon atom of Arg_{395} and the carbonyl carbon atoms, whose resonances are shifted up field, of Lys_{316} and Phe_{416} in carp 4.3 MCBP. Removal of only one Ca^{2+} ion by EGTA titration causes a change in the environment of the Arg_{395}-Glu_{401} pair and in the Lys_{316}. Removal of the Ca^{2+} ion shifts the Phe_{416} resonance. Consistent with the earlier results of Donato and Martin,[58] Nelson et al.[174] concluded from their CD spectra that a "twenty fold excess of EGTA removes but one of two Ca(II) from carp parvalbumins" in the absence of Mg^{2+}. Removal of both Ca^{2+} ions increases the rate of the sulfhydryl reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB).[58]

The structural changes that accompany the loss of calcium are unclear. Parello et al.[179] deduced from their ^1H NMR spectra that removal of both Ca^{2+} ions leads "to a structure very similar to that obtained by chemical or thermal denaturation." In contrast, Donato and Martin[58] found that removing one Ca^{2+} ion results in "little or no change in the helical content of 47%." Removal of the second Ca^{2+} reduces the α-helical content to 39% and "corresponds to disruption of one of the six helical regions found in the native protein." Closset and Gerday[38] presented a third interpretation based on their CD spectra from pike 5.0 and whiting parvalbumins. They suggested that removal of Ca^{2+} ions "induces changes primarily in the tertiary structure, leading to a still ordered but less compact molecule." Many groups have reported a significant reduction in solubility and a tendency to aggregate associated with calcium removal. We have failed to crystallize the calcium-free form.

The structural changes that accompany the loss of calcium are unclear. Parello et al.[179] deduced from their ^1H NMR spectra that removal of both Ca^{2+} ions leads "to a structure very similar to that obtained by chemical or thermal denaturation." In contrast, Donato and Martin[58] found that removing one Ca^{2+} ion results in "little or no change in the helical content of 47%." Removal of the second Ca^{2+} reduces the α-helical content to 39% and "corresponds to disruption of one of the six helical regions found in the native protein." Closset and Gerday[38] presented a third interpretation based on their CD spectra from pike 5.0 and whiting parvalbumins. They suggested that removal of Ca^{2+} ions "induces changes primarily in the tertiary structure, leading to a still ordered but less compact molecule." Many groups have reported a significant

Table 1
PARVALBUMIN CONTENTS OF TISSUES

Species	Muscle	Wet weight (g/kg)	Ref.
Hake	White skeletal	11.5	182
Coelacanth	White skeletal	11.3	93
Turtle	White skeletal	10.0	14
Frog	Skeletal	3.5	190
Mouse	White skeletal	1.6	3
Rabbit	White skeletal	0.8	14
Rabbit	Cruris (red)	0.48	8
Rabbit	Psoas (white)	0.39	8
Rabbit	Psoas (white)	0.3	138
Chicken	White skeletal	0.3	14
Rabbit	Diaphragm	0.047	8
Chicken	Leg	0.04	98
Limulus	White leg	0.04	6
Rabbit	Brain	0.002	8

reduction in solubility and a tendency to aggregate associated with calcium removal. We have failed to crystallize the calcium-free form.

Just as the binding of Ca^{2+} affects the conformation of parvalbumin, so its overall conformation markedly affects its Ca^{2+} affinity. Extending the initial observations of Solano and Coffee [221] Derancourt et al.[57] determined that the isolated EF hand (sequence numbers 76 to 108) and domains 2 and 3 (sequences numbers 1 to 75) have $pK_d(Ca)$ values of 2.5 and 4.1, respectively, in the absence of Mg^{2+}. That is, separation of the two Ca-binding domains (three and four) reduces their Ca^{2+} affinities by 10^4 to 10^6. As noted in the discussion of LC and TNC, the interaction of a calcium-modulated protein with another protein can also alter its calcium affinity and specificity.

The invariance of the unique Arg_{395}-Glu_{401} internal hydrogen bond remains unexplained and may provide a clue to the function of parvalbumin. Even though it is over 25 Å from either Ca^{2+} ion, a modification to that region of the molecule, e.g., derivatization of the Arg with 1,4 cyclohexanedione, greatly reduces the Ca^{2+} affinity.[87] Cys_{209} of carp 3.25 parvalbumin is partially buried on the opposite side of the molecule from the Ca^{2+} binding sites; the S atom is 6 Å from the guanido group of Arg_{395}. Donato and Martin[58] found that removal of both Ca^{2+} ions "frees the single cysteine so that it reacts more rapidly with" DTNB.

The tissue contents of parvalbumin listed in Table 1 indicate that it is not an indispensable component of the contractile mechanism. Further, it is present at levels (probably) less than 0.1 mg/kg wet wt in carp brain;[89] chicken back muscle, heart, stomach, brain, pancreas, small intestine, and kidney, but not spleen, liver, or blood;[98] human heart and uterus;[138] mouse heart;[3] and absent in chicken breast.[14] Baron et al.[8] found less than 0.2 mg/kg in rabbit uterus, bladder, small intestine, spleen, kidney, ovary, adipose tissue, lung, liver, and erythrocytes. The actual values could be in error by a factor of two since some represent yields from parvalbumin preparations; in contrast, Blum et al.[14] quantified the content by densitometry of coomassie blue stained polyacrylamide gels of the initial water extract of muscle. The parvalbumin need not be purified because no other proteins run near it on the gel.

Parvalbumins are highly antigenic and have been implicated in the allergic reaction some people have to fish. Gosselin-Rey and Gerday[88] showed that the antibody-binding ability of MCBP does not change with removal of calcium. Although parvalbumin is evolving slowly (about 9 NRT, Section III.G), there is only limited cross-reactivity

among the various isotypes.[89,147] This antigenicity has been used to localize parvalbumin within tissues and cells.

Lowe et al.[147] found by immunochemical staining that in the mouse parvalbumin is found in the white muscle only, and there in smaller (10 to 35 μm) but not in larger (35 to 60 μm diameter) fibers. It is these larger fibers that are primarily involved in glycogenolysis. The low yields of parvalbumin in red muscle may reflect contamination by white fibers. In contrast, Benzonana et al.[10] reported that cryosections of muscle treated with fluorescent antibodies specific for parvalbumin show a uniform distribution of the marker. Pironi et al.[190] tested single fibers of frog twitch muscle, dissected under oil to prevent diffusion, by double Outcherlony immunodiffusion and found "two types of the frog parvalbumin in each muscle fiber."

Many functions have been suggested for parvalbumin over the past decade:

1. The initial sources of parvalbumin were frog and carp. Hamoir[92] suggested that "they seem to be necessary as long as semi-permeable membranes exist." Only subsequently was parvalbumin isolated from mammals.
2. Parvalbumin is not an enzyme. Its structure has no pits or crevasses characteristic of all of the enzyme structures determined to date. Pechere and Focant[183] tested a variety of possible enzymic functions associated with glycolysis and found none.
3. Following the realization that parvalbumin binds calcium, Pechère et al.[184] suggested that it "might have biochemical effects similar to those of troponin and also that chemical isologies might be detected..." Subsequently, it has been found that the fast, white muscle of fish, where parvalbumin occurs in greatest abundance (up to 10 mg/g wet wt) also contains troponin. It does not replace troponin or myosin light chains in in vitro ATPase assay systems.
4. Blum et al.[13] reported a phosphorylated form of parvalbumin, which might provide a clue to its function. This appears to have been an artifact;[55] any phosphate acceptor protein in the myogen fraction is not parvalbumin.
5. Potter et al.[191] found a Ca^{2+}-dependent activation of brain CNPDE by parvalbumin at 2000 times the molar amount of calmodulin required. Bovine serum albumin, used as a control, does not activate CNPDE. Considering the high levels of MCBP present in fish muscle, they suggested that it "may regulate skeletal muscle phosphodiesterase".
6. Pechère et al.[182] suggested that parvalbumin might interact with a membrane ATPase.
7. Using indirect immunofluorescence, Heizmann et al.[98] saw "regular cross-situation patterns within the I band of isolated myofibrils" of chicken and suggested that "a parvalbumin-like protein may interact with one or more of the protein components of the thin filament." (The three preceding observations infer a direct interaction of parvalbumin with another protein.)
8. Hitchcock and Kendrick-Jones[101] found that neither parvalbumin nor myosin light chains can substitute for TNC in functional assays or in TNI and tropomyosin-binding studies. They suggested that parvalbumin serves as a soluble store of bound calcium in the sarcoplasm. At this same meeting, Pechère et al.[182] elaborated this theme and argued that parvalbumin serves as a Ca^{2+} dependent H^+ buffer following ATP hydrolysis. That is, during muscle activity parvalbumin would be in the MCBP-Ca_0 form and, in this form, would have an increased proton affinity. Pechère et al.[185] subsequently withdrew this interpretation when they found no pH dependence of calcium binding. Briggs[20] identified parvalbumin as the soluble relaxing factor of muscle.

Gerday and Gillis[79] and Blum et al.[14] determined that fragmented sarcoplasmic reticulum (SR) could deplete parvalbumin of calcium, and parvalbumin, in turn, could deplete myofibrils of calcium. These observations are consistent with a "trap or carrier" idea of Demaille et al.[56] The affinity of parvalbumin for calcium is greater than that of troponin but less than that of SR (Ca^{2+}) ATPase. However, the rate of Ca^{2+} binding by troponin may exceed that of parvalbumin. Hence, upon release from the SR, calcium would first bind to troponin and activate myosin ATPase. It would then be soaked up quickly by parvalbumin and be stored there during muscle relaxation while the SR ATPase is pumping away the calcium.[82] The implication that the fastest muscles have the greatest need for a "kinetic sink" is only partially supported by the observed tissue distribution of parvalbumin (Table 1).

This kinetic model of parvalbumin function requires knowledge of the following 13 parameters plus the solution of three simultaneous differential equations. The concentrations of three classes of sites — R (sarcoplasmic reticulum), P (parvalbumin), and T (troponin) — can be approximated from published values. A fourth parameter — CF, calcium free — is required to describe the total calcium (ΣC) in the system: $\Sigma C = CF + CR + CP + CT$. (An estimate of cytosol volume is required to calculate the concentration of these components, e.g., [CF] = CF/vol). The three equilibrium constants describing Ca^{2+} affinities (in the presence of m*M* Mg^{2+} have been published — K_r, K_p, and K_t.

The eighth through tenth parameters are the Ca^{2+} release rates — k_r, k_p, k_t. These will be difficult to determine experimentally because calcium is spectrally inert; one must find for SR, parvalbumin, and troponin spectral signals associated with protein conformation changes. The 11th and 12th parameters are most critical to the model and are particularly difficult to determine. The SR must have two release rates — one during excitation and a second during calcium uptake and quiescence. If the "on rate" is assumed constant, then the assumption of an excited release rate k_r will also yield an excited equilibrium constant — excited $K_r = k_r/k_{on}$; quiescent $K_r = k_r/k_{on}$. The 12th parameter is the duration of time, t_{ex}, during which k_r obtains. In order to see the cyclic nature of the calcium distribution, one must also designate t_q, the quiescent period when k_r obtains. This formulation assumes very short diffusion distances and noncooperativity in binding and release.

In addition to the calcium conservation equation, one must solve the following simultaneous equations:

$$dCR/dt = -k_r[CR] + K_r \cdot k_r \cdot [R] \cdot [CF]$$

$$dCP/dt = -k_p[CP] + K_p \cdot k_p \cdot [P] \cdot [CF]$$

$$dCT/dt = -k_t[CT] + K_t \cdot k_t \cdot [T] \cdot [CF]$$

F. Intestinal Calcium-Binding Protein (ICBP)

The amino acid sequence (Section III.B) of porcine intestinal calcium-binding protein[103] indicates that it contains two EF hands. A 9700-mol wt form is found in mammalian intestine and in bovine and guinea pig kidney.[249] A second form, mol wt 28,000 is found in chick gut, kidney, shell gland, and brain as well as in bovine and rat brain and rat kidney. Within the latter group, the ICBPs are immunologically cross-

reactive; however, there is no cross-reaction between the two groups. Even so, the two are generally assumed to be functionally and evolutionarily related. Immunological staining indicates that ICBP is localized in the brush border region and goblet cells. It is readily extracted from homogenized tissue and is thought to be in the cytosol. Wasserman and Feher[249] suggested that the 5% of chick gut ICBP that is associated with a membrane component might provide a clue to its function.

Calcium transport in various tissues requires 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). Rachitic animals lack ICBP. Following treatment of these animals, or of rachitic tissue cultures, with $1,25(OH)_2D_3$, increased calcium transport is observed as well as an increase in levels of ICBP. It has been assumed that $1,25(OH)_2D_3$ somehow controls the synthesis of ICBP and that ICBP is involved in transcellular transport of calcium. However, Spencer et al.[225] and Toffolon et al.[233] have argued that increased calcium uptake precedes ICBP synthesis following $1,25(OH)_2D_3$ injection into rachitic animals.

Weissman et al.[253] reported the presence in rat kidney of a 26,000-mol wt calcium-binding protein whose yield is not significantly altered by the depletion or addition of vitamin D. Vitamin D may also stimulate the synthesis of other proteins involved in calcium transport, e.g., the (Ca^{2+}), also referred to as alkaline phosphotase, ATPase of chick intestinal brush borders.[160] The mechanism of transcellular, as opposed to transmembrane, transport may involve the Golgi system. The amount of calcium associated with these membrane vesicles is reduced in the intestine of rachitic rats.[76]

The two forms have the following properties:

	mol wt	pI	$pK_d(Ca^{2+})$	$n(Ca^{2+})$	α-Helix (%)
Chick gut	28,000	4.2	6.3	4	~30
Bovine gut	9,700	4.7	~6.5	2	~30

As discussed in Section III.C, the binding of two Ca^{2+} ions by bovine gut ICBP is particularly interesting since one of the two putative calcium-binding loops has a three-residue insertion and might not be expected to bind Ca^{2+}. Moffat et al.[164] reported the crystal unit cell ($P2_12_12$) (56.3 × 43.0 × 29.4 Å) of bovine gut ICBP; hopefully the structure will soon be solved.

III. STRUCTURAL AND EVOLUTIONARY RELATIONSHIPS BETWEEN EF-HAND PROTEINS

A. Overview

The focus of the discussion in this section is to examine the known sequences of calcium-modulated proteins with the goals of (1) gaining insights into their structures, (2) understanding their calcium affinities and selectivities, (3) understanding other functional properties, (4) suggesting additional experiments, and (5) establishing their evolutionary relationships.

Hopefully the crystal structures of bovine ICBP,[164] of rabbit muscle TNC,[162] and of bovine brain CDR (Schatz & Kretsinger, ms. in preparation) will soon be determined. However, at this time the structure of carp parvalbumin, isotype 4.25,[163] provides the sole structural precedent for interpreting the sequence data and various chemical experiments. It consists of six regions of α-helix (A through F). There is a nonhelical N terminal region and five loops between the six α-helices. A calcium ion is coordinated by six amino acids in loop CD (between helix C and helix D); a second calcium ion is coordinated in the homologous loop EF. As seen in Figure 1, regions

CD and EF resemble a pair of right hands related by a twofold axis with the forefinger corresponding to helix E (or C), the thumb symbolizing helix F (or D), and the middle finger indicating the loop around the calcium ion. The palmar surfaces of the forefinger and thumb contribute side chains to the hydrocarbon core. Region AB resembles the EF hand; however, loop AB does not bind calcium and has apparently suffered a three-amino acid deletion. Kretsinger[127] postulated that parvalbumin evolved by gene triplication and splicing.

Collins et al.[43] recognized four EF-hand homolog regions in TNC, and Weeds and McLachlan[251] and Tufty and Kretsinger[237] recognized four EF hands in ELC L1. Kretsinger and Barry[129] presented a model for TNC consisting of two pairs of EF hands, each pair as found in parvalbumin. The two pairs share a common twofold axis and are related to one another by two twofold axes, thereby generating point group symmetry 222. The sequences are discussed in terms of these four, three, and two EF-hand models.

B. Sequences

Tables 2 and 3 list the available sequences of calcium-modulated proteins. I advocate the following numbering and alignment scheme because it makes optimal use of available structural and evolutionary information. Each homology domain, or EF-hand-containing region, consists of 31 residues numbered 00 through 30; the hands themselves are numbered 1 through 4. For example, the fourth hand residues are numbered 400 through 430. Within a hand, deletions are indicated by "—" and inserted residues by "+"; for instance, Leu_{100+} in cardiac TNC. The one-letter code is A Ala, B Asx, C Cys, D Asp, E Glu, F Phe, G Gly, H His, I Ilu, K Lys, L Leu, M Met, N Asn, P Pro, Q Gln, R Arg, S Ser, T Thr, V Val, W Trp, Y Tyr, Z Glx.

The (inferred) hydrophobic or inner aspects of helix E (residues 02, 05, 06, and 09) and of helix F (22, 25, 26, and 29) are under column headings "n". The (inferred) calcium ligands can be assigned to the vertices of an octahedron — X(10), Y(12), Z(14), −Y(16), −X(18), −Z(21).

For example, domain three of bovine brain CDR (referred to as domain CDR bov brain 3; see Table 3) is comprised of residues 83 through 113, in absolute numbering. Helix E-3 has I_{302}, A_{305}, F_{306}, and F_{309} on the inside (domain numbering); helix F-3 has L_{322}, V_{325}, M_{326}, and L_{329} designated as "n". Calcium ligands are provided by side chains of D_{310}, D_{312}, N_{314}, S_{316}, and E_{321} at octahedral vertices X, Y, Z, −X, and −Z, respectively.

The interdomain loops vary in length and should be considered with respect to both the preceding and following hand. For instance, in cardiac TNC (bov heart) the residues Lys·Asp·Asp·Ser·Lys·Gly·Lys·Ser·Glu can be numbered 231 through 239 if they are being considered relative to hand 2 or they can be numbered 291 through 299 relative to hand 3. The C terminal residues count up from 430 and the N terminal down from 99. In order to facilitate reference to other publications, the absolute number of each residue is indicated at both ends of each line.

Ser_{088} of the various RLCs can be phosphorylated. Residue 396 (= 332) of CDR is trimethyllysine. The N termini of these proteins are blocked, where determined, by acetyl groups. The sole exception is coelacanth 4.52 MCBP, whose N terminal Ala is unblocked.[56] The generation of nodal sequences, those inferred to exist in evolutionary precursors, is discussed in Section III.G.

The N terminal sequences that exceed the table length are 14 RLC Rabb Skel $_1$PKKAKRRAAAEG$_{12}$; 13 RLC Chick Skel $_1$PKKAKRRAAEG$_{11}$; and 3 ELC Rabb Skel (A1) $_1$PKKNVKKPAAAAAPAPKAPAPAPAPAPAPKEEKID$_{35}$. The sequence of ELC Rabb Skel A4 is identical to that of A1 except that its N terminus is 41 residues

Table 2

AMINO ACID SEQUENCES OF PARVALBUMINS

	0	1	2	3	4	5	6	7	8	9	20 0	1	2	3	4	5	6	7	8	9	30 0	1	2	3	4	5	6	7	8	9	40 0	1	2	3	Ref.
													n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n				
1 Coel	₁T	K	K	M	S	E	I	L	K	A	E	D	I	D	K	A	L	N	T	F	K	E	A	G	S	–	–	F	D	H	H	K	F	–	F	N	L	V	G	L	K	G	K	P_{41}	181
2 Rabb	₁A	–	–	M	T	E	L	L	N	A	E	D	I	K	K	A	I	G	A	F	A	A	A	E	S	–	–	F	D	H	K	K	F	–	F	Q	M	V	G	L	K	K	K	S_{39}	66
3 Pike	₁A	K	–	–	–	D	L	L	K	A	D	D	I	K	K	A	L	D	A	V	K	A	E	G	S	–	–	F	N	H	K	K	F	–	F	A	L	V	G	L	K	A	M	S_{38}	73
4 Ray	₁S	S	K	I	T	S	I	L	N	P	A	D	I	T	K	A	L	E	Q	C	–	A	A	G	–	–	–	F	H	H	T	A	F	–	F	K	A	S	G	L	S	K	K	S_{39}	231
5 Coel	₁A	–	–	V	A	K	L	L	A	A	A	D	V	T	A	A	L	E	G	C	K	A	D	D	S	–	–	F	N	H	K	V	F	–	F	Q	K	T	G	L	A	K	K	S_{39}	111
6 Frog	₁S	–	–	I	T	D	I	V	S	E	K	D	I	D	A	A	L	E	S	V	K	A	A	G	S	–	–	F	N	Y	K	I	F	–	F	Q	K	V	G	L	A	G	K	S_{39}	30
7 Hake	₁A	–	–	F	A	G	I	L	A	D	A	D	I	T	A	A	L	A	A	C	K	A	E	G	S	–	–	F	K	H	G	E	F	–	F	T	K	I	G	L	K	G	K	S_{39}	31
8 Cod	₁A	–	–	F	K	G	I	L	S	N	A	D	I	K	A	A	E	A	A	C	F	K	E	G	S	–	–	F	D	E	D	G	F	–	Y	A	K	V	G	L	D	A	F	S_{39}	65
9 Pike	₁S	–	–	F	A	G	–	L	K	D	A	D	V	A	A	A	L	A	A	C	S	A	A	D	S	–	–	F	K	H	K	E	F	–	F	A	K	V	G	L	A	S	K	S_{38}	79
10 Whit	₁A	–	–	F	A	G	I	L	A	D	A	D	A	C	A	A	V	K	A	C	E	A	A	D	S	–	–	F	S	Y	K	A	F	–	F	A	K	C	G	L	S	G	K	S_{39}	112
11 Chub	₁A	–	–	F	–	G	–	L	K	E	A	D	I	T	A	A	L	E	A	C	K	A	A	D	S	–	–	F	N	H	K	A	F	–	F	A	K	V	G	M	S	A	K	S_{37}	80
12 Carp	₁A	–	–	F	A	G	V	L	N	D	A	D	I	T	A	A	L	E	A	C	K	A	A	D	S	–	–	F	N	H	K	T	F	–	F	A	K	V	G	L	T	S	K	S_{39}	40
13 Carp	₁A	–	–	F	A	G	V	L	N	D	A	D	I	A	A	A	L	E	A	C	K	A	A	D	S	–	–	F	N	H	K	A	F	–	F	A	K	V	G	L	T	S	K	S_{39}	39
14 Carp	₁A	–	–	Y	G	G	I	L	N	D	A	D	I	T	A	A	L	E	A	C	Z	A	Z	D	S	–	–	F	N	A	K	S	F	–	F	A	K	V	G	L	S	A	K	T_{39}	40
Node	A	K S K	K	I M	T	5	I	L	4	A	E A	D	I	D K T	K	A	L	4	A	C F	K	A	A	G	S	–	–	F	H N D	H	K	6	F	–	F	Q	4	V	G	L	K S A	K	K	S		
							6	7	8	9	30 0	1	2	3	4	5	6	7	8	9	40 0	1	2	3	4	5	6	7	8	9	50 0	1	2	3	
													n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n				
1 Coel	5.40						$_{39}$G	K	P	D	D	T	L	K	E	V	F	G	I	L	D	Q	D	K	S	G	Y	I	E	E	E	E	L	K	F	V	L	K	G	F	G	A	A	G_{76}	
2 Rabb	5.50						$_{37}$K	K	S	T	E	D	V	K	K	V	F	H	I	L	D	K	D	K	S	G	F	I	E	E	E	E	L	G	F	I	L	K	G	F	S	P	D	A_{74}	
3 Pike	5.00						$_{36}$A	M	S	A	N	D	V	K	K	V	F	K	A	I	D	A	D	A	S	G	F	I	E	E	E	E	L	K	F	V	L	K	S	F	A	A	D	G_{73}	
4 Ray	4.45						$_{37}$K	K	S	D	A	E	L	A	E	I	F	D	V	L	D	G	D	Q	S	G	Y	I	E	V	E	E	L	K	N	F	L	K	C	F	S	D	G	A_{74}	
5 Coel	4.52						$_{37}$K	K	S	D	E	Q	L	E	A	I	F	K	I	L	D	Q	D	K	S	G	F	I	E	D	E	E	L	E	L	F	L	Q	N	F	S	A	G	A_{74}	

