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Review

Ion binding to calmodulin

A comparison with other intracellular calcium-binding proteins

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Summary

Over the past few years calcium has emerged as an important bioregulator. Upon external stimulation, the cell generates a transient Ca^{2+} increase, which is transformed into a cellular event through a molecular cascade. The first step in this cascade is the binding of calcium to proteins present in the cytosol. These proteins capable of binding Ca^{2+} under physiological conditions all belong to the same evolutionary family that evolved from a common ancestor. However, they strongly differ in the properties of their calcium binding sites. Calmodulin, the ubiquitous calcium binding protein present in all eukaryotic cells, is very close to the ancestor protein, presents four calcium binding sites which bind calcium, magnesium and monovalent ions competitively and is involved in the triggering of cellular processes. Parvalbumin, another member of the family, is more specialized and found mostly in fast-twitch skeletal muscle. It binds calcium and magnesium with high affinity and seems to be involved in muscle relaxation. On the other hand, troponin C which confers Ca^{2+} sensitivity to acto-myosin interaction exhibits both triggering and relaxing sites. The study of intracellular Ca^{2+} binding proteins has shown that calcium binding proteins have evolved from a simple common structure to fulfill different functions.

Abbreviations

CaBP, calcium-binding protein
ICaBP, the vitamin D-dependent intestinal Ca^{2+}-binding protein
S-100, the glial S-100 protein
RLC, the phosphorylatable myosin regulatory light chain
CaM, calmodulin
Pa, parvalbumin
TnC, troponin C
TnI, troponin I
Hepes, N-2-hydroxyethylpipezarine, N′-2-ethane-sulfonic acid
W7, N-(6-Aminohexyl)-5-chloro-1-Naphtalene sulfonamide
SDS, sodium dodecyl sulfate
NMR, nuclear magnetic resonance

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Introduction

Within the past decade calcium emerged as an important bioregulator. In a normal eukaryotic cell, the cytosolic free calcium concentration ranges from $10^{-8} \cdot 10^{-7}$ M to 10^{-5} M (1). Calcium increase or decrease is under the control of calcium channels and calcium pumps localized either in the plasma membrane or internal membranes (e.g. reticular). The presence of these two systems allows external stimuli to modify the internal calcium concentration of a given cell. Indeed, hormones such as

alpha$_1$-adrenergic agonists bind to receptors of a target cell, the occupancy of which triggers the opening of a calcium channel through which Ca^{2+} ions flow from the external medium (pCa $\cong$ 3) to the cytosol (pCa $\cong$ 7). Cytosolic Ca^{2+} concentration rises to 10^{-5} M. After a kinetic delay, the channel closes and the calcium pump restores the initial calcium concentration (10^{-7} M). Thereby an external stimulus induces a transitory calcium increase. During such an increase, calcium binds to intracellular calcium binding proteins which interact with target proteins, stimulate their enzymatic activities and trigger cellular events such as muscle contraction, or secretion (Fig. 1). The aim of this paper is to review some steps of these regulatory mechanisms and more specifically the interaction of calcium with intracellular calcium binding proteins, and the subsequent activation of target proteins.

Calcium regulatory mechanisms

For more than thirty years, calcium has been known to trigger muscular contraction. In 1968, Ebashi showed that this regulation was due to the binding of calcium to troponin C, a subunit of a protein complex (troponin) localized on the actin filament (2). Since then, research carried out in several laboratories have established Ca^{2+} as the regulatory ion in almost all cellular movements.

It has also been discovered that calcium modulates energy-providing metabolic pathways such as glycogenolysis. That is not surprising in view of the synergy between energy-dependent movements and energy production. Calcium also controls the concentration of cAMP and its own intracellular concentration. Moreover it regulates the metabolism of first messengers such as dopamine, serotonine and prostaglandins. Regulatory roles of calcium are summarized in Table 1.

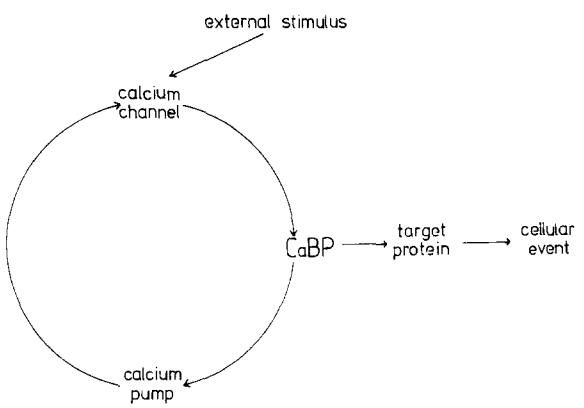

Fig. 1. Calcium regulation in a eukaryotic cell.

Intracellular calcium binding proteins

In a eukaryotic cell, any protein capable of binding calcium in the range of 10^{-7} M to 10^{-5} M under physiological conditions is able to participate in a calcium regulation. Therefore, methods to detect and purify such proteins have been developed. They are based on two principles:

- the first one is the ability of these proteins to bind calcium in a medium containing μM Ca^{2+} concentration.
- the other one is the discovery that intracellular CaBP, which are acidic, low molecular weight proteins, are stable upon heat or acid treatment.

Detection was first achieved by means of counter ion electrophoresis (3). Cytosolic proteins were separated by electrophoresis in a polyacrylamide gel, in the presence of ^{45}Ca and under non denaturing conditions. Then CaBP's were shown to be present by the peak of radioactivity found in gel slices.

Table 1. A survey of calcium-dependent physiological processes.

Cell movement	CaBP involved
Fast skeletal muscular contraction	Skeletal troponin C Parvalbumin
Post tetanic potentiation	Calmodulin
Cardiac contraction	Cardiac troponin C
Smooth muscle contraction	Calmodulin, RLC
Invertebrate muscle contraction	Myosin regulatory light chain
Ciliary movement	Calmodulin
Mitosis	Calmodulin
Secretory mechanisms	Calmodulin
Energy production	
Glycogenolysis	Calmodulin (delta subunit of phosphorylase kinase)
Messenger modulation	
Calcium transport	I CaBP
Calcium fluxes	Calmodulin
cAMP metabolism	Calmodulin
Prostaglandin production	Calmodulin
Dopamine production	Calmodulin
Serotonine production	Calmodulin

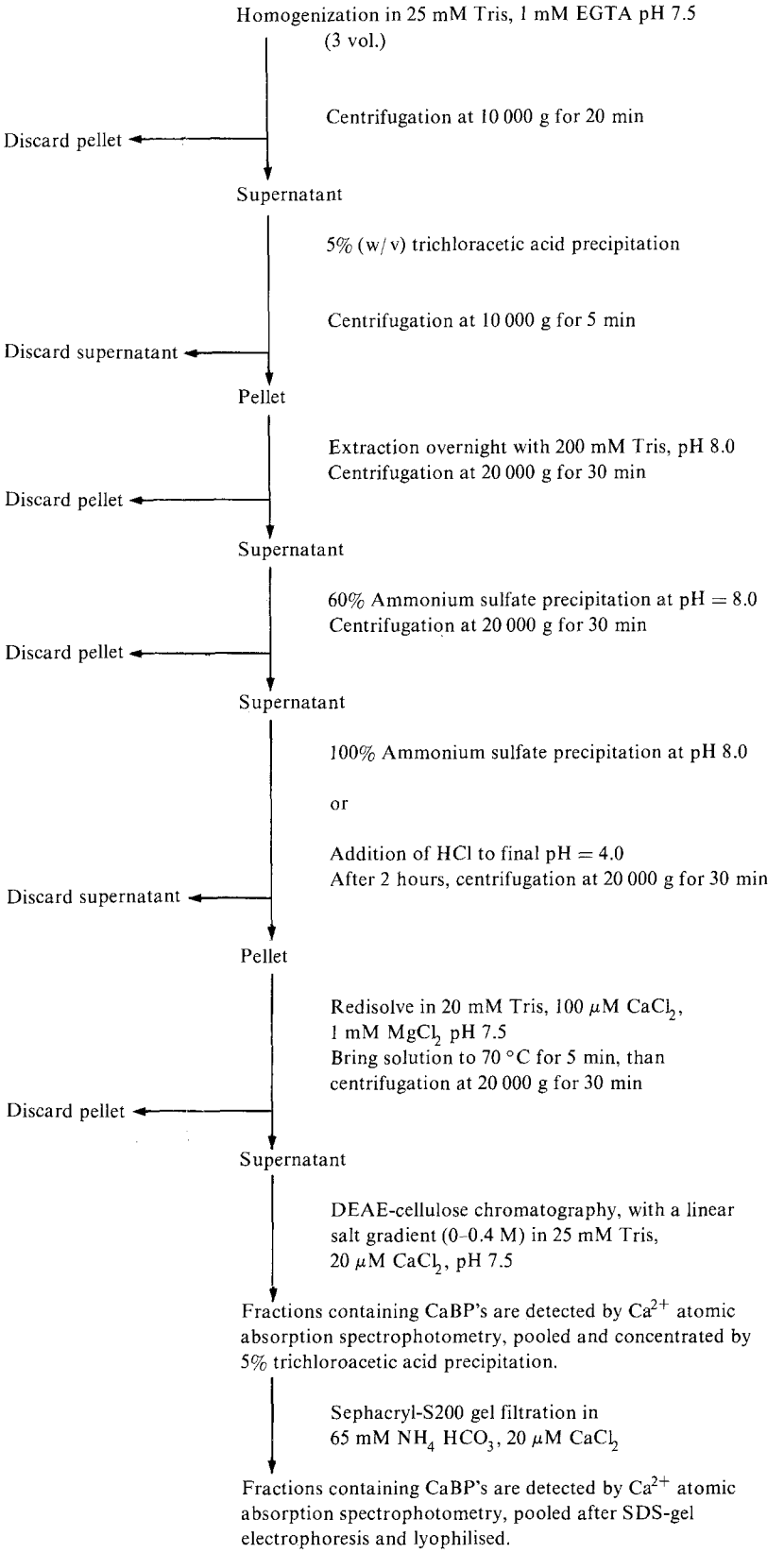

Fig. 2. A general purification scheme for CaBP's.

Based on the same principle, a generally applicable purification procedure (4) was described in which the Ca^{2+} binding activity was simply followed by Ca^{2+} atomic absorption spectrophotometry. Outline of a typical CaBP purification scheme is presented in Fig. 2.

Extensive search for CaBPs in different tissues and species have shown that different CaBPs were present in different cells. Table 2 reviews the known CaBPs and their distribution. More than 25 different Ca^{2+} binding proteins have been sequenced. All these proteins show an internal homology in their primary structure suggesting that they have evolved from a primordial gene coding for a one-domain, 36-residue-long peptide. The gene underwent two tandem duplications leading to a four domain protein which is the ancestor of the whole family.

By analogy with the known crystal structure of parvalbumin (5), each domain contains a Ca^{2+} binding site composed of two alpha helices surrounding a twelve residue loop. In this loop the six Ca^{2+} coordinating ligands are termed X, Y, Z, -Y, -X, -Z (Fig. 3), (see also Fig. 5).

During the course of evolution, changes have taken place with either deletion of one or two domains, or loss of the calcium binding properties for some or all domains.

Calmodulin, the ubiquitous Ca^{2+}-binding protein of eukaryotic cells, is in terms of evolution the closest protein to the four domain ancestor (Fig. 4). In addition, it is the only known CaBP involved in many different metabolisms and devoid of tissue specificity (see Tables 1 and 2). Therefore calmodulin can be considered as the non specialized protein of the CaBP family.

Table 2. Distribution of intracellular low molecular weight calcium binding proteins.

CaBP	Tissue
Calmodulin	ubiquitous
Parvalbumin	fast skeletal muscle and nervous tissue
Skin CaBP[a]	Epidermis and Malpighian epithelia
I CaBP[b]	gut (duodenum), kidney, cerebellum
S100[c]	glial cells
Troponin C	skeletal and cardiac muscle
Myosin regulatory light chain	ubiquitous
Oncomodulin[d]	Hepatoma cell line

[a] Ref. 105; [b] Ref. 106, 107; [c] Ref. 108; [d] Ref. 109.

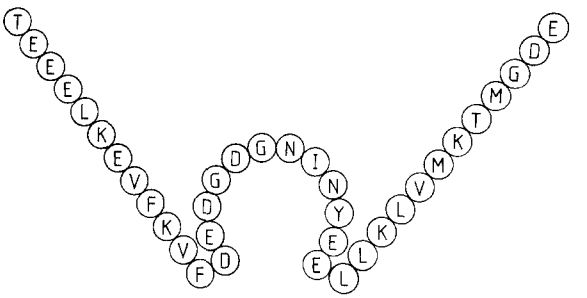

Fig. 3. The amino-acid sequence of the reconstructed primordial one-domain polypeptide (110). The central 12-residue long binding loop is flanked on each side by a short alpha-helical segment.

Calmodulin

Purification and assay

Calmodulin, originally discovered as a calcium-dependent activator of cyclic nucleotide phosphodiesterase (6, 7) is a multifunctional modulator protein mediating the effects of Ca^{2+} in a variety of cellular reactions and processes (for a review, see 8–11).

It was first purified from brain (12) but today a number of procedures exist for preparing calmodulin easily from different tissues and species (13). In our laboratory, calmodulin is obtained from ram testis by a technique using ammonium sulfate precipitation, heat treatment followed by chromatography on DEAE-cellulose and gel filtration of Sephacryl S-200 (14). This method yields about one hundred milligrams of protein per kilogram of tissue. Since 1979, affinity chromatography has been extensively used in the preparation. Exploiting the binding of calmodulin to antipsychotic drugs (such as trifluoperazine) in the presence of calcium (15, 16), Charbonneau et al. used a fluphenazine-Sepharose column in the purification of calmodulin from plants (17).

Three approaches have been used to assay calmodulin.

1) Enzymatic assays are based on the ability of calmodulin to activate various enzymes namely cyclic nucleotide phosphodiesterase (18) and myosin light chain kinase (19). Myosin light chain kinase is particularly interesting because its activity is entirely dependent upon calmodulin. This technique however presents some pitfalls due to the presence,