Table 2 (continued)

AMINO ACID SEQUENCES OF PARVALBUMINS

		6	7	8	9	30	1	2	3	4	5	6	7	8	9	40	1	2	3	4	5	6	7	8	9	50	1	2	3	4	5	6	7	8	9	60	1	2	3
								n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n				
6 Frog	4.50	$_{37}$G	K	S	A	A	D	A	K	K	V	F	E	I	L	D	R	D	K	S	G	F	I	E	Q	D	E	L	G	L	F	L	Q	N	F	R	A	S	A$_{74}$
7 Hake	4.36	$_{37}$G	K	S	A	A	D	I	K	K	V	F	G	I	I	D	Q	D	K	S	D	F	V	E	E	D	E	L	K	L	F	L	Q	N	F	S	A	G	A$_{74}$
8 Cod	4.75	$_{37}$A	F	S	A	D	E	L	K	K	L	F	K	I	A	D	E	D	K	E	G	F	I	E	E	D	E	L	K	L	F	L	I	A	F	A	A	D	L$_{74}$
9 Pike	4.10	$_{36}$S	K	S	L	D	D	V	K	K	A	F	Y	V	I	D	Q	D	K	S	G	F	I	E	E	D	E	L	K	L	F	L	K	N	F	S	P	S	A$_{73}$
10 Whit	4.50	$_{37}$G	K	S	A	D	D	I	K	K	A	F	V	F	I	D	Q	D	K	S	G	F	I	E	E	D	E	L	K	L	F	L	Q	V	F	K	A	G	A$_{74}$
11 Chub		$_{35}$A	K	S	A	G	D	V	K	K	A	F	E	I	I	D	E	D	K	S	G	F	I	E	E	E	E	L	K	L	F	L	Q	N	F	K	A	G	A$_{72}$
12 Carp	4.47	$_{37}$S	K	S	A	D	D	V	K	K	A	F	A	I	I	D	Q	D	K	S	G	F	I	E	E	D	E	L	K	L	F	L	Q	N	F	K	A	G	A$_{74}$
13 Carp	4.25	$_{37}$S	K	S	A	D	D	V	K	K	A	F	A	I	I	D	Q	D	K	S	G	F	I	E	E	D	E	L	K	L	F	L	Q	N	F	K	A	D	A$_{74}$
14 Carp	3.95	$_{37}$A	K	T	P	D	D	I	K	K	A	F	A	V	I	D	Q	D	K	S	G	F	I	E	E	D	E	L	K	L	F	L	Q	N	F	S	A	G	A$_{74}$
Node		K	K	S	D	40	D	L	K	K	I	F	K	I	L	D	Q	D	K	S	G	F	I	E	E	E	E	L	K	F	F	L	K	G	F	S	A	G	A	G
		2	3	4	5	6	7	8	9	50	1	2	3	4	5	6	7	8	9	60	1	2	3	4	5	6	7	8	9	70	1	2	3	4	5	6	7	8	9	80	1	2	3
								n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n				
1 Coel	5.40	$_{74}$A	A	G	R	E	L	T	A	N	E	T	K	A	L	L	K	A	G	D	Q	D	G	D	D	K	I	G	V	D	E	F	T	N	L	V	K	A	A$_{111}$			
2 Rabb	5.50	$_{72}$P	D	A	R	D	L	S	V	K	E	T	K	T	L	M	A	A	G	D	K	D	G	D	G	K	I	G	A	D	E	F	S	T	L	V	S	E	S$_{109}$			
3 Pike	5.00	$_{71}$A	D	G	R	D	L	T	D	A	E	T	K	A	F	L	K	A	A	D	K	D	G	D	G	K	I	G	I	D	E	F	E	T	L	V	H	E	A$_{108}$			
4 Ray	4.45	$_{72}$D	G	A	R	V	L	N	D	K	E	T	S	N	F	L	A	A	G	D	S	D	G	D	H	K	I	G	V	D	E	F	K	S	M	A	K	M	T$_{109}$			
5 Coel	4.52	$_{72}$A	G	A	R	T	L	T	K	T	E	T	E	T	F	I	K	A	G	D	S	D	G	D	G	K	I	G	V	D	E	F	Q	K	L	V	K	A$_{108}$				
6 Frog	4.50	$_{72}$A	S	A	R	V	L	S	D	A	E	T	S	A	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	E	E	F	Q	A	L	V	K	A$_{108}$				
7 Hake	4.36	$_{72}$A	G	A	R	A	L	T	D	A	E	T	A	T	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	E	E	F	A	A	M	V	K	G$_{108}$				
8 Cod	4.75	$_{72}$A	D	L	R	A	L	T	D	A	E	T	K	A	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	D	E	F	G	A	L	V	D	K	W	G	A	K	G$_{113}$
9 Pike	4.10	$_{71}$P	S	A	R	A	L	T	D	A	E	T	K	A	F	L	A	D	G	D	K	D	G	D	G	M	I	G	V	D	E	F	A	A	M	I	K	A$_{107}$				
10 Whit	4.50	$_{72}$A	G	A	R	A	L	T	D	A	E	T	K	A	F	L	K	A	G	D	S	D	G	D	G	A	I	G	V	E	E	W	V	A	L	V	K	A$_{108}$				

		2	3	4	5	6	7	8	9	40	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3
										00																							
												n			n	n			n	X		Y		Z	G		I	–X		–Z	n			n	n			n				
11 Chub		$_{70}$A	G	A	R	A	L	T	D	A	E	T	K	I	F	L	K	A	G	D	A	D	G	D	G	K	I	G	I	D	E	F	A	A	L	V	K	A_{106}			
12 Carp	4.47	$_{72}$A	G	A	R	A	L	T	D	G	E	T	K	T	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	D	E	F	T	A	L	V	K	A_{108}			
13 Carp	4.25	$_{72}$A	D	A	R	A	L	T	D	G	E	T	K	T	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	D	E	F	T	A	L	V	K	A_{108}			
14 Carp	3.95	$_{72}$A	G	A	R	A	L	T	D	A	E	T	K	A	F	L	K	A	G	D	S	D	G	D	G	K	I	G	V	D	E	F	A	A	L	V	K	A_{108}			
			G	A		V				A			S		F						S																W		
Node		A	D	G	R	D	L	T	D	K	E	T	K	A	L	L	K	A	G	D	K	D	G	D	G	K	I	G	V	D	E	F	5	4	L	V	K	A	T	G	
			A			E															Q																	A			

Table 3

AMINO ACID SEQUENCES OF OTHER EF-HAND DOMAIN PROTEINS

```
                                                             1
    1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  Ref.
                                                             0
                                                                   n        n  n        n  X     Y     Z  G     I  –X       –Z n        n  n        n

1                                         1S  A  E  K  S  P  A  E  L  K  S  I  F  E  K  Y  A^A K  E  G  D^P N  Q  L  S  K  E  E  L  K  Q  L  I  Q  A^EFPSL  L  K  G40  103
2                                            1A  K  K  S  P  E  E  L  K  G  I  F  E  K  Y  A^A K  E  G  D^P N  Q  L  S  K  E  E  L  K  L  L  L  Q  T^EFPSL  L  K  G39  77
3             33K  I  D  L  S  A  I  K  I  E  F  S  K  E  Q  Q  D  E  F  K  E  A  F  L  L  Y  D  R  T  G  D  S  K  I  T  L  S  Q  V  G  D  V  L  R  A  L  G  T  N  P82  72
4                                  1A  D  Q  L  T  E  E  Q  I  A  E  F  K  E  A  F  S  L  F  D  K  D  G  N  G  T  I  T  T  K  E  L  G  T  V  M  R  S  L  G  Q  N  P43  238
5                                  1A  D  E  L  T  E  E  Q  I  A  E  F  K  E  A  F  S  L  F  D  K  D  G  D  G  T  I  T  T  K  E  L  G  T  V  M  R  S  L  G  Q  N  P43  53
6          1M  D  D  I  Y  K  A  A  V  E  Q  L  T  E  E  Q  K  N  E  F  K  A  A  F  D  I  F  V^L G  A  E  D  G  C  I  S  T  K  E  L  G  K  V  M  R  M  L  G  Q  N  P51  239
7    1A  Q  P  T  D  Q  Q  M  D  A  R  S  F  L  S  E  E  M  I  A  E  F  K  A  A  F  D  M  F  D  T  D  G  G  G  D  I  S  T  K  E  L  G  T  V  M  R  M  L  G  Q  T  P53  240
8  (1S  A  M  T) D  Q  Q  A  E  A  R  A  F  L  S  E  E  M  I  A  E  F  K  A  A  F  D  M  F  D  A  D  G  G  G  D  I  S  T  K  E  L  G  T  V  M  R  M  L  G  Q  N  P53  257
9              (T  D  Q) Q  A  E  A  R  A  Y  L  S  E  E  M  I  A  E  F  K  A  A  F  D  M  F  D  A  D  G  G  G  D  I  S  V  K  E  L  G  T  V  M  R  M  L  G  Q  T  P50  206
10            1D  T  Q  Q  A  E  A  R  S  Y  L  S  E  E  M  I  A  E  F  K  A  A  F  D  M  F  D  A  D  G  G  G  D  I  S  V  K  E  L  G  T  V  M  R  M  L  G  Q  T  P50  43
11         1A  D  K  A  A  S  G  V  L  T  K  L  P  Q  K  Q  I  Q  E  M  K  E  A  F  S  M  I  D  V  D  R  D  G  F  V  S  K  D  D  I  K  A  I  S  E  Q  L  G  R  T  P51  116
12             ?  R  A  T  S  N  V  F  A  M  F  D  Q  S  Q  I  Q  E  F  K  E  A  F  N  M  I  D  Q  N  R  D  G  F  I  D  K  E  D  L  H  D  M  L  A  S  M  G  R  ?     115
13           10E  G  –  S  S  N  V  F  S  M  F  D  Q  T  Q  I  Q  E  F  K  E  A  F  T  V  I  D  Q  N  R  D  G  I  I  D  K  D  D  L  R  E  T  F  A  A  M  G  R^L N  V58  156
14           11E  G  G  S  S  N  V  F  S  M  F  D  Q  T  Q  I  Q  E  F  K  E  A  F  T  V  I  D  Q  N  R  D  G  I  I  D  K  E  D  L  R  D  T  F  A  A  M  G  R^L N  V60  155

                                        Q  L  T  E        I  A                    F        D        G           T  K  E  L     T     M              Q
N1                                4  5  E  F  S  K  E  Q  Q  D  E  F  K  E  A  F  3  L  Y  D  5  T  G  D  S  4  I  T  L  S  Q  V  G  D  V  L  R  3  L  G  T  N  P  137

             E  G     S     N     F  S  M  F  D     T        2     F              T  V        Q  N                 I  I  D           L  R  T  F  A  A  M           N  V
N2           D  K  3  A  S  G  V  L  T  K  L  P  Q  K  Q  I  Q  E  M  K  E  A  F  S  M  I  D  V  D  R  D  G  F  V  S  K  D  D  I  K  3  I  S  E  Q  L  G  R  T  P

                                                             2
                                                4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4  5  6  7  8  9  0  1  2  3  4
                                                             0
                                                                   n        n  n        n  X     Y     Z  G     I  –X       –Z n        n  n        n

1 ICBP pig gut                               38L  K  G  P  R  T  –  L  D  D  L  F  Q  E  L  D  K  N  G  D  G  E  V  S  F  E  E  F  Q  V  L  V  K  K  I  S  E  K  Q75
2 ICBP bov gut                               37L  K  G  P  ?  ?  –  L  D  Z  ?
3 ELC rabb skel                              80T  N  P  T  N  A  E  V  K  K  V  L  G^N P  S  D  E  Q  M  N  A  K^K I  E  F  E  Q  F  L  P  M  L  Q  A  I  S  N  N  K  D119
4 CDR bov brain                              41Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A  D  G  N  G  T  I  D  F  P  E  F  L  T  M  M  A  R  K  M  K  D  T  D80
```

Table 3 (continued)

AMINO ACID SEQUENCES OF OTHER EF-HAND DOMAIN PROTEINS

		5	6	7	8	9	200	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4
									n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n				
5	CDR rat testis	41 Q	N	P	T	(Z	A	Z	L	Z	B)	M	I	D	E	V	B	B	B	G	A	G	T	I	D	F	P	E	F	L	T	M	M	A	R	K	M	K	D	T	D_{80}
6	TNC bov heart	49 Q	N	P	T	P	E	E	L	Q	E	M	I	D	E	V	D	E	D	G	S	G	T	V	D	F	D	E	F	L	V	M	M	V	R	C	M	K	D	D	S_{88}
7	TNC frog skel	51 Q	T	P	T	K	E	E	L	D	A	I	I	E	E	V	D	E	D	G	S	G	T	I	D	F	E	E	F	L	V	M	M	V	R	Q	M	K	Q	D	A_{90}
8	TNC chick skel	51 Q	N	P	T	K	E	E	L	D	A	I	I	E	E	V	D	E	D	G	S	G	T	I	D	F	E	E	F	L	V	M	M	V	R	Q	M	K	E	D	A_{90}
9	TNC human skel	48 Q	T	P	T	K	E	E	L	D	A	I	I	E	E	V	D	E	D	G	S	G	T	I	D	F	E	E	F	L	V	M	M	V	R	Q	M	K	E	D	A_{87}
10	TNC rabb skel	48 Q	T	P	T	K	E	E	L	D	A	I	I	E	E	V	D	E	D	G	S	G	T	I	D	F	E	E	F	L	V	M	M	V	R	Q	M	K	E	D	A_{87}
11	RLC scallop	49 R	T	P	D	D	K	E	L	T	A	M	L	–	–	–	–	K	E	A	P	G	P	L	N	F	T	M	F	L	–	–	–	–	S	D	–	K	L	S_{78} –
13	RLC chick skel	56 R^L	N	V	K	N	E	E	L	D	A	M	I	–	–	–	–	K	E	A	S	G	P	I	N	F	T	V	F	L	T	M	F	–	G	E	–	K	L	K_{85} –
14	RLC rabb skel	58 R^L	N	V	K	N	E	E	L	D	A	M	M	–	–	–	–	K	E	A	S	G	P	I	N	F	T	V	F	L	T	M	F	–	G	E	–	K	L	K_{90} –
	Node ELC-CDR-TNC		Q	N					L	Q		M	I		E	V			D	G		G	T		D			E				M		R		M	K	D		
			T	T	P	T	4	A	E	V	K	4	V	L	4	P	S	D	E	Q	M	N	A	K	I	E	F	E	Q	F	L	3	M	L	3	A	4	S	N	N	3	D
	Node RLC		R	N	V	K	N	E	E	L	D	A	M	3	–	–	–	–	K	E	A	S	G	P	I	N	F	T	V	F	L	T	M	F	–	G	E	–	K	L	K	
			R	T	P	D	D	K	E	L	T	A	M	3	–	–	–	–	K	E	A	P	G	P	L	N	F	T	M	F	L	–	–	–	–	S	D	–	K	L	S	

		3	4	5	6	7	8	9	300	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	
											n			n	n			n	X		Y		Z	G		I	–X			–Z	n			n	n			n					
3	ELC rabb skel	118 K	D	Q	G	T	Y	E	D	–	F	V	E	G	L	R	V	F	D	K	E	D	G	T	V	G	M	G	A	E	L	R	H	V	L	A	T	L	G	E	K	M_{157}
4	CDR bov brain	79 T	D	–	–	–	S	E	E	E	I	R	E	A	F	R	V	F	D	K	D	G	N	G	Y	I	S	A	A	E	L	R	H	V	M	T	N	L	G	E	K	L_{116}
5	CDR rat testis	79 T	D	–	–	–	S	E	E	E	I	R	E	A	F	R	V	F	D	K	D	G	D	G	Y	I	S	A	A	E	L	R	H	V	M	T	D	L	G	E	K	L_{116}
6	TNC bov heart	87 D	S	K	G	K	S	E	E	E	L	S	D	L	F	R	M	F	D	K	N	A	D	G	Y	I	D	L	E	E	L	K	I	M	L	Q	A	T	G	E	T	I_{127}
7	TNC frog skel	89 D	A	E	G	K	S	E	E	E	L	A	E	C	F	R	I	F	D	K	N	A	D	G	Y	I	D	S	E	E	L	G	E	I	L	R	S	S	G	E	S	I_{129}
8	TNC chick skel	89 D	A	K	G	K	S	E	E	E	L	A	D	C	F	R	I	F	D	K	N	A	D	G	F	I	D	I	E	E	L	G	E	I	L	R	A	T	G	E	H	V_{126}
9	TNC human skel	86 D	A	K	G	K	S	E	E	E	L	A	E	C	F	R	I	F	D	R	N	A	D	G	Y	I	D	P	E	E	L	A	E	I	F	R	A	S	G	E	H	V_{126}
10	TNC rabb skel	86 D	A	K	G	K	S	E	E	E	L	A	E	C	F	R	I	F	D	R	N	A	D	G	Y	I	D	A	E	E	L	A	E	I	F	R	A	S	G	E	H	V_{126}

Table 3 (continued)

AMINO ACID SEQUENCES OF OTHER EF-HAND DOMAIN PROTEINS

	3 4 5 6 7 8 9	30	0 1 2 3 4 5 6 7 8 9	40	0 1 2 3 4 5 6 7 8 9	50	1 2 3
			n n n X Y Z G I–X –Z n n n n				
11 RLC scallop	$_{76}$S – G T D S E	E T	I R N A F A M F D E Q E N K K L N I E Y I K D L L E D M G N N				F_{117}
13 RLC chick skel	$_{68}$K – G A D P E	D V	I M G A F K V L D P D G K G S I K K S F L E E L L T T Q C D K				F_{127}
14 RLC rabb skel	$_{90}$K – G A D P E	D V	I T G A F K V L D P E G K G T I K K Q F L E E L L T T Q C D R				F_{139}
15 RLC bov heart						?	L T T Q A E R F
		E E	F G Y I A				
Node ELC-CDR-TNC	3 D 3 G 3 Y E	D –	3 4 E 4 L R V F D K 3 3 3 T V G 3 G A E L R H V L 4 3 L G E K 3				
	K	D V	G K V L P G K G I K K F L E E T T Q C D				
Node RLC	S – G T D 3 E	E T	I 3 N A F A M F D E 3 E N K 3 L N I 3 Y I K D L L E D M G N 3 F				

	5 6 7 8 9	40	0 1 2 3 4 5 6 7 8 9	50	1 2 3 4 5 6 7 8 9	60	1 2 3 4 5
			n n n X Y Z G I–X –Z n n n n				
3 ELC rabb skel	$_{155}$E K M K E	E E	V E A L M – A G Q E D S N G C I N Y E A F V K H I M S	I_{188}			
4 CDR bov brain	$_{114}$E K L T D	E E	V D E M I R E A N I D G D G E V N Y E E F V Q M M T A	K_{148}			
5 CDR rat testis	$_{114}$E K L T D	E E	V D E M I R E A N I (B G B G Z) V D Y Z Z F V E M M T A	K_{148}			
6 TNC bov heart	$_{128}$E T I T E	D D	I E E L M K D G D K N N D G R I D Y D E F L E F M K G V	E_{160}			
7 TNC frog skel	$_{127}$E S I T D	E E	I E E L M K D G D K N N D G K I D F D E F L K M M E G V	Q_{162}			
8 TNC chick skel	$_{127}$E H V T E	E D	I E D L M K D S D K N N D G R I D F D E F L K M M E G V	Q_{162}			
9 TNC human skel	$_{126}$E H V T D	E E	I E S L M K D G D K N N D G R I D F D E F L K M M E G V	Q_{159}			
10 TNC rabb skel	$_{124}$E H V T D	E E	I E S L M K D G D K N N D G R I D F D E F L K M M E G V	Q_{159}			
11 RLC scallop	$_{115}$N N F N K	D E	M R – M T F K F A P V P G G K F D Y V K F T A M I K – G S	G E D E	A_{153}		
13 RLC chick skel	$_{128}$D R F T P	E E	I K N M W A A F P P D V A G N V D Y K N I C Y V I T H G	E D K E	G E_{165}		
14 RLC rabb skel	$_{127}$D R F S Q	E E	I K N M W A A F P P D V G G N V D Y K N I C Y V I T H G	D A K D	E Q_{167}		
15 RLC bov heart	E R F S T	E E	I D Q M F A A F P P D V T G N L D Y K ?				
	T D	E	D E M M				
Node ELC-CDR-TNC	E K 3 K E	E E	V E A L M 3 3 G 3 3 D 3 N G 3 I N Y E A F V K H I 4 3 3 –				
	D	E I	K N W A A P D V N V K N I C Y V T H K				
Node RLC	N 3 F 3 3	D E	M R – M T F K F A P V P G G K F D Y V K F T A M I K – G 3 3	E D E 3			

shorter and its first eight residues — Ser Phe Ser Ala Asp Gln Ilu Ala — have five changes (underlined) relative to A1. In a few instances, private communications have indicated amide assignments or the correct sequence of residues reported only as compositions.