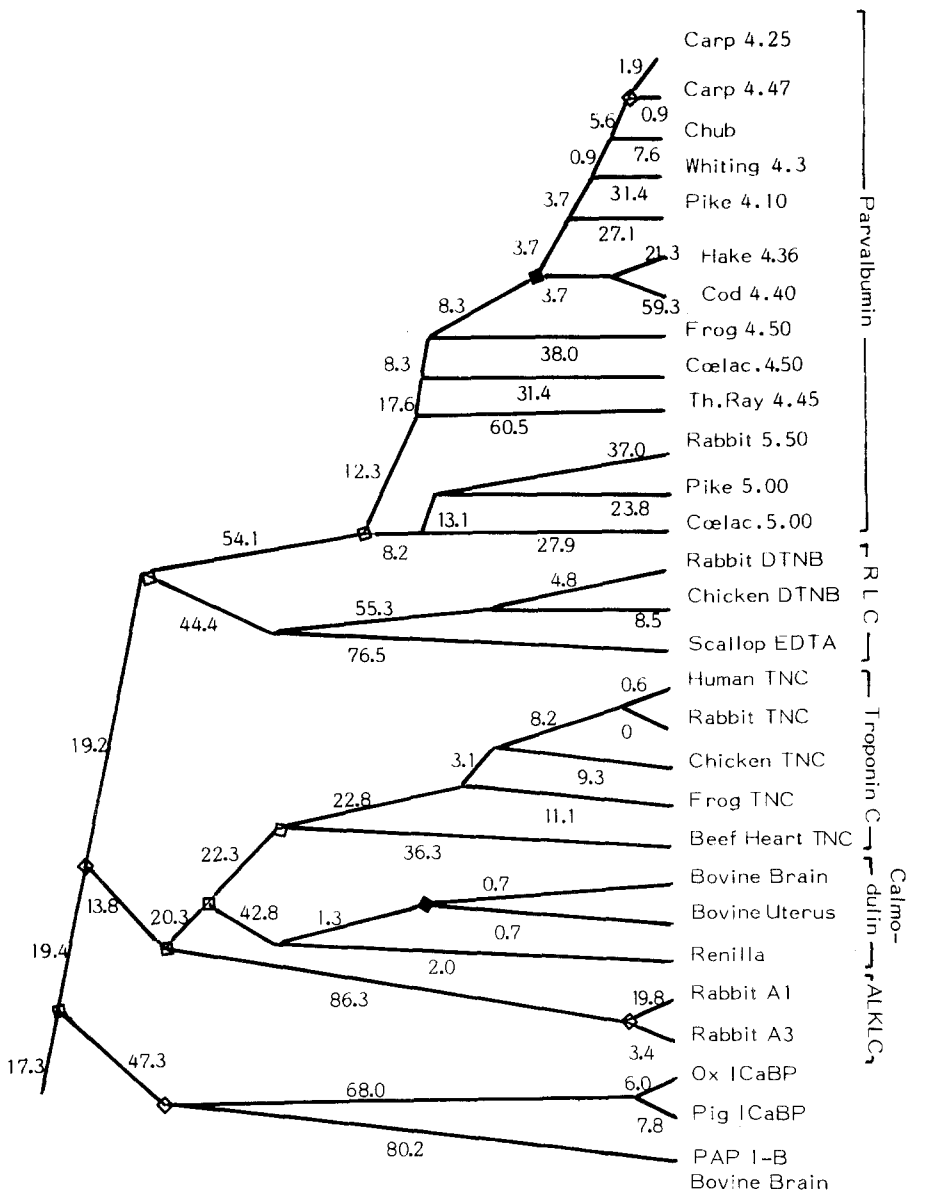

Fig. 4. Evolutionary tree of the calcium binding protein family. Numbers on the links are nucleotide replacements per 100 codons; when the augmentation algorithm for superimposed mutations increased the number of NRs on a link, the augmented value is shown. Symbol for an obvious gene duplication is ◊. ♦ is used whenever there is only circumstantial evidence for gene duplication.

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in most cellular extracts, of activators or inhibitors of the enzyme. In addition, some tissues contain several calmodulin-binding proteins (20, 21, 22, 23, 24, 25, 26, 27) capable of competing with the enzyme for calmodulin. However, the remarkable stability of calmodulin to harsh treatments (such as heat or acid treatment) which denature most proteins can be used to eliminate most if not all interfering proteins.

2) Radioimmunoassay uses anticalmodulin antibodies and radioactively labelled calmodulin to quantify calmodulin in different tissues. Anticalmodulin antibodies are raised either in rabbits or goats following the method of Van Eldik and Wat-

terson (28). The affinity of the antibody for calmodulin is in the nanomolar range, i.e comparable to the affinity of the target enzymes (phosphodiesterase or myosin light chain kinase). Precipitation of the immunocomplex is achieved by either staphylococcal protein A (29) or the antibody raised against goat or rabbit immunoglobulins (30) or by polyethyleneglycol precipitation (M. Petit, personal communication). Radioimmunoassay has the great advantage over enzymatic assay of being much more specific. However, for the time being this method is still expensive and time consuming.

In addition, in these two types of assays, the preparation of the sample is crucial to extract and quantify all the calmodulin.

3) Finally, urea (31) and very resolutive dodecyl sulfate gel electrophoresis have been used to quantify calmodulin. In this technique, proteins with an R_f similar to calmodulin interfere in the assay.

Table 3. Physical properties of calmodulin.

Property	$+ Ca^{2+}$	+ EGTA/+ EDTA
Molecular weight		
Amino Acid Sequence	16 723	
Sedimentation equilibrium	18 700	18 700
SDS gel electrophoresis	16 500	19 000
Hydrodynamic properties		
$S_{20,w}$ (S)	1.87	1.83
Diffusion coefficient (cm^2/s)	$1.09\ 10^{-6}$	
Partial specific volume (ml/g)	0.707	0.712
Stoke radius (Å)	20.9	21.4
Frictionnal coefficient	1.2	1.23
Spectrophotometric properties		
$E_{276\ nm}$ 1%	1.8	2.0
O_{222} (deg cm^2/dmole)	−15 000	−12 000
Fluorescence quantum yield	0.078	0.030
Fluorescence lifetime (ns)	1.7	2.3

Physicochemical properties of calmodulin

The physicochemical properties of the protein have been extensively reviewed by Klee (12). Calmodulin is a small (Mr 16 700), acidic, acid and heat stable protein. It binds calcium in the physiological concentration range. A comparison of its hydrodynamic properties in the presence and absence of calcium (Table 3) suggests that calcium binding to calmodulin does not affect the overall conformation of the protein. By contrast, calcium changes drastically the spectroscopic properties of the protein (Table 3).

Structure of calmodulin

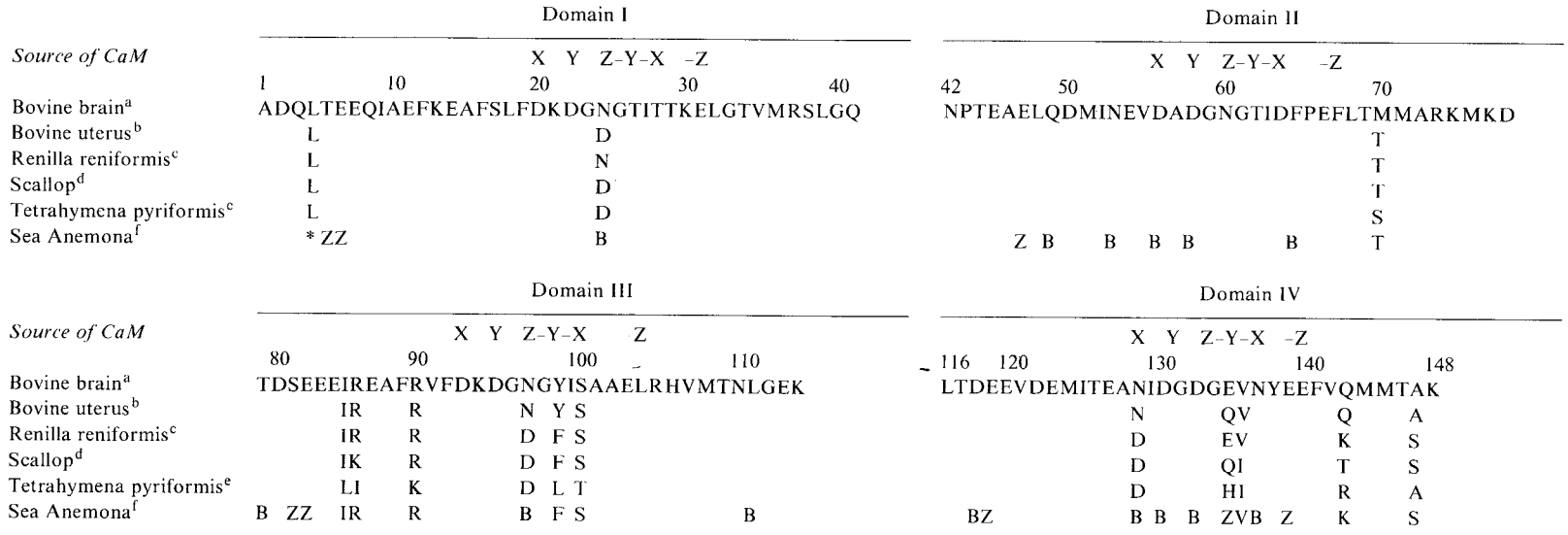
The primary structure of bovine brain calmodulin has been determined by Watterson et al. (32). Calmodulin exhibits four putative calcium binding sites called I, II, III, IV starting from the NH_2-terminal end. The amino acid sequence of calmodulin has been very well conserved during evolution. A comparison of the structure of six calmodulins from different species (Fig. 5) shows that only nineteen residues among the 148 of the sequence have been modified. These modifications include two deletions: one in sea anemona (residue 4) and another one in *Tetrahymena pyriformis* (residue 146). The modified amino acids are located either in the calcium binding loops and especially in the loops of domain III and IV (9 substitutions) or in the peptide connecting site II and III (from residue 70 to 90, 5 substitutions) and in the COOH-terminal part of the molecule (residue 141 to 148, 3 substitutions). Noteworthy is the fact that the calcium binding loop in domain II has been entirely conserved and that in domain I the changes are minimal with replacement of aspartic acid residues by asparaginyl residues and vice versa at the Z position.

Calcium binding to calmodulin

Numerous studies have been performed to determine the calcium binding to calmodulin by either using conventional dialysis techniques or following the changes in the spectroscopic properties of the protein upon calcium addition.

A general agreement exists as to the presence of four calcium binding sites in calmodulin, but some discrepancies relative to the affinity and the specificity of these sites for calcium still remain. In addition, recent reports have described the binding of potassium and magnesium ions to calmodulin, binding which alters the affinity of calcium for the protein.

Before we start the discussion on ion binding to calmodulin, it is necessary to develop the general equations describing the interaction of a macromolecule with one ligand on n sites and introduce



[a] Ref. 32; [b] Ref. 111; [c] Ref. 112; [d] Ref. 113; [e] Ref. 114; [f] Ref. 115.

Fig. 5. Primary structure of the Ca^{2+}-binding domains of six different calmodulin species. X, Y, Z, -Y, X, -Z represent the six Ca^{2+} coordinating ligands.

the concept of macroscopic and microscopic binding constants.

Ion binding to a macromolecule can be described by the equation of Adair-Klotz (33):

$$\nu = \frac{x \dfrac{\partial P(x)}{\partial x}}{P(x)} \qquad (1)$$

where ν represents the average number of ligand I molecules bound per mole of protein for a given concentration x of free ligand I. The expression of P(x), the binding polynomial is the following:

$$P(x) = 1 + K_1 x + K_1 K_2 x^2 + \ldots + K_1 K_2 \ldots K_i x^i + + K_1 K_2 \ldots K_i \ldots K_n x^n$$

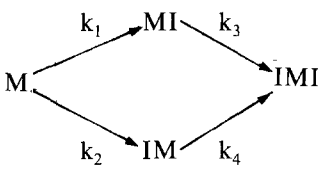
where n is equal to the number of ion binding sites and K_i is called the macroscopic binding constant or Adair constant. This equation is based on the assumption that the ligand binds to the macromolecule in a stepwise manner, and does not take into account allosteric phenomenons.

If M is the macromolecule and I the ion, the following scheme takes place:

$$M + I \underset{}{\overset{K_1}{\rightleftharpoons}} MI \quad \text{and } K_1 = [MI]/[M] \cdot [I]$$

$$MI_{i-1} + I \overset{K_i}{\rightleftharpoons} MI_i \quad \text{and } K_i = [MI_i]/[M_{i-1}] \cdot [I]$$

In the case of a protein with two sites for the ligand I, the binding of I can follow different pathways depicted as follow:



$k_1 = [MI]/[M] \cdot [I]$
$k_2 = [IM]/[M] \cdot [I]$
$k_3 = [IMI]/[MI] \cdot [I]$
$k_4 = [IMI]/[IM] \cdot [I]$
$k_1 k_3 = k_2 k_4$

where k_1 and k_2 represent the intrinsic binding constant of ligand I for site 1 and 2, respectively. In general $k_3 = \delta_1 k_2$ et $k_4 = \delta_2 k_1$; δ_1 and δ_2 being cooperativity factors describing respectively the change in affinity of the ligand for site 2 when site 1 is occupied and for site 1 when site 2 is occupied.

The same binding of I to the protein could be described using the stepwise model:

$$M + I \underset{}{\overset{K_1}{\rightleftharpoons}} MI \quad \text{with } K_1 = [MI]/[M] \cdot [I]$$

$$MI + I \underset{}{\overset{K_2}{\rightleftharpoons}} MI_2 \qquad K_2 = [MI_2]/[MI] \cdot [I]$$

The relationship between the macroscopic constants K_1, K_2 and the microscopic constants k_1, k_2, k_3, k_4 is the following:

$$K_1 = k_1 + k_2 \text{ and } K_2 = k_3 \cdot k_4 / k_3 + k_4$$

The intrinsic binding constant appears in the theory developed by Scatchard. In its initial form, the model of Scatchard describes only systems in which the macromolecule possesses n equivalent and independent sites. This means that in our case (a macromolecule with 2 sites)

$$k_1 = k_2 = k_3 = k_4 = k$$
$$\text{and } P(x) = 1 + K_1 x + K_1 K_2 x^2 = (1 + kx)^2$$

Therefore

$$\nu = \frac{x \dfrac{\partial P(x)}{\partial x}}{P(x)} \quad 2\,kx/1 + kx$$

and

$$\nu / x = k\,(2 - \nu) \qquad (2)$$

In (2) we recognize the expression of Scatchard describing the binding of a ligand to a macromolecule with 2 sites and an affinity constant k for these sites. It appears therefore that the Scatchard model is a specific case of the more general model of Klotz and Adair.

In a general ion binding study, the macroscopic binding constants are the parameters which are directly obtained from the experiment. The microscopic or Scatchard binding constants can be derived from them.

Table 4 summarizes the calcium binding to calmodulin determined in different laboratories using different conditions. It is obvious that the microscopic calcium binding constants depend on the concentrations of magnesium and potassium. With the assumption that potassium modifies the interaction between calcium and calmodulin only by a simple competition phenomenon, it has been possible to calculate the intrinsic binding constants of calcium, magnesium and potassium (Table 5).

The results obtained from our model predict that:

- Calmodulin presents four sites, each of which binds calcium, magnesium and potassium.
- The binding of calcium to calmodulin follows a sequential and ordered pathway; this means that

Table 4. Macroscopic calcium binding constants of calmodulin at different concentrations of K^+ and Mg^{2+} (μM).

Buffer	K^+ mM	Mg^{2+} mM	$1/K_1$	$1/K_2$	$1/K_3$	$1/K_4$	Ref.
10 mM Tris pH = 7.55	0	0	0.13	0.14	0.60	1.3	116
10 mM Tris pH = 7.4	0	0	0.06	0.18	0.45	1.6	36
10 mM Tris pH = 7.55	20	0	0.37	0.46	1.6	8.5	116
10 mM Hepes pH = 7.55	20	0	0.38	0.47	1.85	10.4	116
10 mM Imidazole pH = 6.5	100	0	0.6	1.5	3.5	9.5	38
10 mM Tris pH = 7.55	40	0	0.73	0.79	3.8	31	116
10 mM Hepes pH = 7.55	20	1	1.6	0.66	30	4.3	116
10 mM Tris pH = 7.55	100	0	2	1.9	7.3	61	116
10 mM Tris pH = 7.0	0	0	2	6	18	–	
49 mM $(NH_4)_2$ SO_4							58
10 mM Hepes pH = 7.5	100	0	3.3	1.16	8.3	22	39
10 mM Tris pH = 7.55	200	0	3.7	3.3	22	580	116
100 mM Hepes pH = 7.55	200	0	3.7	12	33	–	116
100 mM Hepes pH = 7.5	20	5	4.3	5.1	79	36	39
10 mM Hepes pH = 7.5	100	3	5.3	4.8	25	38	39
10 mM Mops pH = 7.2	150	1	5.3	3.6	22	83	117
100 mM Hepes pH = 7.55	200	5	7	56	67	–	116
50 mM Hepes pH = 7.5	75	5	7.5	2.7	31	31	56
10 mM Hepes pH = 7.55	20	10	9.2	8.9	170	51	116
100 mM Hepes pH = 7.55	200	10	10	40	160	–	116
100 mM Hepes pH = 7.55	200	25	18	67	630	–	116
100 mM Hepes pH = 7.55	20	25	28	16	560	70	116
100 mM Hepes pH = 7.55	200	50	42	190	–	–	116
10 mM Hepes pH = 7.55	20	50	47	38	480	230	116
100 mM Hepes pH = 7.55	200	100	91	–	–	–	116

calcium binds to a first site, thus modifying the affinity for the second site, then binds to a second site modifying the affinity for the third site and so on.

- Magnesium and potassium bind to the same sites than calcium but their binding occurs randomly.

Our model does not take into account the following three facts:

1. The existence of additional low affinity calcium binding sites which disappear in the presence of potassium.
2. At high concentration (>10 mM), the effect of magnesium on the calcium binding is not dependent any more on the concentration of potassium. At such concentrations, magnesium could bind to the additional low affinity calcium binding sites and modify calcium binding.
3. Anions or zwitterions (such Hepes) may modify calcium binding at high concentration (>100 mM).

However, two main conclusions emerge from our model:

1. Calcium binding is affected by potassium and magnesium ions.
2. Calcium binding follows a sequential pathway.

In addition, the intrinsic binding constants of each site for each ion clearly show that discrepancies between the different affinity constants reported in the literature can be easily explained on the basis of the different experimental conditions used in various laboratories (12, 34–39).

The next paragraph will bring more evidence for sequential calcium binding.

Table 5. Intrinsic dissociation constants of calmodulin for Ca^{2+}, Mg^{2+}, K^+.