C. Structural Interpretations

1. Parvalbumins

The main chains of the various parvalbumins are assumed to be almost isostructural with that of carp 4.25 (Figure 1). Table 4 summarizes its structural characteristics with emphasis on the anticipated effects of alternate sequences. The conclusions of such an examination are summarized and several experiments suggested.

Of the 109 positions, 24 are invariant (Table 6). The side chains of three of these amino acids are exposed to the solvent. Ten are associated with the hydrophobic core of the molecule or have little access to the solvent. I see no reason for the invariance of the surface residues, nor why nine of the core invariants could not have another hydrophobic side chain. Ala_{205} could not be larger without unacceptable van der Waals contacts. Eight invariants are calcium ligands. As discussed in Section III.D, I do not understand why Asp and Glu cannot be replaced by Asn and Gln. The presence of Gly_{418} at X permits H_2O to coordinate the Ca^{2+} ion; however, Ala is small enough to permit H_2O access. The invariance and unique H-bonding network of the buried Arg_{395}-Glu_{401} pair suggest their essential, but not understood, role.

Seventeen positions are designated conservative in that only two different residues have been observed at that particular position. The replacement of Ser_{314} at Z by Glu (cod) will force a conformational change in the main chain and brings a fifth carboxylate into the coordination sphere. This situation is unique among all calcium-modulated proteins. In four of the eight conservative positions of the core, the alternate amino acid occurs in only one sequence. Several pairs differ by three carbon atoms in volume and surely induce a slight rearrangement of core packing. Residue 409 could not be larger than Ala without forcing a significant change in the main chain. At 317 the two Ilu residues (317 and 417) pack together and fix their respective loops to the core. In three of the eight conservative positions on the surface, the second amino acid occurs only once. Gly_{315}, as well as Gly_{415}, is oriented ($\phi \simeq 95°$, $\psi \simeq 10°$) so that any alternate amino acid would force some change. The deletion in ray of 210 and 214 will shorten the loop which covers the Arg_{395}-Gly_{401} H bond. For the other six conservative surface positions, I see no functional reason for their conservation. It is difficult to estimate whether these 13 invariant and 16 conserved positions (exclusive of Ca^{2+} ligands and 395—401) have special functional significance or whether they represent sampling statistics.

Ten core positions are variable; however, nearly all of the variation is confined to hydrophobic amino acids plus Ala and Gly. There will be some compensatory side chain movements associated with these substitutions. Fifty-eight surface residues are designated variable. Substitutions for Gly_{415}, as for Gly_{315}, will necessitate some change in ϕ and ψ angles. Four of these surface variable positions (196, 308, 324, and 416) are solely hydrophobic; I see no reason for this.

Five substitutions must cause changes in the main chain conformation relative to that of carp MCBP. Any substitute (e.g., Asp_{315} [hake], Asp_{415} [coel 5.4], and His_{415} [ray]) for Gly_{315} and Gly_{415} will change the Ca^{2+}-binding loops. The side chain of Gly_{314} (cod) is too large to fit into the space for the Z ligand. The 210 and 214 deletion (ray) in loop AB must make the Arg_{395}-Gly_{401} bond more exposed.

There is space for the extended C terminus of cod in either helical or nonhelical

Table 4
STRUCTURAL INTERPRETATIONS OF PARVALBUMIN SEQUENCES

	Domain 2 (AB)	Domain 3 (CD)	Domain 4 (EF)
31	Surface, variable; Gly-Lys-Ala-Ser parallelism	Surface, variable; ϕ −35°, Pro OK, nonhelical	Only cod has $_{430}$GAKG$_{433}$; room for extended F helix
30	Surface, variable; Lys-Ala-Ser parallelism	Surface, variable; last residue of α-helix	
29 n	Core; only Met of chub	Core; Phe invariant	Does F helix continue?
28	Surface; Gly, ϕ = 54, ψ = 53, why invariant? The terminus of helix D has an unusual distortion	Surface, variable; Cys (ray) exposed	Surface, variable
27	Core; Cys (whiting) should not react	Surface, variable	Surface, variable
26 n	Surface; Leu and Met (α), Ala(ray), Lys (β), cf. 204	Core, Leu invariant	Core; Ala (ray) −2 volume
25 n	Surface; variable	Core Ilu and Val (α); Phe (β)	Core; only Met of hake
24	Core; Phe$_{405}$ ring must move for -OH of cod	Surface; why hydrophobic except Asn (ray)?	Surface, variable
23	Deletion placed here for optimal alignment	Surface, variable	Surface, variable
22 n	Core; Phe invariant	Core; Leu invariant	Core; Trp (whiting) unique spectroscopic probe
21 −Z	Surface; variable; loop 2 not Ca^{2+}	Glu (invariant) length required to Ca^{2+}	Glu (invariant) length required to Ca^{2+}
20	Surface; variable	Surface; only Asp or Glu? Parallelism	Surface; only Glu or Asp? Not near Ca^{2+}
19	Surface; His = Tyr (unique in frog and whit); parallelism	Surface; hydrophilic except Val (ray)	Surface; why hydrophobic?
18 −X	Surface; variable	Glu invariant; Asp long enough?	Gly invariant; room for Ala; H_2O-Ca^{2+} critical?
17 I	Core; Phe (invariant)	Core; critical for loop Val (hake) only change	Core; critical for loop stabilization? Never Leu, Met, Val?
16	Deletion	Tyr (ray and coel. 5.4) spectroscopic probe for EF Ca^{2+}; surface; parallelism	Surface; Met (pike 4.1) unique, Met$_{428}$ (pike 4.1) core
15 G	Deletion	Surface; ϕ 96°, ψ 10°; Asp (hake) change main chain?	surface; ϕ 95°, ψ 14°; His (ray) and Asp (coel 5.4) change main chain?
14 Z	Surface, Ser H-bond donor in modified β-bend covering invariant Arg$_{334}$ -Glu$_{401}$ internal H bond; see ray 210 deletion	Glu (cod) side chain too long for Ca^{2+} coordination, change main chain? Fifth CO_2	Asp invariant
13	Surface, variable; Gly cannot H-bond with Thr$_{337}$ over Arg$_{334}$ as does Asp$_{213}$ in carp MCBP	Surface; ϕ 65°, ψ 18°	Surface; ϕ 47°, ψ 59° allowed region; cf. 313; why Gly invariant?
12 Y	Surface; variable	Asp invariant	Asp invariant
11	Surface; variable	Surface, variable	Surface, variable
10 X	Surface, variable; ray deletions 210 and 214, shortening the loop over the Arg$_{334}$ -Glu$_{401}$ H bond, and exposing it	H bond from Gly$_{318}$ precludes Asn	H bond from Gly$_{418}$ precludes Asn

Table 4 (continued)
STRUCTURAL INTERPRETATIONS OF PARVALBUMIN SEQUENCES

	Domain 2 (AB)	Domain 3 (CD)	Domain 4 (EF)
9 n	Cys in carp two sites of Hg binding; Phe (rabbit and coel 5.4) pack near Phe_{222} and Phe_{217} of core or swing to surface?	Core; Ala (cod) smaller	Core; Gly except Ala pike 5.0) OK, any larger group contacts Leu_{425}
8	Surface, variable	Surface; hydrophobic cavity for N terminus (193)?	Surface Ala except Asp; Asp (pike 4.1)
7	Surface, variable	Surface, variable	Surface; Lys-Ala parallelism
6 n	Core; Glu (cod) turn out to solvent, Leu_{394} compensate by replacing Ala_{394}	Partial core; why Phe invariant?	Partial core, variable
5 n	Partial core; invariant Ala; Val too large	Core	Core
4	Surface; Lys (α and ray), Ala (β), cf. 226	Surface, variable; Lys-Glu parallelism	Surface, variable; Ala-Thr parallelism
3	Surface; parallelism, Asp-Lys-Thr	Surface, variable	Surface, variable; Ser-Lys parallelism
2 n	Partial core	Core; Ala (frog) smaller	Partial core; why Thr invariant?
1	Surface; H bond from Lys_{220} which is variable; why Asp invariant?	Surface; Asp-Glu parallelism	Internal; Glu invariance predicted in all MCBPs, unique H bonds to Arg_{334}
0	Surface, variable	Surface, variable	Surface, variable; Asp-Glu parallelism
199	Surface, variable; ϕ −49°, Pro (ray) OK	Surface, variable; ϕ −43°, Pro (carp 4.4) OK	Surface, variable
98	Surface, variable; Asn-Lys-Ala-Ser parallelism	Surface, variable; ϕ −87°, Pro (coel 5.4) OK	Surface, variable; Ser-Thr parallelism; H bond donor to Asp_{203} (not Gly_{203}?)
97	Core; Leu, Val (frog)	Surface, variable; only Met (of pike 5.0)	Core; invariant Leu
96	Surface, hydrophobic; Ilu-Leu parallelism	Surface, variable; Gly-Lys-Ala-Ser parallelism	Surface, variable
95	Surface, variable; carp Gly, ϕ −137°, ψ 30°, allowed region		Internal; Arg invariance predicted in all MCBPs, unique H bonds to Glu_{401}
94	Partial surface, variable; the four N-terminal residues of carp (AcAsp, Phe, Ala, Gly_{198}) cannot be assigned with certainty in the election density		Internal; Leu (cod) compensatory change, Glu_{206} turn Outward, freeing usual Leu_{206} volume
93	Consistent hydrophophobic suggests that it points inward and is part of the core		Surface, variable
92	The variable length and sequence of the N terminus suggest that the main chain turns outward and the side chain of 193 points inward		Surface, variable; ϕ −35°, Pro OK, nonhelical

conformation. The position of the extended N terminus cannot be predicted, but there is no suggestion that the main chain of the remainder of the molecule is altered.

There are 95 side chain carbon atoms in the hydrophobic core of the carp structure. In addition, there are 22 nonhydrogen atoms on side chains contributing to the core. Several differences in sequence involve changes in core volume equivalent to several atoms. Except for two instances noted below, I cannot identify specific pairs of compensatory changes in sequence. However, even with all of these changes, the total core volume remains remarkably constant ranging from 116 (whiting) to 120 (hake) and an average of 117.6 ± 0.5 nonhydrogen atoms.

At internal position 394, Leu of cod replaces Ala or Gly; the Leu, Ilu, or Val of core position 206 would prevent the Leu_{394} from fitting in. However, 206 in cod is unique; its Glu certainly turns outward, thereby making room for Leu_{394}. The other apparent compensatory change involves positions 204 Lys, α-group (as defined by the cladogram, Figure 2) and ray, Ala elsewhere and 226 hydrophobic, α-group and ray, and Lys elsewhere. In the carp structure, Lys_{226} is H-bonded to the unexplained invariant Asp_{201}. Lys_{204} is in a position to bond to Asp_{201}; both Lys residues are never present in the same molecule. This Lys_{226} or Lys_{204} couple suggests that ray may belong in the α-group.

There are 13 examples of "parallel" evolution (P in Table 6); that is, one can infer from the cladogram that the mutation A → B occurred twice or that A → B → A occurred (Section III.G). Although one anticipates from statistics that this will occur occasionally, this frequency implies some selection. In none of these instances do I see a ready explanation.

2. Others

The four-domain model proposed for TNC[129] should also apply to CDR, and, with modification for insertions and deletions, to the various myosin light chains. Certainly CDR consists of four EF-hand domains. All of the sequence, physical, chemical, and evolutionary data are consistent with our postulate that the four hands occur as two pairs, 1-2 and 3-4, in a conformation similar to that of pair 3-4 in parvalbumin. Less well established is the relationship of pair 1-2 to pair 3-4. We suggested that the twofold axes of both pairs are colinear and that the hydrophobic cores of both pack together with Ca^{2+} ions one and two on the opposite side of the molecule from ions three and four. Table 5 summarizes the amino acid sequences of these proteins in terms of the proposed structure. This discussion focuses on the validity of the model; details of calcium coordination are discussed in Section III.D.

As is often the case with globular proteins, the termini of the α-helices of carp parvalbumin are noncanonical in both ϕ, ψ angles and in hydrogen bonding pattern. The first and last residues are best considered to be:

Helix E3: Ala_{299}-Ilu_{309}	Helix F3: Glu_{319}-Phe_{329}
Helix E4: Asp_{399}-Gly_{409}	Helix F4: Val_{419}-Ala_{428}

The C termini of helices E and the N termini of helices F are fixed by the geometries of the Ca loops; however, the other two termini are expected to vary in the different molecules.

The non-MCBP sequences are generally consistent with the postulate of a helix F. It is difficult to assign the C termini of helix F4 for the RLC; the amino acid compositions suggest residues 429 to 435, the C terminus of the molecule, to be nonhelical. Position 423 is predicted to be on the surface; yet in ELC, CDR, and TNC, it is Val or Leu, and position 223 is Leu, except Gln in ICBP. Helix F3 appears canonical regardless of whether loop 3 binds calcium. Whereas in MCBP position 329 is invariant

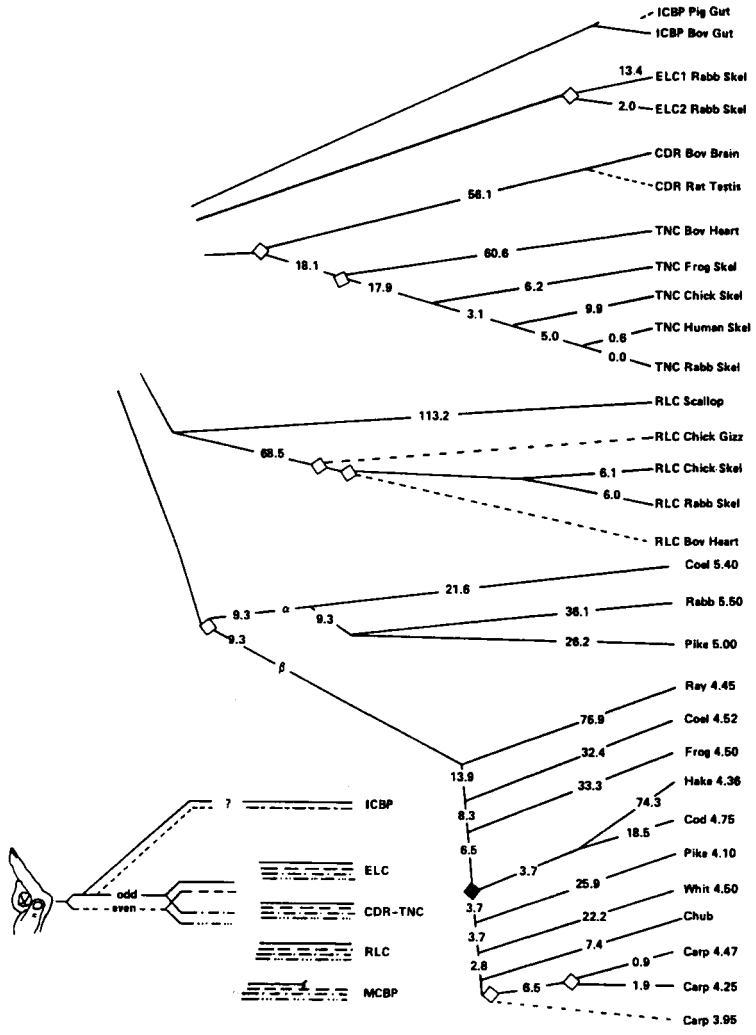


FIGURE 2. Evolution of EF hand domain proteins. The distances, expressed as nucleotide replacements per 10^{10} codon years, were calculated by M. Goodman. Dashed lines are my additions. Within the five groups, the calculated cladogram probably represents the phylogeny. I suggest that four families evolved by a pair of gene duplications with MCBP subsequently deleting domain 1. ICBP may have evolved from the original "odd-even" pair or from the four-domain precursor with a subsequent deletion of domains 1 and 2 or of domains 3 and 4.

Phe, in the other proteins 329 has various hydrophilic residues. In contrast, 129 is conserved Leu or Met. Helix F2 is distorted in the non-calcium-binding domains, by Pro_{224} in ELC and by deletions in the RLCs. Helix F1 appears canonical except for the three residue insertion at the terminus, about 128, of ICBP.

The calcium-binding loops having deletions (RLC-loop 2), insertions (TNC card loop 1 and ELC loop 2), or substitution (RLC Tyr Phe_{321}, RLC Pro_{216}, RLC scal Pro_{214}, RLC skel Pro_{410}) appear not to bind calcium. Loop 1 of ICBP has a two-residue insertion and has lost a ligand (Ala_{110} at X); yet, Levine et al.[145] reported two Ca^{2+}-binding sites on ICBP. The noncalcium-binding loops retain a hydrophobic group at position 17; it supposedly attaches the loop to the core of the molecule. At position 15 Gly occurs in all of the Ca^{2+} loops and in most of the nonbinding loops. Oddly, in

Table 5
STRUCTURAL INTERPRETATIONS OF THE SEQUENCES OF OTHER EF-HAND PROTEINS

	Domain 1	Domain 2	Domain 3	Domain 4
31		C terminus ICBP hydrophilic; Leu, RLC; hydrophilic other loops 2—3		C termini of RLCs extend beyond "helix F" as does cod MCBP; not α-helix
30		Met in TNC and CDR might point inward		Surface, variable; cf. Met_{130}
29 n	Hydrophobic; inside helix; ICBP insertion change helix and loop?	Surface, variable; only Ilu ICBP inside	Leu ELC and CDR inside of helix? Others nonhelical?	Val TNC inside of helix?
28	Surface, variable; why? Met TNC	Surface, variable	Surface, variable	Surface, variable; deletion RLC scal stop helix?
27	Surface, variable	Surface, variable	Surface, variable	Surface, variable
26 n	Core, hydrophobic; except Ser RLC scal	Core, hydrophobic	Core, hydrophobic	Core, hydrophobic
25 n	Core, hydrophobic; the RLC skel exposed	Core, hydrophobic; helix RLC scal deleted	Core, hydrophobic	Core, hydrophobic; except His ELC, buried?
24	Surface, variable	Surface; Thr Val parallelism; Pro ELC distort helix	His ELC and CDR exposed surface, variable	Tyr RLC skel exposed surface, variable
23	Surface, variable	Leu conserved except Gln ICBP; part of core with N terminus helix E1	Surface, variable	Surface? Val Leu CDR ELC and TNC; test Cys RLC skel
22 n	Core, hydrophobic	Phe invariant; also Phe MCBP	Core, hydrophobic, Leu conserved	Core, hydrophobic; Phe conserved
21 −Z	Glu-Ca^{2+}, ICBP, CDR, TNC; Asp-Ca^{2+} RLC	Glu-Ca^{2+}; Gln ELC, no Ca^{2+}	Glu-Ca^{2+}; others Phe or Tyr RLC scal buried	Glu-Ca^{2+}; variable others
20	Surface, variable	Surface variable; Pro CDR OK, $\phi = -47°$	Surface, variable	Surface, Asp or Glu in Ca loop; others vary
19	Surface, variable; as in MCBP, odd hydrophilic, even hydrophobic	Surface, why invariant Phe?	Surface, variable	Surface, why Tyr, Phe? Why hydrophobic?
18 −X	Only use of Thr CDR as ligand	Glu ELC not Ca ligand	Lys RLC, Met ELC not ligands	Asp even in RLC
17 I	Core; Ilu, Val, Leu; crucial loop stabilization	Core; Ilu, Val, Leu; as in MCBP only Ilu Val Ca binding	Core; Ilu, Leu; Gly ELC change loop?	Core; Ilu, Val, Leu, Phe
16	Surface, variable; insertion ICBP	Pro must change RLC loop; $\phi -143°$ $\psi -134°$; Lys insertion ELC	Surface, variable; Phe and Tyr monitor Ca 4	Surface, variable
15 G	Ser ELC non-Ca loop?	Ala ELC non-Ca loop?	Thr ELC, Lys RLCscal non-Ca loop?	Gly invariant $\phi \simeq 95°$, $\psi \simeq 15°$
14 Z	Gly TNC skel, H_2O ligand Ca?	Pro RLC scal change loop; $\phi -113°$; Ala CDR rat?	Lys and Asn RLC non-Ca	Gly and Ala RLC non-Ca
13	Surface, variable; Gly or Arg if Ca loop	Surface, variable; Gly if Ca loop	Surface, variable; Gly or Ala Ca loop	Pro RLC scal change loop; $\phi 65°$; Gly or Asn Ca loop