Sites	1	2	3	4
Ca^{2+} (nM)	67	170	600	900
Mg^{2+} (μM)	70	270	100	90
K^+ (mM)	3.7	10.6	8.7	1.5

The intrinsic binding constants for the different ions have been computed using data from Ref. 116.

Sequence of calcium binding to calmodulin

The experimental data presented in Table 5 do

not fit with the presence on calmodulin of four equivalent or four independent calcium binding sites, but are compatible with a sequential and ordered binding of calcium.

We have determined the affinity of each Ca^{2+}-binding site for potassium. If the affinity of a given calcium binding domain for potassium is assumed to be proportional to the number of carboxyl groups in this domain, the sites can be assigned to primary structure domains on the basis of their affinity for potassium. If I, II, III and IV (from the NH_2-terminus to the COOH-terminus) stand for the four Ca^{2+}-binding domains of calmodulin, the sequential pathway followed by Ca^{2+} binding can be either II → I → III → IV or II → III → I → IV. Domains I, II, III, IV indeed contain 4, 3, 3 and 5 carboxyl groups, respectively (32). This binding sequence has been established, taking the amino acid sequence from bovine brain as reference. It is however necessary to mention that the number of carboxyl groups in each calcium binding domain may vary with different calmodulins.

Terbium has been shown to be a useful tool in the study of calcium binding to proteins (40) in general and to calmodulin in particular. Due to their ionic radius, terbium ions compete with calcium ions for calcium-binding sites. Moreover terbium is as effective as calcium in the activation of the calmodulin-dependent cyclic nucleotide phosphodiesterase (41). When bound to a protein close to an aromatic residue, terbium ions become highly luminescent as a result of a dipole-quadrupole energy transfer process. By contrast, free Tb^{3+} is only poorly luminescent. When metal-free ram testis calmodulin was titrated with terbium, the increase in luminescence at 545 nm (upon excitation at 275 nm) remained weak up to two Tb^{3+} bound per mol of calmodulin. This already indicates that the two first Tb^{3+} ions are bound to sites which lack tyrosyl residues, a confirmation of the sequential and ordered binding. Vertebrate calmodulin contains two tyrosyl residues located in domain III at position 99 and in domain IV at position 138. Domains I and II lack tyrosyl residues and are therefore first occupied by terbium (42). Terbium titration of metal-free octopus calmodulin which appeared to contain only one tyrosyl residue, located at a position homologous to that of tyrosine 138 in domain IV of vertebrate calmodulins, shows that terbium luminescence increased, albeit weakly, only after three terbium were already bound. This means that domain IV has the lowest binding affinity and is the last to be saturated (43).

In conclusion to these experiments, the sequence of terbium binding to calmodulin can be described as follows: domains I and II followed by domain III and finally by domain IV.

The ambiguity in the Ca^{2+} binding sequence presented at the beginning of this paragraph is therefore solved, the final sequence being II → I → III → IV.

Wang and Aquaron (44), when studying terbium binding to bovine brain calmodulin, reached the same conclusions as we did with ram testis calmodulin.

The assignment of site IV as the lowest affinity calcium binding site has been confirmed by nuclear magnetic resonance studies. Using Gd^{3+}-broadening experiments, Krebs and Carafoli have shown that site IV was the site with the highest Gd^{3+}/Ca^{2+} exchange rate and therefore the site with the lowest affinity for calcium (45).

Recently, a study of terbium binding to bovine brain calmodulin has been reported by Wallace et al. (41). Although their results agree with a sequential terbium binding to calmodulin, the sequence of binding they propose (I → III → IV → II) is different from ours. Indeed, the increase of terbium luminescence at 545 nm (upon excitation of the protein at 275 nm) occurs after one mol of terbium has been bound per mol of calmodulin (compared to 2 mol of terbium per mol of calmodulin in our study). Two main differences exist between the two experiments:

1. The presence in their buffer solution of 100 mM KCl. According to Table 5, the affinity of potassium for the first site occupied by calcium (domain II) is greater than its affinity for the second site (domain I). Therefore, potassium ions increase the positive cooperativity existing between the two first sites occupied by calcium. Indeed, in the presence of potassium, the macroscopic calcium binding constants of the first sites occupied by calcium are almost identical (Table 4). As a corollary, when calcium (in the presence of potassium) binds to the first site (domain II), the second site (domain I) is immediately occupied by calcium. Therefore, when the stoichiometry Tb^{3+}/ calmodulin is equal to 1, the Tb^{3+}-calmodulin complexes are mainly calmodulin-Tb_0 and calmodulin-Tb_2. The third site occu-

pied by calcium has a low affinity for potassium and therefore is occupied by terbium before the first and second sites are entirely saturated. As a result of the presence of potassium, the lag phase of Tb^{3+} luminescence increase is reduced. This is in agreement with the result reported by Wallace et al. (41).

If site four (domain IV) is occupied by calcium or terbium (due to its high affinity for potassium) at the end, terbium luminescence is expected to increase between 3 and 4 mol of Tb^{3+} per mol of calmodulin. As shown previously (46), the energy transfer between tyrosine 138 and Tb^{3+} is less efficient than the transfer between tyrosine 99 and Tb^{3+}. Terbium luminescence increase should then be biphasic with:

- a rapid increase between 1–2 and 3 terbium per mol calmodulin,
- and a much slower increase between 3 and 4 terbium bound per mol of protein.

This seems to be the case in the graphs reported in (41). Taking into account the presence of potassium ions, their experiments fit very well with our model.

2. Calmodulin is very stable in the presence of calcium. However, in the metal-free state, the protein loses its properties quite rapidly. It is therefore possible that the extensive dialysis (1 week) used by Wallace et al. to remove calcium from calmodulin has changed the ion binding properties of the macromolecule. Decalcification of calmodulin using trichloracetic acid precipitation has the advantage of being more rapid.

As a conclusion to this paragraph, we can say that today a number of studies favour a sequential and ordered binding of calcium to calmodulin which can be affected by potassium ions and which proceeds along the following pathway:

Domain II → Domain I → Domain III → Domain IV

The conformational changes induced by calcium binding to calmodulin are also sequential

Conformational changes triggered by calcium binding to calmodulin have been studied by a number of techniques including circular dichroism (12, 38), ultraviolet differential spectra (12), tyrosine fluorescence (38, 46, 47, 48) proton nuclear magnetic resonance (49), susceptibility to proteolytic attack (50), reactivity towards various chemical modifications (51, 52, 53, 54, 55). Each of them gives a signal S which can be followed as a function of the added calcium.

If S_0, S_1, S_2, S_3, S_4 are the signals corresponding to the complexes CaM-Ca_0, CaM-Ca_1, CaM-Ca_2, CaM-Ca_3, CaM-Ca_4 then

$$S = \frac{S_0 + K_1 S_1 x + K_1 K_2 S_2 x^2 + K_1 K_2 K_3 S_3 x^3 + K_1 K_2 K_3 K_4 S_4 x^4}{1 + K_1 x + K_1 K_2 x^2 + K_1 K_2 K_3 x^3 + K_1 K_2 K_3 K_4 x^4}$$

Equation (3)

where K_1, K_2, K_3, K_4 represent the macroscopic calcium binding constants previously defined and x, the free calcium concentration. S can be generalized to all kinds of signals and can namely represent the number of ligands bound per mole of calmodulin. In this case S_0, S_1, S_2, S_3, S_4 are respectively equal to 0, 1, 2, 3, 4 and S can be represented by the symbol ν.

Equation 3 becomes then:

$$\nu = \frac{K_1 x + 2K_1 K_2 x^2 + 3K_1 K_2 K_3 x^3 + 4K_1 K_2 K_3 K_4 x^4}{1 + K_1 x + K_1 K_2 x^2 + K_1 K_2 K_3 x^3 + K_1 K_2 K_3 K_4 x^4}$$

which is the Klotz-Adair equation defined in (1).

In experiments where K_1, K_2, K_3, K_4 have been determined, S_0, S_1, S_2, S_3 and S_4 can be calculated by following S as a function of the free calcium concentration. This means that informations are obtained about the conformation of calmodulin with one, two, three or four calcium bound.

In most studies, S has been plotted versus total added calcium C_T at calmodulin concentrations significantly higher than the dissociation constant so that the added calcium can be considered as bound calcium ($C_T = \nu$).