Table 5 (continued)
STRUCTURAL INTERPRETATIONS OF THE SEQUENCES OF OTHER EF-HAND PROTEINS

	Domain 1	Domain 2	Domain 3	Domain 4
12 Y	Ala TNC card not Ca ligand	Surface, variable non-Ca loops	Surface, variable non-Ca loops	Asp in non-Ca loops
11	Surface, variable; Leu TNC card insert, change loop	Surface, variable	Pro RLC skel OK; ϕ −45°	Pro RLC OK; ϕ −40°
10 X	Val TNC card and Ala ICBP not Ca ligand	Asp all except RLC deletion (alter loop and helix E-2)	Asp invariant even non-Ca loop; stop helix?	Pro RLC vert stop helix; ϕ −100°
9 n	Core, hydrophobic; Tyr ELC and ICBP spectroscopic probe	Core; Ser ELC buried? Deletion RLC	Core, hydrophobic	Core, cf. MCBP, Gly near Leu_{425}; Phe RLC alter packing
8	Surface, why hydrophobic? cf. 308 and 308 MCBP	Pro and Asn (insert) break helix ELC	Surface, why hydrophobic? cf. 108 and 308 MCBP	Surface, variable
7	Surface, variable	Surface, variable	Surface, variable; why Arg conserved?	Surface, variable; deletion ELC distort helix and loop
6 n	Partial core; Phe invariant; cf. 306 and 306 MCBP	Partial core, hydrophobic	Partial core, why Phe conserved?	Partial core, hydrophobics; Thr buried? Trp RLC-skel spect. probe RLC scal
5 n	Core; Ala, except Ilu ICBP; cf. 305	Core, hydrophobic	Core, frequent Ala as in MCBP 305	Core; Leu, Met parallelism
4	Surface, variable	Surface, variable	Surface, variable	Surface, variable; deletion RLC scal make 3_{10} helix?
3	Surface, why Lys invariant? cf. MCBP 303 and 304 Lys frequent	Surface, variable	Surface, variable	Surface, variable
2 n	Core, hydrophobic	Core, Leu except Val ELC	Core, hydrophobics	Core, hydrophobics
1	Surface, Glu invariant; cf. 01 others	Glu invariant, except deletion ICBP	Val, Thr ELC alter helix beginning? Deletion ELC affect loop 2 → 3	Surface, Glu or Asp
0	Surface, variable	Surface, variable	Surface; Glu, Asp parallelism	Surface; Glu, Asp parallelism
099	Surface, variable, Ilu still in helix, buried?	Surface, variable, Pro CDR cf. ICBP 099 and RLC chick 399	Surface; invariant? Why Glu	Surface, variable; Pro RLC chick isostructural ?
98	Surface, variable	Surface, variable, Pro ICBP cf. 298 RLC skel	Surface, variable; Tyr spectroscopic probe	Surface, variable
97	Hydrophilic; begin nonhelical N terminus?	Pro buried ?	Hydrophilic; deletion of three CDR, shorten loop 2 → 3	Hydrophobic, buried?
96	The N termini preceding helices E-1 have primarily hydrophilic residue, are probably not helical and cannot be located on the model	Hydrophilic, variable	Hydrophilic, variable	Hydrophilic; Hls RLC and trimethyllysine CDR spectroscopic probe of loop 3 → 4

Table 5 (continued)
STRUCTURAL INTERPRETATIONS OF THE SEQUENCES OF OTHER EF-HAND PROTEINS

	Domain 1	Domain 2	Domain 3	Domain 4
95		Hydrophilic; Leu RLC skel insert alter loop 1 → 2	Hydrophilic, variable	Hydrophilic, variable
94	Hydrophobics, except Ser ICBP; buried?	Gly_{194} critical for loop 1 → 2? cf. 394	Hydrophilic, variable; deletion RLC shorten loop 2 → 3	Gly_{394} critical for loop 3 → 4? Cys or Ala in RLC
93	Tyr, TNC human and rabb skel, spect. probe		Hydrophilic, variable	
92			$Leu_{232 \text{ or } 293}$ RLC correlate deletions 230 and 234	
91			Hydrophilic; Lys except Glu, Asn	
90			Met_{230} TNC, CDR buried? still in helix F-2	

3 of 28 Ca loops of parvalbumin, Gly_{315} or $_{415}$ is changed with an obligatory main chain movement. In parvalbumin, position 413 is invariant Gly, yet 313 is usually Lys. Among the nonparvalbumins, Gly_{13} always occurs in the Ca-binding loops of domains 1 and 2 and in CDR loops 3 and 4. Yet in TNC domains 3 and 4 there is an Ala_{313} and Asn_{413}.

Most of the residues 99 through 09 are consistent with the pattern expected from helix E of parvalbumin. Two helices have single deletions, domains RLC scal 4 and ELC 4, and one, ELC 2, a single insertion; it appears that these helices are retained. The RLCs have four residue deletions, 207 through 210, as well as deletions in their helix F2 regions. The entire domain 2 of RLC may contain no α-helix. None of the domains with insertions or deletions appears to bind calcium with the possible exception of ICBP 1. At surface positions 108 and 308 there are hydrophobic residues, as at MCBP 308, but hydrophilic ones at 208 and 408. However, in ICBP, a two-domain molecule, position 108 is Lys. Position 106 is invariant Phe and 306 all Phe, except Leu in ELC. Position 103 is invariant Lys, while 203, 303, and 403 are variable. Position 101 is invariant Glu, as is MCBP 401, and 201; 301 and 401 are frequently Glu. In parvalbumin this invariance is due to the Arg_{395}-Glu_{401} salt bridge. These nonparvalbumin Glu's may be involved in defining the N termini of helices E.

The three loops 1 → 2, 2 → 3, and 3 → 4 connecting the four domains are the least understood parts of the model. Loops 1 → 2 and 3 → 4 are postulated to be similar, at least in TNC and CDR. Helices F1 and F3 appear to include residues 29, and helices E2 and E4 include residues 01. Although parvalbumin has ten intervening residues, the seven intervening residues of TNC and CDR provide enough length to connect helices F_{odd} to E_{even}. Hydrophobic 397 of MCBP corresponds to hydrophobic 397 of all non-MCBPs and to Pro_{197} of CDR and TNC. If one assumes that the side chains of residues 397 and 197 occupy the same hydrophobic pocket between helices F_{odd} and E_{even} as does invariant Leu_{397} of parvalbumin, then the three-residue deletion cuts out the β-bend at the C terminus of helices E_{even} (Lys_{330}-Ala-Asp_{332}) in the carp structure. Leu residues 129 and 339 would retain a parvalbumin-like position as would Pro_{197} and $hydrophobic_{397}$. With only slight change and employing the ϕ, ψ range of Gly residues 130 and 330, the three residues, e.g., in CDR $_{330}$-Gly, Glu, $Lys_{332 \text{ or } 396}$, could

Table 6
CLASSES OF PARVALBUMIN SEQUENCE CHANGES

Surface							Core			Ca²⁺ and Ara-Glu	
Variable					Conserved	Invariant	Variable	Conserved	Invariant	Conserved	Invariant
429	399	311	226	207	420	413	426	425	417	314 M	421
428	398 P	308	225	203 P	408	228	406	422	402		418
427	396	307	221	200	407 P	201	394	409	397		414
424	393	304 P	220	199	320		325	405	329		412
423	331	303	219 P	198 P	316 P		309	317	326		410
419	330	301 P	218	196 P	315 M		305	229	322		401
416	328	300	213	195	214 M		302	224	306		395
415 M	327	299	212	194	204		227	197	222		321
411	324	298	211	193			206		217		318
404 P	323	297	210	192			202		205		312
403 P	319	231 P	209								310
400 P	313	230 P	208								

The lower value of 6.7 reported by Benzonana et al.[9] is due to its being determined in the presence of 2 m*M* Mg^{2+}. Potter et al.[194] found that MCBP binds two Mg^{2+} ions with $pK_d(Mg^{2+}) = 4.0$. This implies that in the unstimulated cell MCBP binds two Mg^{2+} ions. Sowadski et al.[224] found that in crystals of MCBP (2.8 *M* ammonium sulfate) Tb^{3+} first replaces the EF Ca^{2+} ion, which has one H_2O ligand, and then the CD Ca^{2+} ion. Various groups reported that both Ca^{2+} ions are bound with the same affinity; however, Pechère[186] reported that in the absence of Mg^{2+} there are two values of $pK_d(Ca^{2+})$, 8.3 and 6.7. Moews and Kretsinger[163] observed that the CD and the EF calcium-binding loops are connected by a hydrogen bond in a short stretch of antiparallel β-pleated sheet and that the CD and the EF hands are intimately opposed. This would seem an ideal structure for intramolecular cooperativity of Ca^{2+} binding. Benzonana et al.[9] and Pechère[186] felt their data might indicate slight cooperativity; however, Potter et al.[194] found none in their equilibrium dialysis studies. ^{13}C NMR studies[178] indicate that a one-Ca^{2+} ion form is present at levels higher than anticipated if Ca^{2+} binding were cooperative.

Intestinal calcium-binding protein (ICBP) from pig,[59] cow,[77,145] and rat[26] appear to bind two Ca^{2+} ions. This is particularly surprising since the loop of hand 1 has a two-residue insertion and has Ala at the X vertex. Chick ICBP with mol wt 27,000 binds four Ca^{2+} ions[18] and might be anticipated to be a four-hand homolog.

Most recent observations of TNC are consistent with the conclusions of Potter and Gergely[192] that skeletal TNC has two high-affinity sites $pK_d(Ca^{2+})$ 7.3, which also bind Mg^{2+}, pK_d 3.7, and two low-affinity sites $pK_d(Ca^{2+})$ 5.3, which do not bind Mg^{2+}. In whole troponin, these Ca^{2+} affinities increase to 8.7 and 6.7, respectively. Leavis et al.[136] reported that hands 3 and 4, isolated as a fragment following limited trypsin digestion, bind Ca^{2+} strongly (pK_d 7.8) and with positive cooperativity. Various other experiments support the interpretation that the high-affinity sites are in hands 3 and 4. It is generally agreed that in the resting muscle sites 1 and 2 bind no cation; however, sites 3 and 4 in whole troponin might contain either two Ca^{2+} ions or two Mg^{2+} ions.

Cardiac TNC has two high-affinity Ca^{2+} - Mg^{2+} sites in loops 3 and 4 and one lower affinity Ca^{2+} site, pK_d 4.3, in loop 2.[194] Loop 1 has Val and Ala at the X and Y vertices and does not bind calcium.

CDR binds four Ca^{2+} ions with a reported pK_d 5.6[194] or pK_d 6.0, n = 1 and pK_d 6.8, n = 3.[263] Further, Wolff et al. found that all four sites bind Mg^{2+} and suggested that, in the unstimulated cell, CDR is in the four-Mg^{2+} form. One site, $pK_d(Mg^{2+})$ 4.7, may retain Mg^{2+} during cell excitation. Proteins that appear to be CDR from bovine adrenal medulla[133] and from *Lumbricus*[245] have been reported to bind only two equivalents of calcium.

Calcium binding by myosin light chains is even more dependent on its association with myosin than is that of TNC upon association with TNI and TNT. In summary, it appears that the two ELC residues required for enzymic activity (e.g., alkali extractable light chains of skeletal muscle) do not bind Ca^{2+} either as isolated or as part of the myosin hexamer.

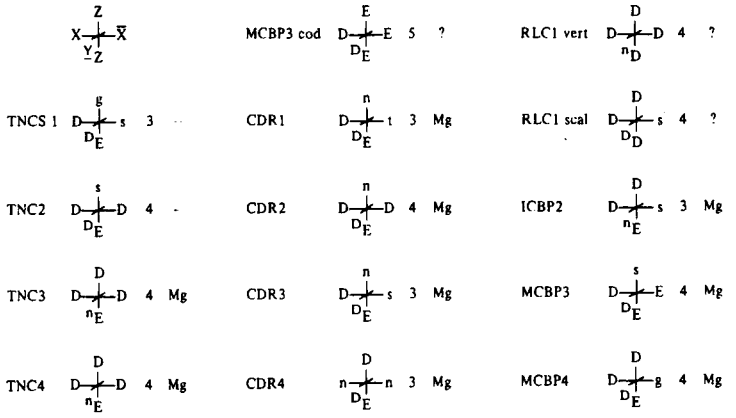
The regulatory light chains (RLC) each bind one Ca^{2+} ion when associated with the heavy chain. The DTNB-LC of chicken gizzard and the EDTA-LC of scallop striated muscle restore calcium binding to desensitized scallop myosin. The two DTNB-LCs of rabbit skeletal muscle are responsible for binding two Ca^{2+} ions in the myosin hexamer.[69]

The following correlations come from an examination of these Ca^{2+}-binding characteristics in terms of the available sequence information:

1. In order to bind Ca^{2+}, a loop needs side chain oxygen atoms at five vertices —

X, Y, Z, −X, −Z. Recall a peptide oxygen coordinates at −Y. One vertex can lack a side chain ligand if Gly is at that position, thereby allowing H_2O to coordinate Ca^{2+} (Gly_{418} at −X of MCBP and, by inference, Gly_{114} at Z of skeletal TNC). This correlation suggests that the Ca^{2+} ion is bound in loop 1 of RLC and that loop 1 of cardiac TNC does not bind Ca^{2+}. However, it would also suggest that ELC would bind two Ca^{2+} ions, whereas it binds none. Two other inconsistencies should be emphasized. The reported sequence of testis CDR is identical (save for amide assignments) to that of brain CDR, except that whereas Vanaman et al.[238] reported Ala · Asp · Gly · Asn_{214}, Dedman et al.[53] found Asx · Asx · Gly · Ala_{214} in testis CDR. This implies that Ala is at the Z vertex and that the loop coordination of the Ca^{2+} ion is significantly different from the MCBP EF-hand pattern. The second inconsistency is loop 1 of ICBP where Ala_{110} is at the X vertex.

2. In order to bind Ca^{2+}, a loop cannot contain deletions, as in loop 1 of MCBP and loop 2 of RLC, or insertions, as in loop 1 of cardiac TNC and loop 2 of ELC. The sole exception appears to be loop 1 of ICBP which includes a Pro and a two-residue insertion. If loop 1 does indeed bind Ca^{2+}, the coordination number and pattern may be entirely different from that observed in MCBP.



3. The "established" calcium-binding loops — MCBP 3 and 4; ICBP 2; CDR 1, 2, 3, and 4; TNC skeletal 1, 2, 3, and 4; TNC cardiac 2, 3, and 4; and RLC 1 — have the following distribution of ligands. (I indicate the number of carboxylate ligands and the ability of the loop to bind Mg^{2+}.) Ser occurs at only Z and −X and Thr at only −X in CDR loop 1. Thr and Ser are the shortest ligands with oxygen bonded to the β-carbon atom; they never occur twice in the same loop. Asp is always found at X, except for Asn in CDR loop 4. Glu, with oxygen bonded to the δ-carbon atom, is always found at −Z, except for Asp in RLC loop 1. MCBP has Glu at −X in loop 3 (Gly in loop 4), while the others have shorter side chains. Cod MCBP is unique, being the only Ca^{2+}-modulated protein with Glu at Z; the other parvalbumins have Ser, two carbons shorter, at this position. Also, it is the only Ca^{2+}-modulated protein with five liganding carboxylate groups. ICBP loop 1 contradicts the pattern of ligand distribution noted above by having Glu at Y. ELC loop 1 has Thr at Y and Gln at −Z.

4. The sum of carboxylate groups in the "established" loops is usually 4 or 3. In cod MCBP there are five carboxylates. ICBP loop 2 has only three carboxylate ligands at (potential) vertices, although Glu_{120} is adjacent to Glu_{121} at Z. ELC loop 1, which does contain five oxygen (potential) ligands but appears not to

bind Ca^{2+}, has only two carboxylate groups. I see no correlation between total charge and calcium Ca^{2+} affinity or selectivity.

5. The total charge distribution within the loops (residues 10 through 21) for ligands and nonligands ranges from six carboxylate groups and zero Lys or Arg (TNC loop 2) and eight carboxylate groups and one Lys (cod MCBP loop 3) to three carboxylate groups and two Lys (CDR loop 1). I see no correlation between net charge of the loop and calcium affinity or selectivity.
6. All Ca^{2+}-binding loops have Ilu or Val at position 17. The hydrophobic side chain appears to attach the loop to the hydrophobic core.
7. All Ca^{2+}-binding loops have Gly at position 15 except for hake MCBP Asp_{315}, coelacanth 5.40 MCBP Asp_{415}, and ray MCBP His_{415}. In the carp MCBP crystal structure, the ϕ, ψ values 96°, 10° of Gly_{315} and 95°, 14° of Gly_{415} indicate some change in loop structure associated with any substitution for Gly. Neither ELC loop 1 nor ICBP loop 1 have Gly at 15.
8. Potter et al.[194] suggested that the occurrence of Gly at 13 correlates with Ca^{2+} specificity, while any other residue indicates Mg^{2+} binding as well. Although their suggestion is valid for cardiac and skeletal TNC, the subsequent report of Mg^{2+} binding by CDR[263] contradicts the suggestion. The available data indicate no correlation between Mg^{2+} binding and glycine at position 13.

Gly_{13}:	CDR-1	CDR-2	CDR-3	CDR-4	MCBP-4	ICBP-2	TNC-1	TNC-2
Mg^{2+}:	+	+	+	+	+	?	—	—
$Other_{13}$:	RLC-1	TNC-3	TNC-4	MCBP-3				
Mg^{2+}:	?	+	+	+				

Two interesting questions follow from their suggestion. Why are TNC-1 and TNC-2 the only loops that do not bind Mg^{2+} with $pK_d(Mg^{2+})>3$? What, if any, metal ions are bound to the various calcium-modulated proteins in the resting cell?

E. Functional Properties

Descriptions of crystal structures and amino acid sequences inevitably impart a static image. To the contrary, spectral studies indicate significant vibrations and oscillations within the structure. Further, all five — ICBP, MCBP, RLC, TNC, and CDR — undergo significant conformational changes upon binding calcium. As with the non-EF-hand calcium-binding proteins the calcium bound structure is more resistant to thermal denaturation and to proteolysis.

Levine et al.[144] followed the calcium titration of rabbit skeletal TNC by ^1H NMR spectroscopy. The conformation of apo-TNC is generally similar but not identical to that of TNC-Ca_4. A significant change in main chain conformation accompanies the TNC-Ca_0 → TNC-Ca_2 transition. Subsequent calcium binding at the low-affinity sites, domains 1 and 2, causes changes in side chain conformation. In contrast, Potter et al.[194] suggested that their circular dichroism measurements showed that 35% of the change in main chain conformation results from the TNC-Ca_2 → TNC-Ca_4 transition. Potter et al.[193] suggested that the large entropic change (14.7 kcal/deg · mol) associated with calcium binding to the high-affinity sites, domains 3 and 4, is due to water displacement associated with α-helix formation.

Levine et al.[145] interpreted their ^1H NMR spectra of bovine ICBP as indicating that, even in the no-Ca^{2+} state, there is enough defined structure to perturb the Tyr resonance. The first bound Ca^{2+} ion has a slow (10/sec) on-off rate and affects the Tyr_{109} signal of domain 1. I would have anticipated that domain 2 would have the stronger

calcium affinity. The second Ca^{2+} is less tightly bound and has an exchange rate $>10^2$/sec. Three Phe side chains (196, 206, and 222) and probably a fourth (129) are predicted to be buried, but not the fifth (219) and, in fact, four show increasing interactions with addition of two equivalents of Ca^{2+}.

There is yet no firm evidence that either ICBP or MCBP function by binding to other proteins. The myosin light chains probably never occur free of myosin nor TNC free of the troponin trimer — TNC, TNI, TNT. The CDR occurs free in the cytosol in the no-Ca^{2+} form and complexed with one of its targets in the four-Ca^{2+} form. All three proteins — RLC, TNC, and CDR — undergo conformational changes upon interaction with the other proteins of their respective heterooligomeric complexes.

Potter et al.[195] found that the binding of a spin label or a fluorescent probe to Cys_{305} of rabbit skeletal TNC did not change its calcium-binding affinity. Ca^{2+} or, to a lesser extent, Mg^{2+} binding reduces the mobility of the spin label and increases the fluorescence of the probe. Both of these effects, as well as calcium affinity, are enhanced by the interaction of TNC with TNI, which binds to domain 4, and with TNT, which binds to domain 3.[136]

The dynamic aspects of the parvalbumin structure were discussed in Section II.E in the context of its possible function(s). One general and very important question has been asked particularly of the parvalbumins. To what extent does the conformational stability and the calcium affinity depend on the sequence within a single EF-hand domain or a pair of hands, and to what extent on the total protein? The Arg_{395} modification[87] and Cys_{209}[58] response to calcium binding support the interpretation that the entire structure is responsive to and necessary for high-affinity Ca^{2+} binding. Maximov et al.[157] synthesized an EF-hand domain (tabulated below)

	012345678901n34nn78nX1Y3ZG6I$\bar{X}$90$\bar{Z}$n34nn78n012345678901
Synthetic	EQTDDEIKEVLKAFDKDGGGRIDFEEFVKLILGVTGEGAR
MCBP 4	ALTDGETKTFLKAGDSDGDGKIGVDEFTALVKA
TNC 3	MKEDAKGKSEEELAECFRIFDRNADGYIDAEELAEIFRASGEHVTDEEIESL
TNC 4	ASGEHVTDEEIESLMKDGDKNNDGRIDFDEFLKMMEGVQ

and found that it retains $^{45}Ca^{2+}$ in G-25 chromatography under conditions where domain 4 (residues 396 through 428) of carp 4.25[221] does not. Leavis et al.[136] found that domain 3 of rabbit skeletal TNC retains its calcium affinity (pK_d 5.4, cf. holo TNC pK_d 7.3) and its ability to interact with TNT but loses its Mg^{2+} affinity. Domain 4 also retains its calcium affinity (pK_d 4.4, cf. holo TNC pK_d 7.3) and its ability to interact with TNI. The domains 3 and 4 fragment retains full Mg^{2+} and Ca^{2+} affinity (pK_d 7.7). These results and the four sequences indicate that calcium affinity depends on both the domain sequence and the interdomain interactions.