In such plots, the signal S observed at molar ratios Ca^{2+}/CaM equal to 1 does not necessarily correspond to S_1, the signal given by calmodulin with one calcium ion bound. Depending on K_1, K_2, K_3, K_4, S could represent the average signal given by a mixture of various calcium-calmodulin complexes.

A simple interpretation of these plots can only be given in certain cases, where the observed signal is very specific (in NMR for example).

Although an exhaustive analysis of the conformational changes induced by calcium has never been done, the following results have been reported.

Calcium binding to calmodulin does not drastically alter the overall shape of the protein. Indeed, the hydrodynamic parameters of calmodulin are not changed upon calcium binding (12). However the alpha-helicity of the protein was found to increase by about 15% and the environment of residues such as tyrosines 99 and 138, lysine 77, methionines 71, 72 and 76 and histidine 107 was found to be modified (45, 49, 55).

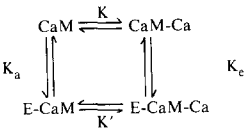
Moreover, these changes are associated with at least a two step process. When calmodulin binds the two first calcium ions, most of the conformational changes occur. Tyrosine fluorescence (46, 47) as well as circular dichroism (12) changes have been shown to be complete after two mol of calcium are bound per mol of calmodulin. During this first step both tyrosyl residues 99 and 138 are affected (46). Therefore binding of calcium to domains I and II induces an overall conformational change which affects domain III and IV. Subsequently binding of calcium to domains III and IV changes only locally the structure of the protein; in NMR, phenylalanine and tyrosine resonances sharpen and the residues move to their final positions (45). However, these minor conformational changes of calmodulin are critical for the activation of the target enzymes.

These studies show that the conformational changes induced by calcium is sequential, with a first overall change obtained at two calcium bound per mol of calmodulin and minor changes occurring when successively the third and the fourth calcium binds to the protein. According to our model, domain II plays the most important role in that it is the starting point to the sequential binding mechanism. This should be put together with the fact that domain II has been very well conserved during evolution (see paragraph: structure of calmodulin).

Why a sequential binding to four calcium sites?

The advantages of having four calcium binding sites on calmodulin have been exhaustively discussed by Huang et al. (56). Let us compare two situations: one in which calmodulin would have only one calcium binding site and the other one where it has four sites.

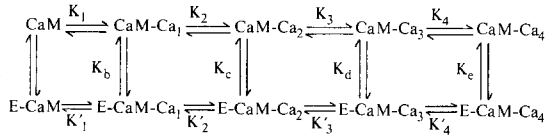
With only one calcium binding site on calmodulin, the enzyme (E) – calmodulin (CaM) – calcium system would be thermodynamically represented as follows:



For simple thermodynamic reasons $K \cdot K_e = K_a \cdot K'$.

As $K = 10^6\ M^{-1}$, $K_e = 10^9\ M^{-1}$ and $K_a < 10^5\ M^{-1}$ (56), the affinity constant K' of calcium for the complex E-CaM is greater than $10^{10}\ M^{-1}$. This means that once the system has been switched on, it will be very difficult and time consuming to remove calcium and dissociate the E-CaM-Ca complex. Such a tight binding reduces the efficiency of the control process.

As calmodulin has four sites for calcium, the following scheme occurs in the interaction with an enzyme:



Equation (4)

K'_1, K'_2, K'_3 and K'_4 have not been determined. However, as $K_a = 10^{-4} K_e$, we can roughly estimate that $K'_i \sim 10\ K_i$, i.e. $K'_i \sim 10^7\ M^{-1}$.

In this case, the tremendous affinity difference between enzyme and calmodulin in the absence of calcium ($K_a < 10^5\ M^{-1}$) and at saturating levels of calcium ($K_e = 10^{10}\ M^{-1}$) can be overcome relatively easily through a 10 to 20 fold decrease in Ca^{2+} affinity for the enzyme-calmodulin complex in each of the four steps.

Why is the binding of calcium sequential?

Let us assume that the different calmodulin-calcium complexes (CaM-Ca_1, CaM-Ca_2, . . .) activate different calmodulin-dependent enzymes. Then the sequential binding of calcium to calmodulin would theoretically generate a sequential activation of the different enzymes (57). A first subset of enzymes would then be activated upon a limited entry of calcium, yielding essentially the CaM-Ca_2 complex

(due to the K^+-induced cooperativity between the two first sites). Then as calmodulin binds more calcium, other enzyme subsets would be activated.

Thus the sequential binding to four calcium binding sites may allow calmodulin to transform a quantitative entry of calcium into qualitatively different responses and to activate different enzymes in an efficient and reversible way.

Enzyme–calmodulin interaction

Two different appraoches have been used to investigate the interaction of calmodulin with its target proteins.

The first approach was a kinetic study of the mechanism of calmodulin-dependent enzyme activation. This has been done using cyclic nucleotide phosphodiesterase (56, 58) brain adenylate cyclase (59) and myosin light chain kinase (60, 61).

The second approach was the determination of the sites or domains of calmodulin that interact with the enzymes.

Concerning the kinetic study of the interaction, all reports except one (61) use the scheme (4).

In addition, in all experiments, the concentration of enzyme (E_T) was much lower than the calmodulin concentration (C_T).

If S is the observed enzymatic activity and S_L the enzymatic activity of the complex E-CaM-Ca_L ($0 \leqslant L \leqslant 4$), S can be expressed as a function of the free calcium concentration x and the free enzyme concentration y.

$$S = C_T \frac{\begin{array}{c} S_0K_ay + S_1K_1K_bxy + S_2K_1K_2K_cx^2y \\ + S_3K_1K_2K_3K_dx^3y + S_4K_1K_2K_3K_4K_ex^4y \end{array}}{\begin{array}{c} 1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4 \\ + K_ay + K_1K_bxy + K_1K_2K_cx^2y + K_1K_2K_3K_dx^3y + K_1K_2K_3K_4K_ex^4y \end{array}}$$

As $E_T \ll C_T$ and $E_T \ll K_a^{-1}$.

$$S = C_T \frac{\begin{array}{c} S_0K_ay + S_1K_1K_bxy + S_2K_1K_2K_cx^2y + S_3K_1K_2K_3K_dx^3y \\ + S_4K_1K_2K_3K_4K_ex^4y \end{array}}{1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4}$$

The kinetic study of the interaction enzyme-calmodulin can be analyzed using the equation of the Adair-Klotz in its more general form. If x, y represent the free concentration of calcium and enzyme, respectively, and S a signal (spectroscopic, enzymatic), in the case of scheme 4, S can be written under the general form.

$$S = \frac{\begin{array}{c} S_0 + S_1K_1x + S_2K_1K_2x + S_3K_1K_2K_3x^3 + S_4K_1K_2K_3K_4x^4 + S_5K_ay \\ + S_6K_bK_1xy + S_7K_cK_1K_2x^2y + S_8K_dK_1K_2K_3x^3y + S_9K_eK_1K_2K_3K_4x^4y \end{array}}{\begin{array}{c} 1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4 + K_ay + K_bK_1xy \\ + K_cK_1K_2x^2y + K_dK_1K_2K_3x^3y + K_eK_1K_2K_3K_4x^4y \end{array}}$$

The association constants K_i are those defined in scheme 4 and S_L is the signal corresponding to the complex E-CaM-Ca_L ($0 \leqslant L \leqslant 4$).

In all experiments performed, the concentration of enzyme E_T was much lower than the total calmodulin concentration C_T and $E_T \ll K_a^{-1}$. Therefore:

$$S = \frac{S_0 + S_1K_1x + S_2K_1K_2x^2 + \ldots + S_9K_eK_1K_2K_3K_4x^4y}{1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4}$$

If A is the enzymatic activity of the enzyme-calmodulin complex, then $A = C_T \times S$.

In addition, as the complex CaM-Ca_i is devoid of enzymatic activity $S_0 = S_1 = S_2 = S_3 = S_4 = 0$ and S_5, S_6, S_7, S_8, S_9 can be termed respectively a_0, a_1, a_2, a_3, a_4.

Let us call $Q_1(x) = a_0K_a + a_1K_bK_1x + a_2K_cK_1K_2x^2 + a_3K_dK_1K_2K_3x^3 + a_4K_eK_1K_2K_3K_4x^4$

and $Q_2(x) = 1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4$

Then $A = y\,C_T \frac{Q_1(x)}{Q_2(x)}$

Using the equation of Adair Klotz, we can determine the molar ratio of enzyme ν_y in the complex E-CaM-Ca_i.

$$\nu_y = \frac{K_ay + K_bK_1xy + K_cK_1K_2x^2y + K_dK_1K_2K_3x^3y + K_eK_1K_2K_3K_4x^4y}{1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4}$$

In addition $E_T = y + \nu_y \cdot C_T$ (as $E_T \ll C_T$, the total concentration of E-CaM-Ca_L complex is equal to C_T).

If $Q_3(x) = K_a + K_bK_1x + K_cK_1K_2x^2 + K_dK_1K_2K_3x^3 + K_eK_1K_2K_3K_4x^4$.

$$E_T = y + y\,C_T \frac{Q_3(x)}{Q_2(x)}$$

$$y = \frac{E_T}{1 + C_T \dfrac{Q_3(x)}{Q_2(x)}}$$

$$\text{Therefore } A = \frac{E_T}{1 + C_T \frac{Q_3(x)}{Q_2(x)}} C_T \frac{Q_1(x)}{Q_2(x)}$$

$$\text{and } \frac{E_T}{A} = \frac{1}{C_T} \frac{Q_2(x)}{Q_1(x)} + \frac{Q_3(x)}{Q_1(x)}$$

If the free calcium concentration (x) is kept constant, Q_1, Q_2 and Q_3 are constant and a plot of E_T/A versus $1/C_T$ gives a straight line. This has been experimentally observed by Blumenthal and Stull (60), Huang et al. (56), Cox et al. (58) and Malnoe et al. (60) with three different enzymes. As K_1, K_2, K_3, K_4 and thus $Q_2(x)$ can be determined from direct ion binding studies to calmodulin, $Q_1(x)$ and $Q_3(x)$ can be determined from the slope and the intercept. Thus theoretically a_0, a_1, a_2, a_3, a_4, K_a, K_b, K_c, K_d, K_e could be determined. However, the precision of the experiments did not allow measurement of K_a, K_b, K_c and K_d. Thus only a_0 K_a, a_1 K_b, a_2 K_c, a_3 K_d, K_e, a_4 could be determined. Huang et al. (56) showed that in the interaction of calmodulin with cyclic nucleotide phosphodiesterase $a_0 = a_1 = a_2 = a_3 = 0$, $a_4 \neq 0$ and $K_e = 10^{10}$ M^{-1}. This means that only calmodulin with four bound calcium is able to activate the enzyme and that the affinity constant of phosphodiesterase for the complex CaM-Ca_4 is 10^{10} M^{-1}.

With cyclic nucleotide phosphodiesterase and brain adenylate cyclase, Cox et al. (58) and Malnoe et al. (59) found that $a_0 = a_1 = a_2 = 0$ but a_3 and $a_4 \neq 0$. The discrepancy could be relevant to the fact that the determination of K_1, K_2, K_3, K_4 has been done under conditions slightly different from those existing in the assay medium. It appears therefore that the complex able to activate cyclic nucleotide phosphodiesterase or adenylate cyclase is probably CaM-Ca_4.

Using a slightly different method of analysis, Blumenthal and Stull (60) reached the same conclusions with skeletal muscle myosin light chain kinase.

The interaction between myosin light chain kinase and calmodulin has also been investigated using the fluorescence technique (62). The authors came to the conclusion that binding of one mole of calcium per mole of calmodulin is enough to activate skeletal muscle myosin light chain kinase. However, analysis of the data was performed by taking only into account the calmodulin–calcium system, thereby neglecting the interaction between the enzyme and the different calmodulin–calcium complexes as well as the perturbation induced by the enzyme on the calcium–calmodulin system. Refering to the most exhaustive studies realized by Huang et al. (56) and Cox et al. (59, 60) it seems that for the time being the complexes CaM-Ca_4 (and perhaps CaM-Ca_3) are the active species in the stimulation of various enzymes.

To determine the domain of interaction between calmodulin and the target proteins, three different methods have been used:

1) the chemical modification of calmodulin on specific residues and the comparison of the ability of these calmodulin derivatives to activate calmodulin-dependent enzymes (Table 6),

2) the use of drugs known to interact with calmodulin and to prevent the activation of enzymes (15),

3) the activation of calmodulin-dependent enzymes by proteolytic fragments of calmodulin (62).

Table 6. Chemical modifications of calmodulin and their effects on the activation of target enzymes (55, 118).

Residue	Reagent	Important modified residue	Phosphodiesterase activity	Interaction with Tn I	Adenylate cyclase	Synaptosomal binding
Histidine	Diethylpyrocarbonate	His 107	+	+	+	
Arginine	Cyclohexanedione	4 Arg out of 6	+	–	±(76%)[a]	±(64%)[a]
Tyrosine	Tetranitromethane	Tyr 99, Tyr 138	+	+	+	+
Lysine	Potassium isocyanate	2 Lys out of 8	±(41%)[a]	ND[b]	+	+
Lysine	o-methylisourea	2 Lys out of 8	+	ND	ND	ND
Methionine	Chloramine T	Met 71, 72, 76 eventually Met 109	–	–	–	–
	Iodoacetate					

[a] Numbers in parentheses indicate remaining activity.
[b] ND: Not Determined.

Without reviewing all results, the main informations brought about by these techniques can be summarized as follows:

1. The use of various calmodulin derivatives in the interaction with target proteins (Table 6) suggests that calmodulin has several interacting domains. Indeed, chemical modifications of arginyl or lysyl residues affect differently the interaction between calmodulin and cyclic nucleotide phosphodiesterase, troponin I or adenylate cyclase.

2. Oxidation of methionyl residues (55) and the use of proteolytic fragments of calmodulin (62) suggest that peptide 70–79 may be involved in the interaction of calmodulin with cyclic nucleotide phosphodiesterase.

3. Activation of cyclic nucleotide phosphodiesterase is inhibited competitively by hydrophobic compounds such as trifluoperazine (15) or W7 (63). It seems therefore reasonable to admit that these compounds and the enzyme interact with calmodulin on the same domain.

Recent studies on the binding domain of trifluoperazine have shown the existence of two interacting sites located in the region 70–124 (64) and another one on the COOH-terminal part of the protein (44, 65).

It is worth pointing out that interaction between trifluoperazine and calmodulin only occurs in the presence of calcium (15) and that binding of calcium to calmodulin exposes a hydrophobic region (66). Lysyl residues are extremely important in the activation of phosphodiesterase and there are two lysyl residues in the stretch 70 to 79 (lysine 75 and 77). Taking together these data, it is tempting to speculate that the interaction between cyclic nucleotide phosphodiesterase and calmodulin involves, in domain 70–79, both hydrophobic (via methionyl residues 71, 72, 76) and electrostatic (via lysyl residues 75 and 77) binding.

4. Limited proteolytic attack of calmodulin generates three main peptides: peptides 1–76, 77–148 and 1–106 (63). None of these peptides, despite the fact that it binds calcium, can efficiently activate cyclic nucleotide phosphodiesterase. This is in favour of the presence on calmodulin of more than one binding site for cyclic nucleotide phosphodiesterase and probably for other target enzymes. Additional evidence came from the existence of multiple binding sites between troponin I (one of the target proteins of calmodulin) and troponin C, a protein closely homologous to calmodulin (67).

In addition, the unmasking of the different binding sites could well be correlated with the binding of calcium to calmodulin. In this case, it becomes obvious that the activation of the different calmodulin-dependent enzymes is sequential and modulated by calcium influx. Moreover, one should make a dinstinction between the binding to and the activation of the enzymes. Indeed binding of one or two calcium ions could well unmask specific sites, thus allowing the binding of calmodulin to the enzyme, but not its activation which would require additional calcium binding.

Calmodulin: a protein with four triggering sites

Calmodulin appears as a calcium binding protein able to stimulate various enzymes in response to an increase in the intracellular calcium concentration. The calcium binding sites on calmodulin are therefore called triggering sites as opposed to the relaxing sites present on other calcium binding proteins such as parvalbumins (see next section). Binding of calcium follows a sequential and ordered pathway, the sequence of occupancy of the different domains being: II → I → III → IV. However binding of calcium to the different sites is dependent upon the presence of potassium and magnesium ions.