F. Phosphorylation

There appear to be two, and only two, second messengers in most, if not all, eukaryotic cells. The known interactions between the cyclic nucleotides and the calcium systems involve, in one sense, the regulatory effect of CDR on adenylate cyclase, phosphodiesterase, and myosin light chain kinase (Section II.B). In another sense, it appears that all cAMP effects are realized via activation of cAMP-dependent protein kinase. Of particular relevance to the calcium-modulated processes, the myosin RLCs and both TNT and TNI, but not TNC, are phosphorylated. Earlier reports of phosphorylation of parvalbumin have been refuted,[55] and those of CDR-like protein phosphorylation[22,262] have not been confirmed.

RLC from various vertebrate sources (human platelets,[1] guinea pig vas deferens,[33] rabbit white skeletal muscle,[75,169] and pig stomach[217]) can be phosphorylated at Ser_{088}

in the domain numbering system. Even though position 088 is also Ser in scallop RLC, it is not phosphorylated. Kendrick-Jones and Jakes[116] suggested that the length of the N terminus is related to the suitability of Ser_{088} as a light chain kinase substrate. They removed the N terminus of chicken gizzard RLC through Arg_{085}; "the modified light chain loses its ability to be phosphorylated." In scallop RLC, Ser_{088} is the sixth residue; whereas in chick RLC, it is the 13th and in rabbit RLC the 15th (Table 3). The Ser_{088} (37th residue) of ELC A1 is not phosphorylated. Ser_{093} is the N terminal residue of ELC A2.

Experiments concerning the physiological significance of the vertebrate RLC phosphorylation are complicated by the existence of both CDR-activated myosin light chain kinase and by endogenous phosphatases and kinases of unknown specificity. Morgan et al.[169] treated the myosin of rabbit skeletal muscle with myosin light chain phosphatase (70,000 mol wt); removal of all phosphate produced no change in either basal or actin-activated myosin of HMM ATPase activity due to phosphorylation. In contrast, Chacko et al.[33] found a fivefold increase in actin-activated ATPase activity from vas deferens muscle. Small and Sobieszek[217] also found phosphorylation of myosin (RLC) necessary for actin activation of myosin. Whereas Chacko et al.[33] reported that Ca^{2+} increased the actin-activated ATPase activity of previously phosphorylated guinea pig vas deferens myosin, Small and Sobieszek[217] and Hartshorne et al.[95] found no such effect with gizzard myosin. Hartshorne et al. suggested that in smooth muscle phosphorylation is essential for activity and that calcium exerts its regulatory effect solely via phosphorylation and dephosphorylation each contraction cycle.

As extracted, vertebrate RLC is usually a mixture of the phosphorylated (called ML3) and nonphosphorylated (ML2) forms. It is not clear which form is effective in resensitizing desensitized scallop myosin, nor is it clear as to how phosphorylation of RLC Ser_{088} effects skeletal or smooth muscle activity.

Both TNT and TNI are phosphorylated to varying extents as isolated from muscle. Both serve in vitro as substrates for cAMP-dependent protein kinase and phosphorylase kinase as well as for phosphatase. The physiological significance of this phosphorylation remains unknown. England[67] showed that in rat hearts both the force of contraction and the extent of TNI phosphorylation increase in parallel in response to perfusion with adrenaline over the range 3×10^{-8} to 3×10^{-6} *M*. Wilkinson and Grand[258] recently determined the amino acid sequence of chicken fast skeletal muscle (CF) TNI and discussed the three other known TNI sequences — rabbit fast (RF), rabbit slow skeletal (RS), and rabbit cardiac (RC) — using the numbering of the longest sequence, RC. RC-TNI has 26 additional N terminal residues relative to CF, RF, and RS. Ser_{20} of RC-TNI is phosphorylated, but position 20 is missing in CF, RF, and RS. Ser_{146} is phosphorylated in RF and in RC-TNI; Ser_{146} in CF and in RS-TNI is assumed to be phosphorylated as well. Ser_{116} of RF-TNI is phosphorylated, but neither Thr_{116} of CF nor Asn_{116} of RS or RC-TNI appears to be modified. Thr_{116} and Ser_{46} can be phosphorylated in RF-TNI, and the same residues are assumed to be phosphorylated in CF and in RS-TNI. However, Ser_{37} and Thr_{46} of RC-TNI are not phosphorylated.

Several phosphorylation-blocking and fragment-binding studies are summarized by Wilkinson and Grand[259] as indicating that the TNC-binding region spans residues 26 to 75 and the actin-binding region spans residues 123 to 145, i.e., up to Ser_{146}. Solaro et al.[223] showed that Ser_{201}, which is unique to RC-TNI, is phosphorylated by cAMP protein kinase. Moir and Perry[166] suggested that Ser_{20} and Ser_{140} of RC-TNI are the normal sites for cAMP-dependent protein kinase. As isolated, RC-TNI has 1.9 equivalents of bound phosphate; RF-TNI has 0.5.

The significance of TNT phosphorylation is even less well understood. It has three

sites of potential phosphorylation — Ser_1, Ser_{149}, or Ser_{150}, and Ser_{156} or Ser_{157} — by phosphorylase kinase. As isolated, only Ser_1 is phosphorylated, at 60 to 70%. All three sites are protected from in vivo phosphorylation by TNC.[165]

G. Cladogram and Evolutionary Implications

Figure 2 is a cladogram showing the taxonomic relationships of the sequenced EF-hand domain proteins. It is based on those published by Goodman and Pechère,[85] Pechère,[186] and Barker et al.[7] I am particularly grateful to Dr. Goodman for giving me his most recent calculations and for discussing its implications with me. He bears no blame for my more speculative interpretations concerning the origin of the EF hand and its pattern of gene duplication nor for my inclusion of 2 (ICBP bov gut), 12 (RLC chick gizz), and 15 (RLC bov heart) based on their partial sequences.

The actual computational techniques are complicated and are described or cited in the above references. However, several of the underlying principles should be kept in mind when considering such models.

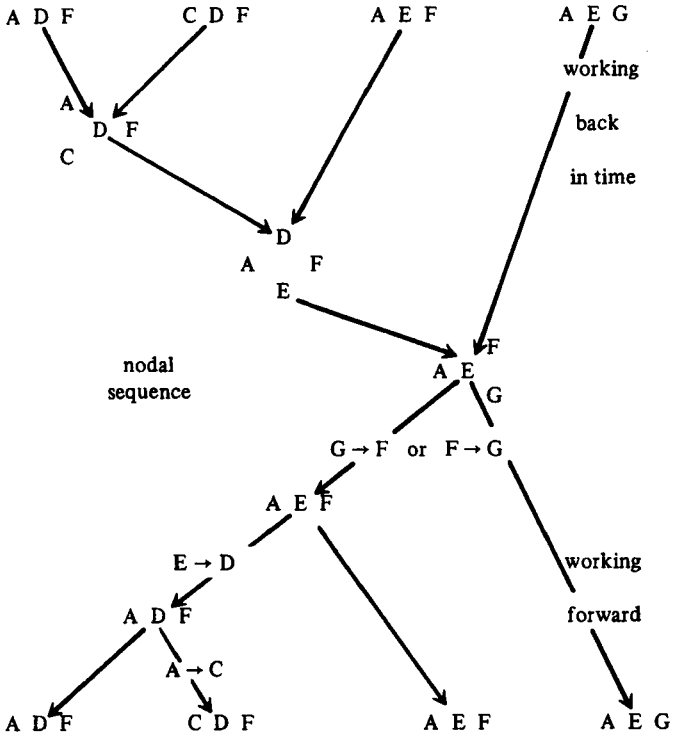
1. The distances on the maximum parsimony cladogram or "tree" represent the minimum number of nucleotide replacements per 100 codons required to go from one external point or node to another.

2. Deletions or insertions are scored as a single mutational event, even though one cannot assess relative frequencies or structural impacts of these events.

3. In order to infer phylogeny from the taxonomy of the proteins, one assumes that the proteins accept point mutations randomly at a constant average rate, characteristic of that protein. This appears to be the case for a family of proteins such as the cytochromes c, which serve similar functions in similar molecular environments in different organisms. This constancy of rate may obtain within the parvalbumins but certainly does not between the various EF-hand proteins. Further, the rates of accepting point mutations and deletions vary for different EF-hand domains within one protein and at different residues within one domain.

4. The distances associated with the branches of the tree, even if there is a constant rate of accepting point mutations, have large but difficult-to-calculate statistical errors associated with them. Frequently the topography of a tree is critically dependent on a short branch length with a large error.

5. The most probable nodal sequence lends additional insight into distantly related homologs, into essential structural features, and into the actual evolutionary pathway. The basic idea of minimizing the required number of mutational events and generating a nodal sequence is illustrated. These conclusions are most reliable when there are many observations and short branch lengths. Note also that the most divergent protein exerts the greatest leverage on the earliest nodal sequence. Although one cites an average rate of evolution for each protein family, in fact each amino acid position has its own characteristic rate. Some are invariant; some are subject to great and rapid variation. The nodal residues deduced at rapidly varying positions are much less reliable. As will be discussed, the degree of invariance is assumed to be related to the functional importance of a particular residue. In the nodal sequences of Tables 2 and 3, an underlined residue is invariant; a single residue is deduced as described above. Twofold ambiguities are indicated by listing both residues, higher-fold ambiguities by the symbols "3" or "4".



6. Since the nodal sequence represents a protein that existed earlier in time, it is more meaningful to compare, for instance, the nodal sequences of the parvalbumin precursor and the TNC precursor than to compare a specific parvalbumin and a specific TNC.

The cladogram is a reasonably accurate representation of the phylogeny of the parvalbumins, of the regulatory light chains (RLC), and of the TNC-CDR group. However, the evolutionary relationships among the different groups of EF-hand proteins are less certain.

Goodman (manuscript in preparation) has calculated that the rate of evolution of parvalbumins from the earliest gnathostomes to the teleosttetrapod α and β subfamily ancestor (424 to 400 × 10^6 years ago) was rather high — 90 nucleotide replacements per 10^{10} codon years (NRT). From the teleost-tetrapod divergence to the present the rate is about 9 NRT. Similarly, for the RLC-ELC-TNC group, Goodman has calculated a marked slowing in the evolutionary rate — 18 NRT, vertebrate-invertebrate branch to the amniote ancestor for RLC (680 to 300 × 10^6 years ago) compared with 3 NRT for the amniote ancestor to the present for TNC. We do not know whether the functions of these EF-hand proteins have changed during these periods, but their rates of evolution have slowed significantly.

The EF-hand family is unique in its diversity. Representatives bind from zero to four calcium ions and contain two, three, or four domains. They interact with a wide variety of other proteins — TNT, TNI, myosin heavy chain, phosphodiesterase, adenylate cyclase, membrane (Ca^{2+}) ATPase, and light chain kinase, none of which appear to be homologous to one another.

A comparison of individual domains, as opposed to entire proteins, shows very clearly that the even hands (2 and 4) are more closely related to one another than they

are to the odd hands (1 and 3). This observation is consistent with a primordial odd-even pair; a subsequent duplication produced a pair of pairs — domains 1 and 2 and domains 3 and 4. There is no compelling evidence that the pair duplication occurred more than once in evolutionary history. The parvalbumin structure shows excellent steric fit between the two hands of the pair. Nonetheless, domain 2 of MCBP, which has lost its ability to bind calcium, has assumed a slightly different conformation and a very different packing relative to hands 3 and 4. I regard the suggestion by Derancourt et al.[57] that "after the first duplication, it (parvalbumin) will have reached a state comparable to the 1-75 fragment" (domains 2 and 3) to be very improbable. One might anticipate altered hand conformation and interdomain packing in the non-calcium-binding loops of the light chains. The deletions in domain 2 of ELC, which is postulated to pair with the calcium-binding hand 1, cast doubt on the generality of the four-hand model which appears valid for TNC and CDR. Also, the hydrodynamic studies of Stafford and Szent-Györgyi[226] suggest an elongated molecule very different from that of the TNC, CDR model.

The evolution of ICBP is unclear. As MCBP evolved from a four-domain precursor by deletion of domain 1, so ICBP may have evolved by deletion of a pair of hands. Domain comparisons indicate a slightly, and probably not significant, closer relationship of ICBP to pair 1-2 than to pair 3-4 of the four-hand proteins. Alternatively, ICBP may have diverged prior to the basic pair duplication event and evolved directly from the original odd-even pair.

It is fascinating to contemplate the variety of structures and functions assumed by the EF-hand proteins. Yet all of these proteins, even those such as ELC which has lost the ability to bind calcium, share the common property of being associated with calcium-modulated processes. I have argued that the evolution of these calcium-modulated processes and of the EF-hand proteins are strongly coupled. It will be interesting to test the generality of this theory and to discover whether any calcium-modulated processes (Section IV.D) do not involve EF-hand proteins or whether any calcium-modulated proteins do not contain EF hands.

IV. OTHER POSSIBLE CALCIUM-MODULATED PROTEINS

A. Overview

I have suggested two criteria defining a calcium modulated protein. It is present in the cytosol or on a membrane bounding the cytosol. Its calcium affinity, under physiological conditions — pH $\sim$ 7, $pMg^{2+} \sim 3$, $pK^{+} \sim 1$ — is in the range $pK_a(Ca) \sim 5.0$ to 6.5. That is, in the quiescent cell ($pCa \simeq 7.5$) it does not bind, Ca^{2+}; in the "excited" cell ($pCa \simeq 4.0$) it does bind Ca^{2+}.

The following proteins may be calcium modulated. For some, the calcium affinity is not well determined; for others, the cellular location is unknown. The amino acid sequences are not known for any of these possibly calcium-modulated proteins. None of the established EF-hand proteins (CDR, TNC, RLC, ELC, MCBP, or ICBP) are enzymes. I have predicted that some of the calcium-modulated enzymes contain EF hands and that they have evolved by splicing the gene for an EF hand with the gene for a nonhomologous protein. This does not imply that the Ca^{2+} ion is near the active site. Indeed, precedents from extracellular calcium-binding enzymes[130] indicate that the Ca^{2+} ion is seldom, if ever, bound at the active site.

Finally, I emphasize that there are many extracellular and mitochondrial enzymes that bind calcium but are not calcium modulated. None of them appears to contain EF hands (see review by Kretsinger[124]).

B. Enzymes

1. Adenosine Triphosphatase (Ca^{2+}) of the Sarcoplasmic Reticulum

All eukaryotic cells, and probably all prokaryotes as well, have mechanisms for extruding Ca^{2+} from the cytosol. Some depend on exchange with a preformed Na^+ gradient, e.g., squid axon,[11] others upon a preformed H^+ gradient, e.g., mitochondria[171] or bacteria.[235] Many cells, including prokaryotes,[120] have a (Ca^{2+}) ATPase associated with either their plasma membrane or with the membrane of an internal membrane system such as the endoplasmic reticulum. The evolutionary or functional relationship among these ATPases is not known; however, it is assumed that most of them are associated with calcium pumping. In Section IV.B.2, I list these various ATPases; here I describe in more detail the structure of the (Ca^{2+}) ATPase from sarcoplasmic reticulum (SR), the best characterized active transport system.

Martonosi and Jilke[154] have summarized evidence that SR ATPase functions as an oligomer. Reconstitution experiments (reviews by MacLennan and Holland[149] and Hasselbach[97]) indicate that no additional proteins are required for Ca^{2+} transport. The ATPase accounts for 50% of the SR mass, and phospholipid accounts for 30%. Although the ATPase binds several equivalents of phospholipid with high affinity, these can be replaced with various phosphatidylserines or phosphatidylethanalamines in synthetic liposome systems with little loss of function capacity. The net equation is as follows (review of suggested steps, Kretsinger[124]):

$$ATP^{4-}{}_{out} + 2\,Ca^{2+}{}_{out} + Mg^{2+}{}_{in} + 2K^{+}{}_{in} \rightleftarrows ADP^{3-}{}_{out} + P^{-}{}_{out} + 2\,Ca^{2+}{}_{in} + Mg^{2+}{}_{out} + 2K^{+}{}_{out}$$

Makinose[151] demonstrated that this reaction can be reversed in vitro, as it is in vivo, to produce ATP from a Ca^{2+} gradient. The energy used in a cycle of Ca^{2+} release and uptake is partially recovered; it is not all dissipated as heat. The intact system appears to pump as long as ATP and Ca^{2+} ($pCa^{2+} < 7.5$) are present; calcium release requires ADP and phosphate. It is not understood how a nerve impulse initiates the release of Ca^{2+}. The unstimulated calcium efflux is very low, 3×10^{-13} $M/cm^2 \cdot sec$.

Limited trypsin digestion cleaves the 115,000 mol wt ATPase into fragments B (mol wt 55,000) and A, which can be further cleaved to yield A_1, (mol wt 33,000) and A_2 (mol wt 24,000). Fragment A_1 has ATPase activity and is phosphorylated, supposedly in conjunction with Ca^{2+} transport. However, Schwartz et al.[214] have reported a phosphorylase b kinase-dependent phosphorylation of the SR (see Section IV.B.3. Fragment A_2, at the N terminus, functions as a Ca^{2+} ionophore. Fragment B, at the C terminus, is the most hydrophobic of the three.

Allen[4] and Green[266] have tentatively determined the amino acid sequence of five segments accounting for 658 of 1000 residues in the molecule (Table 8). I cannot detect an EF hand.

2. Other (Ca^{2+}) ATPases

Calcium ATPase activity has been identified in a variety of tissue extracts; it is usually associated with a particulate fraction. It is generally inferred that such activities are associated with calcium pumps; however, one must exclude the possibilities of membrane-associated, or entrapped, myosin and of active transport systems for other entities.

There are about 4000 erythrocyte (Ca^{2+}) ATPase molecules per cell; it has a monomer mol wt $\sim$ 150,000;[61] either one[210] or two[209] Ca^{2+} ions are transported per ATP hydrolyzed. As is the case in SR, the ATPase can be dephosphorylated by ADP gen-

Table 8
AMINO ACID SEQUENCES OF FIVE FRAGMENTS OF SARCOPLASMIC RETICULUM ATPASE

```
         1         2         3         4         5         6         7         8         9         0
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890

MEAAHSKSTZZCLAYFGVSETTGLTPDQVKRHK

LRNAENAIZALKEYEPEMGKVYRADRKSVZRIKARDIVPGDIVEVAVGBKVPABIRILSIKSTTLRVBZSILTGQSVSVIKHTZPVPBPGRAVBZBK

MAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTBZMSVCKMFIIDKVBGBFCSLBZFITGSTYAPZGZVLPKBVBBIRSGQFDGLVELATICALCBBSSL

DFBZTKGVYEKVGZATZTALTTLVZKMBVFBTZVRNLSKVZRANACNSVIRQLMKKZFTLZFSRDRKSMSVYCSPAKSSRAAVGBKMFVKGAPZGVIBRC

NYVRVGTTRVPMTGPVKZKILSVIKEWGTGRDTLRCLALATRNTPPKRZZMVLBBSSRFMEYETDLTFVGVVGMLNPPRKEVMGSIELCRDAGIRVIM

IGIFGENEEVANRAYTGRZFBBLPLAZZREACRRACCFARVZPSKHSKIVZYLZSYBZITAMTGBGVBBAPALKKAZIGIAMGSGTAVABTASZMVLAB

BFSTIVAAVEEGRAIYBBMKZF

IARNYLEG (C terminus)
```

erating ATP in the presence of Ca^{2+}.[202] An endogenous CDR activates the ATPase and is responsible for its calcium sensitivity (Section II.B).

I list a few more recent examples. The source may be either plasma membrane or internal vesicles. The relationships of these various ATPases to one another or to those of SR or erythrocytes is unknown.

Tissue	Organelle	Ref.
Tetrahymena pyriformis	Granules in cytoplasm	37,176
Amoeba proteus	Granules in cytoplasm	204
Dog brain	Microsomes	234
	Coated vesicles	12
Sea urchin egg	Vesicles with spindle	158
Rat adipocytes	Endoplasmic reticulum	27
Human placenta	Plasma membrane	216
Bovine adrenal medulla	Plasma membrane	143
Rat kidney prox. tubule	Plasma membrane	119
Rat mast cell	Plasma membrane	44

3. ATP: Phosphorylase Phosphotransferase (EC 2.7.1.38)

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the phosphorolysis of glycogen to glucose 1-phosphate. Phosphorylase b is a dimer, mol wt 250,000. The active form, a, is phosphorylated and is a tetramer. Phosphorylase b kinase (PbK) has twelve subunits — α(4 × mol wt 145,000), β(4 × 128,000), and γ (4 × 45,000). Khoo[117a] reported the requirement for micromolar levels of free Ca^{2+} in crude extracts of chicken adipose tissue for PbK activity and cited similar results in vertebrate skeletal, smooth and cardiac muscle, insect flight muscle, and guinea pig brain. Waisman et al.[244] found that PbK is phosphorylated by a CDR-dependent protein kinase and suggested that this is the mechanism for mediating calcium regulation of glycogenolysis (Section II.B). Kiliman and Heilmeyer[118] reported that micromolar Ca^{2+} increases the "affinity for glycogen phosphorylase b about 20-fold" and that it "may also phosphorylate troponin and its own subunit α." Keppens et al.[117] observed that, in liver slices, calcium is required in the incubation medium for angiotensin, vasopressin, or phenylephrine to activate glycogen phosphorylase. They argued that Ca^{2+} functions as a second messenger.*

4. Tyrosine-3-monoxygenase (EC 1.14.16.2)

Repeated stimulation of sympathetic nerves results in increased synthesis of norepinephrine. The rate-limiting step is the hydroxylation of tyrosine to 3,4 dihydroxyphenylalanine. Morgenroth et al.[170] observed a reversible fourfold increase in tyrosine hydroxylase activity in extracts from rat brain noradrenergic tissue with an apparent $pK_d(Ca^{2+})$ of 5.9. Roth et al.[207] summarized their in vitro studies and suggested "that changes in calcium fluxes in the dopaminergic nerve terminals might be responsible for altering tyrosine hydroxylase activity in vivo."

5. Calcium-Activated Proteases

There are several systems in which the addition of calcium to a crude extract causes an increase in some enzymic activity. Without purifying and characterizing the enzyme(s) involved it is difficult to determine whether the calcium acts directly on the

* Cohen et al.[41] reported that CDR imparts calcium sensitivity to PbK, functioning as the fourth subunit, $(\alpha\beta\gamma\delta)_4$.

enzyme, via a CDR, via a phosphorylase-phosphatase system or, as discussed in this section, via a calcium-activated protease.

Inoue et al.[108] characterized a calcium-dependent protease from rat brain — mol wt 93,000, pI 4.8, $pK_d(Ca^{2+})$ 3.5 — that converts a cAMP-independent protein kinase proenzyme to its active form. Phillips and Jakábová[189] described the activity of a calcium-dependent protease of human platelets that seems to resemble the myofibrillar protease, called calcium-activated factor (CAF). Dayton et al.[48] reported that CAF is present in the cytosol at 3.4 mg/kg wet wt and is a heterodimer with subunits of mol wt 80,000 and 30,000. They found that its in vitro myofibril hydrolysing activity is optimal at pCa 3 and concluded that "it seems likely that Ca^{2+} is involved in controlling CAF activity in vivo". They suggest that this CAF is the same protein as that involved in Z-line removal[172,201] and in phosphorylase b kinase activation.[62,123]

6. Aequorin

Hydrozoa such as *Aequorea* contain a luciferase that strongly binds luciferin, a substituted imidazolopyrazinone, and (probably) molecular O_2. Upon subsequent binding of three equivalents of Ca^{2+} per 20,000 mol wt monomer, the luciferin is oxidized and a photon, λ = 469 nm, is emitted. (See review by Prendergast et al.[196]). The high calcium affinity, $pK_d(Ca^{2+}) \simeq 6.0$, and selectivity of aequorin have allowed scientists to determine cytosol concentrations of free Ca^{2+} by microinjection.

C. Nonenzymes

1. Luciferin-Binding Protein

Anthozoa such as *Renilla* also contain a luciferase as do the hydrozoa. However, in the unstimulated cell the luciferin is bound to luciferin-binding protein (LBP), mol wt 18,500, pI = 4.3. Upon binding two equivalents of calcium, $pK_d(Ca^{2+})$ = 6.8, the luciferin is transferred to luciferase where it is oxidized with the emission of a photon, λ = 490. Cormier and Charbonneau[45] noted the similarity of LBP to CDR.

2. Spasmin

Routledge and Amos,[208] in whose laboratory much of the research was done, reviewed the spasmoneme-based contraction of ciliated protozoa. The spasmoneme organelle is responsible for shortening the body of heterotrich ciliates such as *Stentor* and *Spirostomum* and for shortening the stalks of peritrich ciliates such as *Vorticella* and *Zoothamnium* at rates up to 170 stalk lengths per second. The work of contraction is entropic and derives from the energy expended by the vesicles in the cytoplasm removing Ca^{2+} following the previous contraction cycle. Spasmin, which accounts for some 60% of the protein of the *Zoothamnium* spasmoneme, consists of at least two isotypes — both, mol wt 20,000, pI 4.7, and $pK_d(Ca^{2+})$ = 6.7 with n = 2 or 3 Ca^{2+} per molecule.

It is assumed to be globular and somehow arranged into filaments. Addition of calcium causes a large change in conformation, resulting in a coiling of the filaments and a loss of form birefringence. In contrast, Ettiene[68] has isolated a Ca^{2+}-activated, nonenzyme, contractile protein from *Spirostomum* of mol wt 100,000 and $pK_d(Ca^{2+}) \simeq 3.6$ with n = 8 or 9 Ca^{2+} per molecule. Its relationship to the spasmins is not clear. Contraction based on the conformational change of spasmin represents a form of calcium-regulated cell motility fundamentally different from those based on actomyosin or on microtubules.

3. S-100

Moore[168] first described a calcium-binding protein unique to the vertebrate nervous

system. Unfortunately, subsequent work has failed to determine its function, amino acid sequence, or subunit composition (see review by Calissano et al.[29]) S-100 is present in the aqueous extract of nervous tissue and is soluble in 100% saturated ammonium sulfate at neutral pH. Immunofluorescent studies indicate that it is associated primarily with glial cells; however, a small fraction may be associated with the postsynaptic membrane.[107] S-100 has pI 4.1 and a subunit composition either α_2 (mol wt 14,000/2), β_1 (mol wt 7000)[50] or α_2 (mol wt 8,000/2), β_2 (mol wt 10,600/2).[227] The 20,000 mol wt oligomer binds four Ca^{2+} ions with $pK_d(Ca^{2+})$ 4.2.[29] Isobe and Okuyama[109] determined the sequence of an S-100 isomer 91 residues long. It contains an EF-hand domain:

$_{52}$V	V	D	K	V	M	E	T	L	D	S	D	G	D	G	E	C	D	F	Q	E	F	M	A	F	V	A	M	I	T_{91}
	n			n	n			n	X		Y		Z	G			–X			–Z	n			n	n			n	

4. Invertebrate Sarcoplasm Calcium-Binding Protein (ISCP)

Cox et al.[46] and Kohler et al.[121] have characterized a group of calcium-binding proteins from the sarcoplasm of invertebrate muscle. Their function(s) and evolution remain unknown; however, they do resemble parvalbumins. In contrast to the parvalbumins, some have unblocked N terminal residues and high Tyr contents, as well as higher molecular weights.

Source	Yield (g/kg)	MW/oligomer	pI	$pK_d(Ca^{2+})$	$n(Ca^{2+})$/monomer	$pK_d(Mg^{2+})$	$n(Mg^{2+})$	Comments
Crayfish *Astacus pentastacus*	2.7 tail 0.07 heart	44,000/2	5.1	~8.3	3	4.7	2	45% helix dimer α_2, $\alpha\beta$, or β_2
Sand worm *Nereis virens*		17,000/1	4.3	8.2	3		3	
Amphioxus		22,000/1	4.9	7.9	2	4.8	1	

5. Tubulin

Tubulin is the main but not sole constituent of the microtubule polymer (review by Snyder and McIntosh[219]). Either in the helical or in the depolymerized condition (in vitro $pCa^{2+} \simeq 4$ or cooling to 4°C) it exists as the $\alpha\beta$ heterodimer. The two subunits have mol wt 55,000 and are very similar, but not identical, in sequence. Weisenberg[252] and others[78,211] suggested that increased cytosolic levels of free Ca^{2+} might cause microtubule depolymerization with resultant motion, for example, separations of metaphase chromosomes. This hypothesis is not yet confirmed or refuted. Strapans et al.[228] deduced from fluorescence studies that chick brain "tubulin has no significant affinity for calcium." Solomon[222] found one high-affinity site per dimer, $pK_d(Ca^{2+})$ 5.5 and $pK_d(Mg^{2+})$ 4.3. Even if calcium does not bind to tubulin at physiological concentrations, it might bind to one of the microtubule associated proteins; or, as suggested by Welsh et al.,[255] the effects of Ca^{2+} on the mitotic apparatus might be mediated by CDR (Section II.B).

6. Synexin

Creutz et al.[47] characterized synexin from bovine adrenal medulla as having mol wt 47,000 and binding Ca^{2+} specifically and cooperatively with $pK_d = 3.7$. At high concentrations, synexin activity, assayed as aggregation of chromaffin granules, is first observed at pCa 5.2. At saturating Ca^{2+} concentrations, half-maximal aggregating activity occurs at a ratio of 30 synexin molecules per granule.

D. Functions in Search of a Protein

The third postulate presented in Section I is, "The target of Ca^{2+}, functioning as a second messenger, is a protein in the cytosol." Just as there are several proteins (MCBP, ICBP, and S-100) whose functions remain unknown, so there are many calcium-modulated functions whose associated proteins remain to be identified.

The involvement of calcium in "stimulus-secretion coupling" was postulated by Douglas in the early 1960s.[60] Although the mechanism(s) of vesicle formation, filling, translocation, fusion, and recycling are not known, the involvement of microfilaments is fairly well established.[28,241] Calcium may affect other aspects of the process, such as synaptosomal protein phosphorylation[54,131] and microtubule formation.[232] Fast axonal transport may involve calcium, possibly by activation of a protease in the presynaptic terminal (review by Lasek & Hoffman[135]).

Calcium also functions as a second messenger in secretion of salt solutions, e.g., from fly salivary gland,[197] from vertebrate intestine,[15,74] from frog bladder,[94] and from sweat gland.[198] Exocytosis appears not to be the mechanism; ion-specific channels may be modulated by calcium.

Several other important processes might result from calcium modulation of permeability. When a single photon is absorbed in a disc of the rod outer segment, calcium is released from the disc interior to the cytosol[70,218] where it supposedly blocks a sodium channel, thereby perturbing the dark current (review by Hagins[91]).

Calcium also appears to modulate K^+ channels in *helix* neurons[159] and in the postsynaptic region.[63] Calcium uptake correlates well with glucose transport in thymocytes.[256] The size of molecule passing through junctional membrane channels is critically dependent on cytosol-free Ca^{2+} levels.[146,187] The ciliary beating of *Paramecia* is somehow regulated by calcium. Near the ciliary base there are calcium dense plaques.[71] The so-called "pawn" mutant appears to be deficient in its Ca^{2+} gate.[132] Intracellular free Ca^{2+} is critical in maintaining flagellar beat symmetry in sea urchin spermatozoa[21] and in *Chlamydomonas.*[175]

Calcium injection will activate most eggs and, in amphibians, leads to the development of viable haploids. Gilkey et al.[81] have recorded by aequorin luminescence the "free calcium wave traversing the activating egg of the medaka". Cell proliferation and, in particular, the mitogenic response seem critically dependent on the extracellar concentration of Ca^{2+} and/or ionophore.[17,99]Somehow, calcium is involved in mitosis[105,188] and in cleavage furrow formation.[104]

Extracellular Ca^{2+} levels are critical in cell adhesion and fusion, e.g., chick myoblasts[212] and chick erythrocytes;[242] however, it is not known whether Ca^{2+} functions as a second messenger. The mechanism(s) of both transcellular calcium transport and of mineralization may involve the translocation through the cytosol of microcrystals of calcium phosphate.[134] Transport of calcium inward across the plasma membrane may well involve calcium-binding (not -modulated) proteins such as the vitamin K-dependent protein of the chick chorioallantois membrane.[236]

One can confidently predict that new calcium-modulated proteins will be discovered.

V. CONCLUSION

I have argued that "calcium-modulated proteins contain EF hands." This postulate has provided a unifying concept for research in the field of calcium physiology. Calcium-dependent regulator, troponin-C, myosin light chains, parvalbumin, S- 100 and intestinal calcium-binding protein are homologous to one another.

One of the fascinating problems of the 1980s will be to understand how the evolution of the EF-hand proteins has been associated with the evolution of the role of calcium

as a second messenger. This goal will require both the further characterization of more calcium-modulated proteins and the molecular definition of calcium-modulated processes.

Although the EF-hand postulate is an attractive generalization, its limitations and exceptions should be reemphasized. During evolution, some EF-hand domains have lost the ability to bind calcium. None of the four domains of the enzymatic light chains bind calcium, nor do domains 2, 3, and 4 of regulatory light chains, domain 1 of cardiac TNC, or domain 2 of parvalbumin.

Further, a few proteins that are calcium modulated may have evolved from other protein domains. Both spasmin and SR ATPase are calcium-modulated, yet the available data do not indicate EF hands.

Regardless of the exceptions to the calcium modulation postulate, the EF-hand domain can already be regarded as one of the fundamental units of protein structure and evolution.

ACKNOWLEDGMENTS

I thank numerous colleagues who gave me unpublished results and who proofread this manuscript. In particular, I thank Dr. Goodman for his unpublished cladograms and Wilson Burgess and Ellen Clark for their bibliophilic endeavors.

REFERENCES

1. **Adelstein, R. S. and Conti, M. A.,** Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity, *Nature (London)*, 256, 597, 1975.
2. **Adelstein, R. S., Conti, M. A., and Anderson, W.,** Phosphorylation of human platelet myosin, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 3115, 1973.
3. **Alaba, O. J. and Fischer, E. H.,** personal communication, 1979.
4. **Allen, G.,** On the primary structure of the Ca^{2+}-ATPase of sarcoplasmic reticulum, *FEBS Proc. Meet.*, 45, 159, 1977.
5. **Amphlett, G. W., Vanaman, T. C., and Perry, S. V.,** Effect of the troponin C-like protein from bovine brain (brain modulator protein) on the Mg^{2+}-stimulated ATPase of skeletal muscle actomyosin, *FEBS Lett.*, 72, 163, 1976.
6. **Anderson, B., Brittain, H. G., Jones, W. C., and Nelson, D. J.,** The Isolation and fluorescence properties of muscular parvalbumins from the ancient anthropod *Limulus polyphemus, Proc. 2nd Maine Biomed. Sci. Symp.*, University of Maine Press, Orono, 1977, 425.
7. **Barker, W. C., Ketcham, L. K., and Dayhoff, M. O.,** Evolutionary relationships among calcium-binding proteins, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 73.
8. **Baron, G., Demaille, J., and Dutruge, E.,** The distribution of parvalbumins in muscle and in other tissues, *FEBS Lett.*, 56, 156, 1975.
9. **Benzonana, G., Capony, J.-P., and Pechere, J.-F.,** The binding of calcium to muscular parvalbumins, *Biochim. Biophys. Acta*, 278, 110, 1972.
10. **Benzonana, G., Wnuk, W., Cox, J. A., and Gabbiani, G.,** Cellular distribution of sacroplasmic calcium-binding proteins by immunofluorescence, *Experientia*, 31, 716, 1975.
11. **Blaustein, M. P.,** Effects of internal and external cations and of ATP on sodium-calcium and calcium-calcium exchange in squid axons, *Biophys. J.*, 20, 79, 1977.
12. **Blitz, A. L., Fine, R. E., and Toselli, P. A.,** Evidence that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum, *J. Cell Biol.*, 75, 135, 1977.
13. **Blum, H. E., Pocinwong, S., and Fischer, E. H.,** A phosphate-acceptor protein related to parvalbumins in dogfish skeletal muscle, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2198, 1974.

14. **Blum, E. H., Lehky, P., Kohler, L., Stein, E. A., and Fischer, E. H.,** Comparative properties of vertebrate parvalbumins, *J. Biol. Chem.*, 252, 2834, 1977.
15. **Bolton, J. E. and Field, M.,** Ca ionophore-stimulated ion secretion in rabbit ileal mucosa: relation to actions of cyclic 3′, 5′-AMP and carbamylcholine, *J. Membr. Biol.*, 35, 159, 1977.
16. **Bond, G. H. and Clough, D. L.,** A soluble protein activator of (Mg^{2+} and Ca^{2+})-dependent ATPase in human red cell membranes, *Biochim. Biophys. Acta*, 323, 592, 1973.
17. **Boynton, A. L. and Whitfield, J. R.,** Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1651, 1976.
18. **Bredderman, P. J. and Wasserman, R. H.,** Chemical composition, affinity for calcium, and some related properties of the vitamin D dependent calcium binding protein, *Biochemistry*, 13, 1687, 1974.
19. **Bremel, R. D.,** Myosin linked calcium regulation in vertebrate smooth muscle, *Nature (London)*, 252, 405, 1974.
20. **Briggs, N.,** Identification of the soluble relaxing factor as a parvalbumin, *Fed. Proc.*, 34, 540, 1975.
21. **Brokaw, C. J., Josslin, R., and Bobrow, L.,** Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa, *Biochem. Biophys. Res. Commun.*, 58, 795, 1974.
22. **Brooks, J. C. and Siegel, F. L.,** Purification of a calcium-binding phosphoprotein from beef adrenal medulla, *J. Biol. Chem.*, 248, 4189, 1973.
23. **Brostrom, C. O., Huang, Y. C., Breckenridge, B. M., and Wolff, D. J.,** Identification of a calcium-binding protein as a calcium-dependent regulator of brain denylate cyclase, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 64, 1975.
24. **Brostrom, M. A., Brostrom, C. O., Breckenridge, B. M., and Wolff, D. J.,** Regulation of adenylate cyclase from glial tumor cells by calcium and a calcium-binding protein, *J. Biol. Chem.*, 251, 4744, 1976.
25. **Brostrom, C. O. and Wolff, D.,** Properties and functions of the calcium-dependent regulator protein, *Adv. Cyclic Nucleotide Res.*, 11, 27, 1979.
26. **Bruns, D. E., Black, B., McDonald, J. M., and Jarett, L.,** Freeze-fracture and enzymatic studies of Ca^{2+} transport ATPase in adipocyte endoplasmic reticulum, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 181.
27. **Bruns, M., Fliesher, E. B., and Avioli, L. V.,** Control of vitamin D-dependent calcium-binding protein in rat intestine by growth and fasting, *J. Biol. Chem.*, 252, 4145, 1977.
28. **Burridge, K. and Phillips, J. H.,** Association of actin and myosin with secretory granule membranes, *Nature (London)*, 254, 526, 1975.
29. **Calissano, P., Alemà, S., and Fasella, P.,** Calcium and monovalent cations as conformational effectors of the brain-specific protein S-100, *FEBS. Proc. Meet.*, 41, 207, 1975.
30. **Capony, J. P., DeMaille, J., Pina, C., and Pechère, J.-F.,** The amino-acid sequence of the most acidic major parvalbumin from frog muscle, *Eur. J. Biochem.*, 56, 215, 1975.
31. **Capony, J. P., Ryden, L., Demaille, J., and Pechère, J.-F.,** The primary structure of the major parvalbumin from hake muscle. Overlapping peptides obtained with chemical and enzymatic methods. The complete amino-acid sequence, *Eur. J. Biochem.*, 32, 97, 1973.
32. **Cave, A., Dobson, C. M., Parello, J., and Williams, R. J. P.,** Conformational mobility within the structure of muscular parvalbumins. An NMR study of the aromatic resonances of phenylalanine residues, *FEBS Lett.*, 65, 190, 1976.
33. **Chacko, S., Conti, M. A., and Adelstein, R. S.,** Effect of phosphorylation of smooth muscle myosin on actin activation and Ca^{2+} regulation, *Proc. Natl. Acad. Sci. U.S.A.*, 7, 129, 1977.
34. **Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., and Tallant, E. A.,** Protein activator of cyclic 3′:5′-nucleotide phosphodiesterase of bovine or rat brain also activates its adenylate cyclase, *Biochim. Biophys. Res. Commun.*, 66, 1055, 1975.
35. **Cheung, W. Y.,** Cyclic 3′,5′-nucleotide phosphodiesterase: preparation of a partially inactive enzyme and its subsequent stimulation by snake venom, *Biochim. Biophys. Acta*, 191, 303, 1969.
36. **Cheung, W. Y., Lynch, T. J., and Wallace, R. W.,** An endogenous Ca^{2+}-dependent activator protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase, *Adv. Cyclic Nucleotide Res.*, 9, 233, 1978.
37. **Chua, B., Elson, C., and Shrago, E.,** Purification and properties of a cytosol Ca^{2+}-activated ATPase from *Tetrahymena pyriformis*, *J. Biol. Chem.*, 252, 7548, 1977.
38. **Closset, J. and Gerday, C.,** Conformational studies on parvalbumins by circular dichroism, *Biochim. Biophys. Acta*, 405, 228, 1975.
39. **Coffee, C. J. and Bradshaw, R. A.,** Carp muscle calcium-binding protein. I. Characterization of the tryptic peptides and the complete amino acid sequence of component B, *J. Biol. Chem.*, 248, 3305, 1973.
40. **Coffee, C. J., Bradshaw, R. A., and Kretsinger, R. H.,** The coordination of calcium ions by carp muscle calcium binding proteins A, B and C, *Adv. Exp. Med. Biol.*, 48, 211, 1974.

41. Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. , and Nairn, C., Identification of the Ca^{2+}-dependent modulation protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase, *FEBS Lett.*, 92, 287, 1978.
42. Collins, J. H., Structure and evolution of troponin C and related proteins, in *Calcium in Biological Systems*, Soc. Exp. Biol. Symp. XXX, Cambridge University Press, Cambridge, 1976, 303.
43. Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., and Jackman, N., The amino acid sequence of rabbit skeletal muscle troponin C: gene replication and homology with calcium binding proteins from carp and hake muscle, *FEBS Lett.*, 36, 268, 1973.
44. Cooper, P. H. and Stanworth, D. R., Characterization of calcium-ion-activated adenosine triphosphotase in the plasma membrane of rat mast cells, *Biochem. J.*, 156, 691, 1976.
45. Cormier, M. J. and Charbonneau, H., Isolation, properties and function of a calcium-triggered luciferin binding protein, in *Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 481.
46. Cox, J. A., Wnuk, W., and Stein, E. A., Isolation and properties of a sarcoplasmic calcium-binding protein from crayfish, *Biochemistry*, 15, 2613, 1976.
47. Creutz, C. E., Pazoles, C. J., and Pollard, H. B., Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules, *J. Biol. Chem.*, 253, 2858, 1978.
48. Dayton, W. R., Reville, W. J., Goll, D. E., and Stromer, M. H., A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme, *Biochemistry*, 15, 2159, 1976.
49. Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., and Hartshorne, D. J., Modulator protein as a component of the myosin light chain kinase from chicken gizzard, *Biochemistry*, 17, 253, 1978.
50. Dannies, P. S. and Levine, L., Structural properties of bovine brain S-100 protein, *J. Biol. Chem.*, 246, 6276, 1971.
51. Dedman, J. R., Potter, J. D., and Means, A. R., Biological cross-reactivity of rat testis phosphodiesterase activator protein and rabbit skeletal muscle troponin-C, *J. Biol. Chem.*, 252, 2437, 1977.
52. Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., and Means, A. R., Physicochemical properties of rat testis^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase. Relationship of Ca^{2+} binding, conformational changes, and phosphodiesterase activity, *J. Biol. Chem.*, 252, 8415, 1977.
53. Dedman, J. R., Jackson, R. L., Schreiber, W. E., and Means, A. R., Sequence homology of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+}-binding proteins, *J. Biol. Chem.*, 253, 343, 1978.
54. DeLorenzo, R. J. and Freedman, S. D., Calcium dependent neurotransmitter release and protein phosphorylation in synoptic vesicles, *Biochem. Biophys. Res. Commun.*, 80, 183, 1978.
55. Demaille, J., Dutruge, E., Baron, G., Pechère, J. F., and Fischer, E. H., A reappraisal of the relationship of phosphate-acceptor protein to parvalbumin, *Biochem. Biophys. Res. Commun.*, 67, 1034, 1975.
56. Demaille, J., Dutruge, E., Capony, J. P., and Pechère, J.-F., Muscular parvalbumins: a family of homologous calcium-binding proteins. Their relation to the calcium-binding troponin component, in *Calcium Binding Proteins*, Drabikowski, W., Strzelecka-Golaszewska, H., and Carafoli, E., Eds., Elsevier, Amsterdam, 1974, 643.
57. Derancourt, J., Haiech, J., and Pechère, J. F., Binding of calcium by parvalbumin fragments, *Biochim. Biophys. Acta*, 532, 373, 1978.
58. Donato, H. and Martin, R. B., Conformations of carp muscle calcium binding parvalbumin, *Biochemistry*, 13, 4575, 1974.
59. Dorrington, K. J., Hui, A., Hofmann, T., Hitchman, A. J. W., and Harrison, J. E., Porcine intestinal calcium-binding protein: molecular properties and the effect of binding calcium ions, *J. Biol. Chem.*, 249, 199, 1974.
60. Douglas, W. W., Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence, *Biochem. Soc. Symp.*, 39, 1, 1974.
61. Drickamer, L. K., The red cell membrane contains three different adenosine triphosphatases, *J. Biol. Chem.*, 250, p.1952, 1975.
62. Drummond, G. I. and Duncan, L., On the mechanism of activation of phosphorylase b kinase by calcium, *J. Biol. Chem.*, 243, 5532, 1968.
63. Dubois, D. M. and Schoffeniels, E., Molecular model of postsynaptic potential, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1749, 1975.
64. Ebashi, S., Third component participating in the superprecipitation of "natural actomyosin", *Nature (London)*, 200, 1010, 1963.

65. Elsayed, S. and Bennich, H., The primary structure of allergen M from cod, *Scand. J. Immunol.*, 4, 203, 1975.
66. Enfield, D. L., Ericsson, L. H., Blum, H. E., Fischer, E. H., and Neurath, H., Amino-acid sequence of the major parvalbumin from rabbit skeletal muscle, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1309, 1975.
67. England, P. J., Correlation between contraction and phosphorylation of the inhibitory subunit of troponin in perfused rat heart, *FEBS Lett.*, 50, 57, 1975.
68. Ettienne, E. M., personal communication, 1979.
69. Fabian, F., Mason, D. T., and Wikman-Coffelt, J., Calcium binding properties of cardiac and skeletal muscle myosins, *FEBS Lett.*, 81, 381, 1977.
70. Fain, G. L., Quandt, F. N., and Gerschenfeld, H. M., Calcium-dependent regenerative responses in rods, *Nature (London)*, 269, 707, 1977.
71. Fisher, G., Kaneshiro, E. S., and Peters, P. D., Divalent cation affinity sites in *Paramecium aurelia*, *J. Cell Biol.*, 69, 429, 1976.
72. Frank, G. and Weeds, A. G., The amino-acid sequence of the alkali light chains of rabbit skeletal muscle myosin, *Eur. J. Biochem.*, 44, 317, 1974.
73. Frankenne, F., Joassin, L., and Gerday, C., The amino acid sequence of the pike (*Esox lucius*) parvalbumin III, *FEBS Lett.*, 35, 145, 1973.
74. Frizzell, R. A., Active chloride secretion by rabbit colon: calcium-dependent stimulation by ionophase A23187, *J. Membr. Biol.*, 35, 175, 1977.
75. Frearson, N., Focant, B. W. W., and Perry, S. V., Phosphorylation of a light chain component of myosin from smooth muscle, *FEBS Lett.*, 63, 27, 1976.
76. Freedman, R. A., Weiser, M. M., and Isselbacher, K. J., Calcium translocation by Golgi and lateral-basal membrane vesicles from rat intestine; decrease in vitamin D-deficient rat, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3612, 1977.
77. Fullmer, C. S. and Wasserman, R. H., Bovine intestinal calcium-binding protein: cation binding properties, chemistry and trypsin resistance, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 303.
78. Gallin, J. I. and Rosenthal, A. S., The regulatory role of divalent cations in human granulocyte chemotaxis: evidence for an association between calcium exchanges and microtubule assembly, *J. Cell Biol.*, 62, 594, 1974.
79. Gerday, C. and Gillis, J. M., The possible role of parvalbumins in the control of contraction, *J. Physiol.*, 258, 96, 1976.
80. Gerday, C., Collins, S., and Piron, L.A., Phylogenetic relationships between *Cyrinidae* parvalbumins. II. The amino acid sequence of the parvalbumin V of chub (*Leciscus cephalis*), *Comp. Biochem. Physiol.*, 618, 451, 1978.
81. Gilkey, J. C., Jaffe, L. F., Ridgway, E. B., and Reynolds, G. T., A free calcium wave traverses the activating egg of the medaka, *Oryzias Latipes*, *J. Cell Biol.*, 776, 448, 1978.
82. Gillis, J. M. and Gerday, C., Calcium movement between myofibrils, parvalbumins and sarcophasmic reticulum in muscle, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 193.
83. Gnegy, M. E., Nathanson, J. A., and Uzunov, P., Release of the phosphodiesterase activator by cyclic AMP-dependent ATP: protein phosphotransferase from subcellular fractions of rat brain, *Biochim. Biophys. Acta*, 497, 75, 1977.
84. Goldberg, A. and Lehman, W., Troponin-like proteins from muscles of the scallop, *Aequipecten irradians*, *Biochem. J.*, 171, 413, 1978.
85. Goodman, M. and Pechère, J.-F., The evolution of muscular parvalbumins investigated by the maximum passimony method, *J. Mol. Evol.*, 9, 131, 1977.
86. Gopinath, R. M. and Vincenzi, F. F., Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+}-Mg^{2+}) ATPase, *Biochem. Biophys. Res. Commun.*, 77, 1203, 1977.
87. Gosselin-Rey, C., Bernard, N., and Gerday, C., Conformation and immunochemistry of parvalbumin III from pike white muscle: modification of the arginine residue with 1,2 Cyclohexanedione, *Biochim. Biophys. Acta*, 303, 90, 1973.
88. Gosselin-Rey, C. and Gerday, C., Parvalbumins from frog skeletal muscle (*Rana temporaria L.*). Isolation and characterization. Structural modifications associated with calcium binding, *Biochim. Biophys. Acta*, 492, 53, 1977.
89. Gosselin-Rey, C., Piront, A., and Gerday, C., Polymorphism of parvalbumins and tissue distribution. Characterization of component I, isolated from red muscles of *Cyprinus carpio L.*, *Biochim. Biophys. Acta*, 532, 294, 1978.

90. **Grandjean, J., Laszlo, P., and Gerday, C.,** Sodium complexation by the calcium binding site of parvalbumin, *FEBS Lett.*, 81, 376, 1977.
91. **Hagins, W. A.,** The visual process: excitatory mechanisms in the primary receptor cells, *Annu. Rev. Biophys. Bioeng.*, 1, 131, 1972.
92. **Hamoir, G.,** The comparative biochemistry of fish sarcoplasmic proteins, *Acta Zool. Pathol. Antverp.*, 46, 69, 1968.
93. **Hamoir, G., Piront, A., Gerday, C., and Dando, P. R.,** Muscle proteins of the coelacanth *Latimeria chalumnae Smith, J. Mar. Biol. Assoc. U.K.*, 53, 763, 1973.
94. **Hardy, M. A.,** Intracellular calcium as a modulator of transepithelial permeability to water in frog urinary bladder, *J. Cell Biol.*, 76, 787, 1978.
95. **Hartshorne, D. J., Gorecka, A., and Aksoy, M. O.,** Aspects of the regulatory mechanism in smooth muscle, in *Excitation-Contraction Coupling in Smooth Muscle*, Casteels, R., Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A., Eds., Elsevier, New York, 1977, 377.
96. **Haselgrove, J. C.,** X-ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle, *J. Mol. Biol.*, 92, 113, 1975.
97. **Hasselbach, W.,** The reversibility of the sarcophlasmic calcium pump, *Biochim. Biophys. Acta*, 515, 23, 1978.
98. **Heizmann, C. W., Häuptle, M.-T., and Eppenberger, H. M.,** The purification, characterization and localization of a parvalbumin-like protein from chicken-leg muscle, *Eur. J. Biochem.*, 80, 433, 1977.
99. **Hesketh, T. R., Smith, G. A., Houslay, M. D., Warren, G. B., and Metcalfe, J. C.,** Is an early calcium flux necessary to stimulate lymphocytes? *Nature (London)*, 267, 490, 1977.
100. **Hitchcock, S. E.,** Regulation of motility in nonmuscle cells, *J. Cell Biol.*, 74, 1, 1977.
101. **Hitchcock, S. E. and Kendrick-Jones, J.,** Myosin light chains, carp calcium binding proteins, and troponin components: do they interact to form functional complexes? in *Calcium Transport in Contraction and Secretion*, Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A., Eds., North-Holland, Amsterdam, 1975, 447.
102. **Ho, H. C., Wirch, E., Stevens, F. C., and Wang, J. H.,** Purification of a Ca^{2+}-activatable cyclic nucleotide phosphodiesterase from bovine heart by specific interaction with its Ca^{2+}-dependent modulator protein, *J. Biol. Chem.*, 252, 43, 1977.
103. **Hofmann, T., Kawakami, M., Morris, H., Hitchman, A. J. W., Harrison, J. E., and Dorrington, K. J.,** The amino-acid sequence of a calcium binding protein from pig intestinal mucosa, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 373.
104. **Hollinger, T. G. and Schuetz, A. W.,** "Cleavage" and cortical granule breakdown in *Rana pipiens* oocytes induced by direct microinjectin of calcium, *J. Cell Biol.*, 71, 395, 1976.
105. **Holmes, R. P. and Stewart, P. R.,** Calcium uptake during mitosis in the myxomycete *Physarum polycephalum, Nature (London)*, 269, 592, 1977.
106. **Huxley, H. E.** Structural changes in the actin and myosin-containing filaments during contraction, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 361, 1972.
107. **Hydén, H.,** "A calcium-dependent mechanism for synapse and nerve cell membrane modulation, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2965, 1974.
108. **Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y.,** Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain, *J. Biol. Chem.*, 252, 7610, 1977.
109. **Isobe, T. and Okuyama, T.,** The amino-acid sequence of S-100 protein (PAP I-b protein) and its relation to the calcium-binding proteins, *Eur. J. Biochem.*, 89, 379, 1978.
110. **Jarrett, H. W. and Penniston, J. T.,** Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes. Its membership in the class of CA^{2+}-binding modulator proteins, *J. Bol. Chem.*, 253, 4676, 1978.
111. **Jauregui-Adell, J. and Pechère, J.-F.,** Parvalbumins from coelacanth III. Amino acid sequence of the major component, *Biochim. Biophys. Acta*, 536, 275, 1978.
112. **Joassin, L. and Gerday, C.,** The amino-sequence of the major parvalbumin of the whiting (*Gadus merlangus*), *Comp. Biochem. Physiol.*, 57B, 159, 1977.
113. **Kakiuchi, S. and Yamazaki, R.,** Calcium-dependent phosphodiesterase activity and its activating factor isolated from brain. Studies on cyclic 3′:5′-nucleotide phosphodiesterase, *Biochem. Biophys. Res. Commun.*, 41, 1104, 1970.
114. **Kendrick-Jones, J., Szentkiralyi, E. M. and Szent-Györgyi, A. G.,** Regulatory light chains in myosins, *J. Mol. Biol.*, 104, 747, 1976.
115. **Kendrick-Jones, J. and Jakes, R.,** Regulatory light chains in myosin, in *Excitation-Contraction Coupling in Smooth Muscle*, Casteels, R., Godfraind, T., and Rüegg, J. C., Eds., North-Holland, New York, 1977, 343.

116. **Kendrick-Jones, J. and Jakes, R.,** Myosin-linked regulation: a chemical approach, in *International Symposium Myocardial Failure*, Riecker, G., Weber, A., and Goodwin, J., Eds., Springer-Verlag, New York, 1977, 28.
117. **Keppens, S., Vandenheede, J. R., and DeWulf, H.,** On the role of calcium as second messenger in liver for the hormonally induced activation of glycogen phosphorylase, *Biochim. Biophys. Acta*, 496, 448, 1977.
117a. **Khoo, J. C.,** Ca^{2+}-dependent activation of phosphorylase by phosphorylase kinase in adipose tissue, *Biochim. Biophys. Acta*, 422, 87, 1976.
118. **Kilimann, M. and Heilmeyer, L. M. G.,** The effect of Mg^{2+} on the Ca^{2+}-binding properties of non-activated phosphorylase kinase, *Eur. J. Biochem.*, 73, 191, 1977.
119. **Kinne-Saffran, E. and Kinne, R.,** Localization of a calcium-stimulated ATPase in the basal-lateral plasma membranes of the proximal tubule of rat kidney cortex, *J. Membr. Biol.*, 17, 263, 1974.
120. **Kobayashi, H., Van Brunt, J., and Harold, F. M.,** ATP-linked calcium transport in cells and membrane vesicles of *Streptococcus faecalis*, *J. Biol. Chem.*, 253, 2085, 1978.
121. **Kohler, L., Cox, J. A., and Stein, E. A.,** Sarcoplasmic calcium-binding proteins in protochordate and cyclostome muscle. Characterization of a new protein from amphioxus, *Mol. Cell. Biochem.*, 20, 85, 1978.
122. **Korn, E. D.,** Biochemistry of actomyosin-dependent cell motility (a review), *Proc. Natl. Acad. Sci., U.S.A.*, 75, 588, 1978.
123. **Krebs, E. G., Huston, R. B., and Hunkeler, F. L.,** Properties of phosphorylase kinase, *Adv. Enzyme Regul.*, 6, 245, 1968.
124. **Kretsinger, R. H.,** Calcium binding proteins, *Annu. Rev. Biochem.*, 45, 239, 1976.
125. **Kretsinger R. H.,** Calcium binding proteins and natural membranes, in *Perspectives in Membrane Biology*, Estrada, S. and Gitler, C., Eds., Academic Press, New York, 1974, 229.
126. **Kretsinger, R. H.,** Evolution of the informational role of calcium in eukaryotes, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H. Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 63.
127. **Kretsinger, R. H.,** Gene triplication deduced from the tertiary structure of a muscle calcium binding protein, *Nature (London) New Biol.*, 240, 85, 1972.
128. **Kretsinger, R. H.,** The informational role of calcium in the cytosol, *Adv. Cyclic Nucleotide Res.*, 11, 1, 1979.
129. **Kretsinger, R. H. and Barry, C. D.,** The predicted structure of the calcium-binding component of troponin, *Biochim. Biophys. Acta*, 405, 40, 1975.
130. **Kretsinger, R. H. and Nelson, D. J.,** Calcium in biological systems, *Coord. Chem. Rev.*, 18, 29, 1976.
131. **Krueger, B. K., Forn, J., and Greengard, P.,** Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synoptosomes, *J. Biol. Chem.*, 252, 2764, 1977.
132. **Kung, C., Chang, S.-Y., Satow, Y., van Houten, J., and Hansma, H.,** Genetic dissection of behavior in paramecium, *Science*, 188, 898, 1975.
133. **Kuo, I. C. Y. and Coffee, J.,** Purification and characterization of the troponin-C-like protein from bovine adrenal medulla, *J. Biol. Chem.*, 251, 1603, 1976.
134. **Landis, W. J., Hauschka, B. T., Rogerson, C. A., and Glimcher, M. J.,** Electron microscopic observations of bone tissue prepared by ultracryomicrotomy, *J. Ultrastruct. Res.*, 59, 185, 1977.
135. **Lasek, R. J. and Hoffman, P. N.,** The neuronal cytoskeleton, axonal transport and axonal growth, *Cell Motil.*, 3, 1021, 1976.
136. **Leavis, P. C., Drabikowski, W., Rosenfeld, S., Grabarek, Z., and Gergely, J.,** Localization of high and low affinity Ca^{2+}-binding sites on fragments of troponin C produced by trypsin or thrombin degradation, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H. Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 281.
137. **Legér J. J. and Elzinga, M.,** Studies on cardiac myosin light chains: comparison of the sequences of cardiac and skeletal myosin LC-2, *Biochem. Biophys. Res. Commun.*, 74, 1390, 1977.
138. **Lehky, P., Blum, H. E., Stein, E. A., and Fischer, E. H.,** Isolation and characterization of parvalbumins from the skeletal muscle of higher vertebrates, *J. Biol. Chem.*, 249, 4332, 1974.
139. **Lehman, W.,** Calcium ion-dependent myosin from decapod-crustacean muscles, *Biochem. J.*, 163, 291, 1977.
140. **Lehman, W.,** Hybrid troponin reconstituted from vertebrate and arthropod subunit, *Nature (London)*, 255, 424, 1975.
141. **Lehman, W., Kendrick-Jones, J., and Szent-Györgyi, A. G.,** Myosin-linked regulatory systems: comparative studies, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 319, 1972.
142. **Lehman, W. and Szent-Györgyi anorgyi, A. G.,** Regulation of muscular contraction. Distribution of actin control and myosin control in the animal kingdom, *J. Gen. Physol.* 66, 1, 1975.

143. Leslie, S. W. and Borowitz, J. L., Evidence for a plasma membrane calcium pump in bovine adrenal medulla but not adrenal cortex, *Biochim. Biophys. Acta*, 394, 227, 1975.
144. Levine, B. A., Mercola, D., Coffman, D., and Thornton, J. M., Calcium binding by troponin-C. A proton magnetic resonance study, *J. Mol. Biol.*, 115, 743, 1977.
145. Levine, B. A., Williams, R. J. P., Fullmer, C. S., an Wasserman, R. H., NMR studies of various calcium-binding proteins, in *Calcium-Binding proteins and Calcium Function*, Wasserman, R. M., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 29.
146. Loewenstein, W. R. and Rose, B., Calcium in (junctional) intercellular communication and a thought on its behavior in intercellular communication, *Ann. N.Y. Acad. Sci.*, 307,285, 1978.
147. Lowe, M. C., Alaba, O. J., Moeschler, H. J., Benditt, E. P., and Fischer, E. H., personal communication.
148. Luthra, M. G., Au, K. S., and Hankan, D. J., Purification of an activator of human erythrocyte membrane (Ca^{2+} and Mg^{2+}) ATPase, *Biochem. Biophys. Res. Commun.*, 77, 678, 1977.
149. MacLennan, D. H. and Holland, P. C. Calcium transport in sarcoplasmic reticulum, *Annu. Rev. Biophys. Bioeng.*, 4, 377, 1975.
150. Mahendran, C. and Berl, S., Isolation of troponin-like complex from bovine brain cortex, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2273, 1977.
151. Makinose, M., The mechanism of rapid calcium exchange between the inside and outside of the sacroplasmic membrane, in *Calcium Transport in Contraction and Secretion*, Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A., Eds., North-Holland, New York, 1975, 367.
152. Mannherz, H. G. and Goody, R. S., Proteins of the contractile systems, *Annu. Rev. Biochem.*, 45, 427, 1976.
153. Margossian, S. S., Lowey, S., and Barshop, B., Effect of DTNB light chain on the interaction of vertebrate skeletal myosin with actin, *Nature (London)*, 258, 163, 1975.
154. Martonosi, A. N. and Jilka, R. L., The effect of calcium ion transport ATPase upon the passive calcium ion permeability of phospholipid vesicles, *Biochim. Biophys. Acta*, 466, 57, 1977.
155. Matsuda, G., Maita, T., Suzuyama, Y., Setoguchi, M., and Umegane, T., Amino acid sequence of the L-2 light chain of rabbit skeletal muscle myosin, *J. Biochem.*, 31, 809, 1977.
156. Matsuda, G., Suzuyama, Y., Maita, T., and Umegane, T., The L-2 light chain of chicken skeletal muscle myosin, *FEBS Lett.*, 84, 53, 1977.
157. Maximov, E. E., Zapevalova, N. P., and Mitin, Y. V., On the calcium-binding ability of the synthetic evolutionary ancestor of calcium-binding proteins, *FEBS Lett.*, 88, 80, 1978.
158. Mazia, D., Petzelt, C., Williams, R. O., and Meza, I., A Ca-activated ATPase in the mitotic apparatus of the sea urchin egg (isolated by a new method), *Exp. Cell Res.*, 70, 325, 1972.
159. Meech, R. W. and Standen, N. B., Potassium activation in *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx, *J. Physiol. (London)*, 249, 211, 1975.
160. Melancon, M. J. and DeLuca, H. F., Vitamin D stimulation of calcium-dependent adenosine-triphosphatase in chick intestinal brush borders, *Biochemistry*, 9, 1658, 1970.
161. Mendelson, R. A. and Cheung, P., Muscle crossbridges: absence of direct effect of calcium on movement away from the thick filaments, *Science*, 194, 190, 1976.
162. Mercola, D., Bullard, B., and Priest, J., Crystallization of troponin-C, *Nature (London)*, 254, 634, 1975.
163. Moews, P. C. and Kretsinger, R. H., Refinement of the structure of carp muscle calcium-binding parvalbumin by model building and difference fourier analysis, *J. Mol. Biol.*, 91, 201, 1975.
164. Moffat, K., Fullmer, C. H., and Wasserman, R. H., Preliminary crystallographic data for a calcium binding protein from bovine intestine, *J. Mol. Biol.*, 97, 661, 1975.
165. Moir, A. J. G., Cole, H. A., and Perry, S. V., The phosphorylation sites of troponin T from white skeletal muscle and the effects of interaction with troponin C on their phosphorylation by phosphorylase kinase, *Biochem. J.*, 16, 371, 1977.
166. Moir, A. J. G. and Perry, S. V., The sites of phosphorylation of rabbit cardiac troponin I by adenosine 3′:5′-cyclic monophosphate-dependent protein kinase, *Biochem. J.*, 167, 333, 1977.
167. Morimoto, K., and Harrington, W. F., Evidence for structural changes in vertebrate thick filaments induced by calcium, *J. Mol. Biol.*, 88, 693, 1974.
168. Moore, B. W., A soluble protein characteristic of the nervous system, *Biochem. Biophys. Res. Commun.*, 19, 739, 1965.
169. Morgan, M., Perry, S. V., and Ottaway, J., Myosin light-chain phosphatase, *Biochem. J.*, 157, 687, 1976.
170. Morgenroth, V. H., Boadle-Biber, M. C., and Roth, R. H., Activation of tyrosine hydroxylase from central nosadrenergic neurons by calcium, *Mol. Pharmacol.*, 11, 427, 1975.
171. Moyle, J. and Mitchell, P., Electric charge stoichiometry of calcium translocation in rat liver mitochondria, *FEBS Lett.*, 73, 131, 1977.

172. **Muguruma, M., Yamada, M., and Fukazawa, T.**, Effect of calcium on extraction of Z-band proteins from I-Z-I brushes of rabbit striated muscle, *Biochim. Biophys. Acta*, 532, 71, 1978.
173. **Muszbek, L., Kuznicki, J., Szabó, T., and Drabikowski, W.**, Troponin C like protein of blood platelet, *FEBS Lett.*, 80, 308, 1977.
174. **Nelson, D. J., Miller, T. L., Martin, R. B.**, Non-cooperative Ca(II) removal and terbium (III) substitution in carp muscle calcium binding parvalbumin, *Bioinorg. Chem.*, 7, 325, 1977.
175. **Nichols, K. M. and Rikmenspoel, R.**, Control of flagellar motion in *Chlamydomonas* and *Euglena* by mechanical microinjection of Mg^{2+} and Ca^{2+} and by electrical current injection, *J. Cell Sci.*, 29, 233, 1978.
176. **Nilsson, J. R. and Coleman, J. R.**, Calcium-rich, refractile granules in *Tetrahymena pyriformis* and their possible role in the intracellular ion-regulation, *J. Cell Sci.*, 24, 311, 1977.
177. **Okamoto, Y. and Yagi, K.**, Ca^{2+}-induced conformational changes of spin-labeled g_2 chain bound to myosin and the effect of phosphorylation, *J. Biochem.*, 80, 111, 1976.
178. **Opella, S. J., Nelson, D. J., and Jardetsky, O.**, Carbon magnetic resonance study of the conformational changes in carp muscle calcium binding parvalbumin, *J. Am. Chem. Soc.*, 96, 7157, 1974.
179. **Parello, J., Cave, A., Puigdomenech, P., Maury, C., Capony, J.-P., and Pechère, J.-F.**, Conformational studies on muscular parvalbumins. II. Nuclear magnetic resonance analysis, *Biochimie*, 56, 61, 1974.
180. **Parry, D. A. D. and Squire, J. M.**, Structural role of tropomyosin in muscle regulation: analysis of the X-ray diffraction patterns from relaxed and contracting muscle, *J. Mol. Biol.*, 75, 33, 1973.
181. **Pechère, J.-F., Capony, J.-P., and Demaille, J.**, Evolutionary aspects of the structure of muscular parvalbumins, *Syst. Zool.*, 22, 533, 1973.
182. **Pechère, J.-F., Demaille, J., Dutruge, E., Capony, J.-P., Baron, G., and Pina, C.**, Muscular parvalbumins. Some explorations into their possible biological significance, in *Calcium Transport in Contraction and Secretion*, Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A. Eds., North-Holland, Amsterdam, 1975, 459.
183. **Pechère, J.-F., and Focant, B.**, Carp myogens of white and red muscles. Gross isolation on sephadex columns of the low-molecular-weight components and examination of their participation in anaerobic glycogenolysis, *Biochem. J.*, 96, 113, 1965.
184. **Pechère, J.-F., Capony, J.-P., and Ryden, L.**, The primary structure of the major parvalbumin from hake muscle isolation and general properties of the protein, *Eur. J. Biochem.*, 23, 421, 1971.
185. **Pechère, J.-F., Derancourt, J., and Haiech, J.**, The participation of parvalbumins in the activation-relaxation cycle of vertebrate fast skeletal muscle, *FEBS Lett.*, 75, 111, 1977.
186. **Pechère, J.-F.**, The significance of parvalbumins among muscular calciproteins, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 213.
187. **Peracchia, C.**, Calcium effects on gap junction structure and cell coupling, *Nature (London)*, 271, 669, 1978.
188. **Petzelt, C. and Auel, D.**, Synthesis and activation of mitotic Ca^{2+}-Adenosine-triphosphatase during the cell cycle of mouse mastocytoma cells, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1610, 1977.
189. **Phillips, D. R. and Jakabova, M.**, Ca^{2+}-dependent protease in human platelets: specific cleavage of platelet pholypeptides in the presence of added Ca^{2+}, *J. Biol. Chem.*, 252, 5602, 1977.
190. **Gillis, J. M., Piront, A., and Gosselin-Rey, C.**, Parvalbumins: distribution and physical state inside the muscle cell, *Biochim. Biophys. Acta*, 585, 444, 1979.
191. **Potter, J. D., Dedman, J. R., and Means, A. R.**, Ca^{2+}-dependent regulation of cyclic AMP phosphodiesterase by parvalbumin, *J. Biol. Chem.*, 252, 5609, 1977.
192. **Potter, J. D. and Gergely, J.**, The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase, *J. Biol. Chem.*, 250, 4628, 1975.
193. **Potter, J. D., Hsu, F.-J., and Pownall, H. J.**, Thermodynamics of Ca^{2+} binding to troponin-C, *J. Biol. Chem.*, 252, 2452, 1977.
194. **Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L., and Means, A. R.**, Calcium binding proteins: relationship of binding, structure, conformation and biological function, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 239.
195. **Potter, J. D., Seidel, J. C., Leavis, P., Lehrer, S. S., and Gergely, J.**, Effect of Ca^{2+} binding on troponin C. Changes in spin label mobility, extrinsic fluorescence, and sulfhydryl reactivity, *J. Biol. Chem.*, 251, 7551, 1976.
196. **Prendergast, F. G., Allen, D. G., and Blinks, J. R.**, Properties of the calcium-sensitive bioluminescent protein aequorin, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennon, D., and Siegel, F., Eds., North-Holland, New York, 1977, 469.

197. Prince, W. T., Rasmussen, H., and Berridge, M. J., The role of calcium in fly salivary gland secretion analyzed with the ionophase A-23187, *Biochim. Biophys. Acta*, 329, 98, 1973.
198. Prompt, C. A. and Quinton, P. M., Functions of calcium in sweat secretion, *Nature (London)*, 272, 171, 1978.
199. Rasmussen, H., Goodman, D. B. P., and Tenenhouse, A., The role of cyclic AMP and calcium in cell activation, *CRC Crit. Rev. Biochem.*, 1, 95, 1972.
200. Rasmussen, H. and Goodman, D. B. P., Relationships between calcium and cyclic nucleotides in cell activation, *Physiol. Rev.*, 57, 421, 1977.
201. Reddy, M. K., Etlinger, J. D., Rabinowitz, M., Fischman, D. A., and Zak, R., Removal of Z-lines and α-actinin from isolated myofibrils by a calcium-activated neutral protease, *J. Biol. Chem.*, 250, 4278, 1975.
202. Rega, A. F. and Garrahan, P. J., Calcium ion-dependent dephosphorylation of the Ca^{2+}-ATPase of human red-cells by ADP, *Biochim. Biophys. Acta*, 507, 182, 1978.
203. Regenstein, J. M. and Szent-Györgyi, A. G., Regulatory proteins of lobster striated muscle, *Biochemistry*, 14, 917, 1975.
204. Reinold, M. and Stockem, W., Darstellung eines ATP-sensitiven membransystems mit Ca^{2+}-transportierendesfunktion bei Amöben, *Cytobiologie Z. Exp. Zellforsch.*, 6, 182, 1972.
205. Rodan, G. A. and Feinstein, M. B., Interrelationships between Ca^{2+} and adenylate and guanylate cyclases in the control of platelet secretion and aggregation *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1829, 1976.
206. Romero-Herrera, A., Castillo, O., and Lehmann, H., Human skeletal muscle proteins, the primary structure of troponin C., *J. Mol. Evol.*, 8, 251, 1976.
207. Roth, R. H., Walters, J. R., Murrin, L. C., and Morgenroth, V. H., Dopamine neurons: role of impulse flow and pre-synaptic receptors in the regulation of tyrosine hydroxylase, in *Pre- and Post-synaptic Receptors*, Bunney, W. E., Ed., Marcel Dekker, New York, 1975, 5.
208. Routledge, L. M. and Amos, W. B., Calcium-binding contractile proteins in protozoa, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds. , North-Holland, New York, 1977, 439.
209. Sarkadi, B., Szász, I., Gerlóczy, A., and Gárdos, G., Transport parameters and stoichiometry of active calcium ion extrusion in intact human red cells, *Biochim. Biophys. Acta*, 464, 93, 1977.
210. Schatzmann, H. J., Active calcium transport and Ca^{2+}-activated ATPase in human red cells, *Curr. Top. Membr. Transp.*, 6, 125, 1975.
211. Schliwa, M., The role of divalent cations in the regulation of microtubule assembly. In vivo studies on microtubules of the heliozoan axopodium using the ionophase A 23187, *J. Cell Biol.*, 70, 527, 1976.
212. Schudt, C. and Pette, D., Influence of the ionophase A23187 on myogenic cell fusion, *FEBS Lett.*, 59, 36, 1975.
213. Schulman, H. and Greengard, P., Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein, *Nature (London)*, 271, 478.
214. Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., van Winkle, W. B., and Bornet, E. P., The rate of calcium uptake into sarcoplasmic reticulum of cardiac muscle and skeletal muscle. Effects of cyclic AMP-dependent protein kinase and phosphorylase b kinase, *Biochim. Biophys. Acta*, 426, 57, 1976.
215. Scordilis, S. P. and Adelstein, R. S., A comparative study of the myosin light chain kinases from myoblast and muscle sources: studies on the kinases from proliferate rat myoblasts in culture, rat thigh muscle, and rabbit skeletal muscle, *J. Biol. Chem.*, 253, 9041, 1978.
216. Shami, Y., Messer, H. H., and Copp, D. H., Calcium uptake by placental plasma membrane vesicles, *Biochim. Biophys. Acta*, 401, 256, 1975.
217. Small, J. V. and Sobieszek, A., Ca-regulation of mammalian smooth muscle actomyosin via a kinase-phosphatase-dependent phosphorylation and dephosphorylation of the 20,000 M, light chain of myosin, *Eur. J. Biochem.*, 76, 521, 1977.
218. Smith, H. G., Fager, R. S., and Litman, B. J., Light-activated calcium release from sonicated bovine retinal rod outer segment disks, *Biochemistry*, 16, 1399, 1977.
219. Snyder, J. A. and McIntosh, J. A., Biochemistry and physiology of microtubules, *Annu. Rev. Biochem.*, 45, 699, 1976.
220. Sobieszek, A. and Small, J. V., Myosin-linked calcium regulation in vertebrate smooth muscle, *J. Mol. Biol.*, 102, 75, 1976.
221. Solano, C. and Coffee, C. J., Preparation and properties of carp muscle parvalbumin fragments A (residues 1—75) and B (residues 76—108), *Biochim. Biophys. Acta*, 453, 67, 1976.
222. Solomon, F., Binding sites for calcium on tubulin, *Biochemistry*, 16, 358, 1977.
223. Solaro, R. J., Moir, A. J. G., and Perry, S. V., Phosphorylation of troponin I and the ionotropic effect of adrenaline in the perfused rabbit heart, *Nature (London)*, 262, 615, 1976.

224. Sowadski, J., Cornick, G., and Kretsinger, R. H., Terbium replacement of calcium in parvalbumin, *J. Mol. Biol.*, 124, 123, 1978.
225. Spencer, R., Charman, M., Wilson, P., and Lawson, E., Vitamin D-stimulated intestinal calcium absorption may not involve calcium-binding protein directly, *Nature (London)*, 263, 161, 1976.
226. Stafford, W. F. and Szent-Györgyi, A. G., Physical characterization of myosin light chains, *Biochemistry*, 17, 607, 1978.
227. Stewart, J. A., Subunit composition of the tissue specific bovine brain S-100 protein, *Biochem. Biophys. Res. Commun.*, 46, 1405, 1972.
228. Strapans, I., Kenney, W. C., and Dirksen, E. R., Calcium affinity of chick brain tubulin, *Biochem. Biophys. Res. Commun.*, 62, 92, 1975.
229. Sutoh, K. and Harrington, W. F., Cross-linking of myosin thick filaments under activating and rigor conditions. A study of the radial disposition of cross bridges, *Biochemistry*, 16, 2441, 1977.
230. Szent-Gyorgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J., The light chains of scallop myosin as regulatory subunits, *J. Mol. Biol.*, 74, 179, 1973.
231. Thatcher, D. R. and Pechere, J.-F., The amino-acid sequence of the major parvalbumin from thornbach-ray muscle, *Eur. J. Biochem.*, 75, 121, 1977.
232. Thoa, N. B., Wooten, G. F., Axelrod, J., and Kopin, I. J., Inhibition of release of dopamine-b-hydroxylase and norepinephrine from sympathetic nerves by colchicine, vinblastine or cytocholasin-b, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 520, 1972.
233. Toffolon, E. P., Pechet, M. M., and Isselbacher, K., Demonstration of the rapid action of pure crystalline 1α-hydroxy vitamin D_3 and 1α 25 dihydroxy vitamin D_3 on intestinal calcium uptake, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 229, 1975.
234. Trotta, E. E. and DeMeis, L., ATP-dependent calcium accumulation in brain microsomes, enhancement by phosphate and oxalate, *Biochim. Biophys. Acta*, 394, 239, 1975.
235. Tsuchiya, T. and Rosen, B. P., Calcium transport driven by a proton gradient in inverted membrane vesicles of *Escherichia coli*, *J. Biol. Chem.*, 251, 962, 1976.
236. Tuan, R. S., Scott, W. A., and Cohn Z. A., Purification and characterization of calcium-binding protein from chick charioallantoic membrane, *J. Biol. Chem.*, 253, 1011, 1978.
237. Tufty, R. M. and Kretsinger, R. H., Troponin and parvalbumin calcium binding regions predicted in myosin light chain and T4 lysozyme, *Science*, 187, 167, 1975.
238. Vanaman, T. C., Sharief, F., and Watterson, D. M., Structural homology between brain modulator protein and muscle TnCs, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 107.
239. van Eerd, J.-P. and Takahashi, K., Determination of the complete amino-acid sequence of bovine cardiac troponin C, *Biochemistry*, 15, 1171, 1976.
240. van Eerd, J.-P., Capony, J.-P., and Pechere, J.-F., Amino acid sequence of frog skeletal troponin C, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corrodino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 232.
241. Vial, J. D. and Garrido, J., Actin-like filaments and membrane rearrangement in oxyntic cells, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 4032, 1976.
242. Volsky, D. and Loyter, A., Rearrangement of intramembranous particles and fusion promoted in chicken erythrocytes by intracellular Ca^{2+}, *Biochim. Biophys. Acta*, 471, 243, 1977.
243. Waisman, D., Stevens, F. C., and Wang, J. H., The distribution of the Ca^{2+}-dependent protein activator of cyclic nucleotide phosphodiesterase in invertebrates, *Biochim. Biophys. Res. Commun.*, 65, 975, 1975.
244. Waisman, D. M., Singh, T. J., and Wang, J. H., The modulator-dependent protein kinase activatable by the Ca^{2+}-dependent modulator protein of the cyclic nucleotide system, *J. Biol. Chem.*, 253, 3381, 1978.
245. Waisman, D. M., Stevens, F. C., and Wang, J. H., Purification and characterization of a Ca^{2+}-binding protein in *Lumbricus terrestris*, *J. Biol. Chem.*, 253, 1106, 1978.
246. Wagner, P. D. and Weeds, A. G., Studies on the role of myosin alkali light chains. Recombination and hybridization of light chains and heavy chains in subfragment-1 preparations, *J. Mol. Biol.*, 109, 455, 1977.
247. Wallace, R. W., Lynch, T. J., Tallant, E. A., and Cheung, W. Y., An endogenous inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase, *Arch. Biochem. Biophys.*, 187, 328, 1978.
247a. Wang, J. H., Calcium regulated protein modulator in cyclic nucleotide systems, in *Cyclic Nucleotides Mechanism of Action*, Cramer, H. and Schultz, J., Eds., John Wiley & Sons, New York, 1977, 37.
248. Wang, J. H. and Desai, R., Modulator binding protein bovine brain protein exhibiting the Ca^{2+}-dependent association with the protein modulator of cyclic nucleotide phosphodiesterase, *J. Biol. Chem.*, 252, 4175, 1977.

249. **Wasserman, R. H. and Feher, J. J.**, Vitamin D-dependent calcium-binding proteins, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corrodino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 293.
250. **Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., and Vanaman, T. C.**, Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase, *J. Biol. Chem.*, 251, 4501, 1976.
251. **Weeds, A. G. and McLachlan, A. D.**, Structural homology of myosin alkali light chains, troponin C and carp calcium binding protein, *Nature (London)*, 252, 646, 1974.
252. **Weisenberg, R. C.**, Microtubule formation in vitro in solutions containing low calcium concentration, *Science*, 177, 1104, 1972.
253. **Weissman, J., Schneider, L. E., and Schedl, H. P.**, Renal calcium binding protein in the diabetic and vitamin D-depleted rat, *Biochim. Biophys. Acta*, 497, 358, 1977.
254. **Wells, J. N. and Hardman, J. G.**, Cyclic nucleotide phosphodiesterase, *Adv. Cyclic Nucleotide Res.*, 8, 119, 1977.
255. **Welsh, M. J., Dedmam, J. R., Brinkley, B. R., and Means, A. R.**, Calcium-dependent regulator protein: localization in mitotic apparatus of eukaryotic cells, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1867, 1978.
255a. **Werber, M. M.**, Metal binding to myosin and to myosin DTNB-light chain, *Experentia*, in press, 1978.
256. **Whitesell, R. R., Johnson, R. A., Tarpley, H. L., and Regen, D. M.**, Mitogen-stimulated glucose transport in thymocytes: possible role of Ca^{++} and antagonism by adenasin 3′:5′-monophasphate, *J. Cell Biol.*, 72, 456, 1977.
257. **Wilkinson, J. M.**, The amino acid sequence of troponin C from chicken skeletal muscle, *FEBS Lett.*, 70, 254, 1976.
258. **Wilkinson, J. M. and Grand, R. J. A.**, The amino acid sequence of chicken fast-skeletal-muscle troponin I., *Eur. J. Biochem.*, 493, 1978.
259. **Wilkinson, J. M. and Grand, R. J. A.**, Comparison of amino acid sequence of troponin I from different striated muscles, *Nature (London)*, 271, 31, 1978.
260. **Winkelman, L. and Bullard, B.**, Phosphorylation of a light subunit of locust myosin, in *Insect Flight Muscle*, Tregear, R. T., Ed., Elsevier, Amsterdam, 1977, 285.
261. **Winstanley, M. A., Trayer, H. R., and Trayer, I. P.**, Role of the myosin light chains in binding to actin, *FEBS Lett.*, 77, 239, 1977.
262. **Wolff, D. J. and Brostrom, C. O.**, Calcium-binding phosphoprotein from pig brain: identification as a calcium-dependent regulator of brain cyclic nucleotide phosphodiesterase, *Arch. Biochem. Biophys.*, 163, 349, 1974.
263. **Wolff, D. J., Brostrom, M. A., and Brostrom, C. O.**, Divalent cation binding sites of CDR and their role in the regulation of brain cyclic nucleotide metabolism, in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R. H., Corrodino, R., Carafoli, E., Kretsinger, R. H., MacLennan, D., and Siegel, F., Eds., North-Holland, New York, 1977, 97.
264. **Yagi, K., Yazawa, M., Kakinchi, S., Ohshima, M., and Uenishi, K.**, Identification of an activator protein for myosin light chain kinase as the Ca^{2+}-dependent modulator protein, *J. Biol. Chem.*, 253, 1338, 1978.
265. **Bagshaw, C. R. and Reed, G. H.**, The significance of the slow dissociation of divalent metal ions from myosin "regulatory light chains," *FEBS Lett.*, 81, 386, 1977.
266. **Green, N. M.**, personal communication, 1978.