Upon calcium binding, calmodulin exhibits domains that interact with target proteins. For the time being, we do not know how many such domains are present on calmodulin, nor do we know the relationship between the unmasking of these domains and the occupancy of the calcium binding sites. However, it seems likely that calmodulin does not interact in the same way with all the enzymes. Also one of the two hydrophobic domains present on calmodulin (region 70–79) may well be involved in the binding to cyclic nucleotide phosphodiesterase.

It is predictable that determination of these binding domains will be the purpose of numerous studies in the coming years.

Parvalbumin: properties and function

Parvalbumins form a class of closely homologous low molecular weight (ca. 12 000), acidic and highly soluble calcium-binding proteins, which are present in high concentration in the sarcoplasm of vertebrate fast-twitch skeletal muscles (68, 69) and

Table 7. Calcium binding to parvalbumins.

Source of parvalbumin	Conditions		Affinity constants for Ca^{2+}		Reference
	pH	Mg^{2+} (mM)	K_1 (M^{-1})	K_2 (M^{-1})	
Hake pI 4.36	6.7	2	$2 \cdot 10^7$	$5 \cdot 10^6$	103
Frog pI 4.88	6.7	2	$2 \cdot 10^7$	$5 \cdot 10^6$	103
Carp pI 4.25	7.0	0	$5 \cdot 10^8$	$1.25\ 10^8$	98
Carp pI 4.25	7.5	3	$1.5\ 10^7$	$3.9\ 10^6$	98
Whiting pI 4.44	8.8	0	$5 \cdot 10^8$	$6 \cdot 10^6$	78
Hake pI 4.36	7.55	0	$4 \cdot 10^8$	$5 \cdot 10^7$	75
Hake pI 4.36	8	0	$4 \cdot 10^8$	$5 \cdot 10^7$	75
Hake pI 4.36	6.8	0	$3 \cdot 10^8$	$1.6\ 10^7$	75
Rabbit pI 5.55	7.55	50	$1.3\ 10^5$	$1.7\ 10^4$	75
Rabbit pI 5.55	7.55	100	$5.3\ 10^4$	$1.3\ 10^4$	75
Rabbit pI 5.55	7.55	150	$3.6\ 10^4$	$9.2\ 10^3$	75
Frog pI 4.88	7.55	50	$1.8\ 10^5$	$4.6\ 10^4$	75
Frog pI 4.88	7.55	100	$6.5\ 10^4$	$1.6\ 10^4$	75
Frog pI 4.88	7.55	150	$4.2\ 10^4$	$1.0\ 10^4$	75
Frog pI 4.5	7.55	50	$3.3\ 10^5$	$8.4\ 10^4$	75
Frog pI 4.5	7.55	100	$2 \cdot 10^5$	$5 \cdot 10^4$	75
Frog pI 4.5	7.55	150	$1.4\ 10^5$	$3.6\ 10^4$	75

in specific neuronal cells (70). Parvalbumin distribution is very specific when compared to the ubiquitous repartition of calmodulin. Therefore, it is interesting to study the role of this protein and its calcium binding mechanism. Large amounts of parvalbumins can be obtained very easily using fish muscle as starting material. The denomination of the different parvalbumins is based upon their origin and their isoelectric point (for instance carp Pa, pI 4.25). Among the parvalbumins, carp parvalbumin has been the most extensively studied and has been taken as a model of the parvalbumin class.

Crystallographic studies of parvalbumin have shown the existence of three domains called AB, CD, EF (5). Each of these domains is composed of two alpha-helices surrounding a twelve-residue loop, thus repesenting a putative calcium binding site. However, in the case of parvalbumins, domain AB has lost its ability to bind calcium, so that parvalbumins exhibit only two calcium binding sites.

Ion binding to parvalbumin

Ion binding to parvalbumin has been studied by using the same techniques as for calmodulin. Two sites, with high affinity for both calcium and magnesium have been shown to be present. Table 7 compares the calcium binding constants of different parvalbumins under various conditions. Calcium binding is very sensitive to the presence of magnesium ions. Changes in pH on the other hand do not markedly affect the affinity of calcium for its sites. The macroscopic binding constants for calcium and magnesium are given in Table 8. We suppose that magnesium binds to parvalbumin on

Table 8. Ion binding to parvalbumins.

Source of parvalbumin	K_1 (Ca^{2+}) (M^{-1})	K_2 (Ca^{2+}) (M^{-1})	K_1 (Mg^{2+}) (M^{-1})	K_2 (Mg^{2+}) (M^{-1})
Whiting pI 4.44	$5 \cdot 10^8$	$6 \cdot 10^6$	–	–
Hake pI 4.36	$4 \cdot 10^8$	$5 \cdot 10^7$	–	–
Rabbit pI 5.55	$3 \cdot 10^8$	$1 \cdot 10^8$	$1.3\ 10^5$	$3 \cdot 10^4$
Frog pI 4.88	$3 \cdot 10^8$	$6 \cdot 10^7$	$9.5\ 10^4$	$2.4\ 10^4$
Frog pI 4.50	$9 \cdot 10^8$	$2 \cdot 10^8$	$7.4\ 10^4$	$1.9\ 10^4$
Carp pI 4.25	$5 \cdot 10^8$	$1 \cdot 10^8$	–	–

Binding constants were determined using data from Table 7.

two independent and equivalent sites. For most parvalbumins, the affinity constants for calcium range from $3 \cdot 10^8$ M^{-1} to $9 \cdot 10^8$ M^{-1} for one site and from $5 \cdot 10^7$ M^{-1} to $2 \cdot 10^8$ M^{-1} for the second site. In whiting parvalbumin, the affinity for the second site is much lower ($6 \cdot 10^6$ M^{-1}). This can probably be explained by the mutation affecting the calcium binding residue -Y in domain EF of whiting parvalbumin, lysine which generally occupies position -Y being replaced by an alanyl residue in this parvalbumin (Table 9). The crystallographic study of the Ca^{2+}/Tb^{3+} exchange shows that calcium in site EF is exchanged first with terbium (71, 72, 73). The assignment of site EF to the lowest affinity calcium binding site is also corroborated by ^{113}Cd NMR spectroscopy (74). Therefore, in Table 8 the macroscopic calcium binding constants K_1 and K_2 can be correlated respectively to the binding of calcium to domain CD and domain EF.

Potassium ions bind only weakly to parvalbumin and do not compete with the binding of calcium or magnesium ions.

Is calcium binding to parvalbumin sequential?

Parvalbumins have been first presented as a class of proteins with two independent calcium-binding sites, which bind randomly both calcium and magnesium in a competitive manner (76).

However, a sequential binding could explain the binding results as well and therefore cannot be totally ruled out.

Using binding studies, a distinction between the two models can only be made if one can find a perturbating system which affects the binding sites in a different manner.

For calmodulin for example, the discrimination could be done because potassium ions were binding to the same sites than calcium, but with an affinity different for each site. In the case of parvalbumins, magnesium binds to the same sites than calcium, but has an equal affinity for each site. Moreover, as changes in pH do not affect calcium binding, this technique cannot be used to induce differences between the sites. Therefore, as pointed out by Lee and Sykes (76), the sequential binding versus independent sites question is in general irrelevant.

Recently Permyakov et al. (77) have studied by fluorescence the calcium binding to whiting parvalbumin. Using an analysis method quite similar to the one described in the previous section, they were able to obtain the spectrum corresponding to parvalbumin with one calcium or two calcium bound. As the spectra of the apoparvalbumin and the forms with one or two bound calcium are different they assumed that the binding of calcium was sequential. However, their results do also fit with the model of two independent sites if one assumes that the spectrum corresponding to the conformation with one calcium is in fact the combination of the spectra of two classes of parvalbumin, with one calcium in the CD site and one in the EF site, respectively.

More evidence in favour of a sequential binding

Table 9. Amino acid sequence in the calcium binding loop of the two domains of parvalbumin.

Source of Pa	X		Y		Z		-Y		-X			-Z
						Domain CD						
Whiting pI 4.44	D	Q	D	K	S	G	F	I	E	E	D	E
Hake pI 4.36	D	Q	D	K	S	D	F	V	E	E	D	E
Rabbit pI 5.55	D	K	D	K	S	G	F	I	E	E	E	E
Frog pI 4.50	D	R	D	K	S	G	F	I	E	Q	D	E
Frog pI 4.88	D	Q	D	Q	S	G	F	I	E	K	E	E
Carp pI 4.25	D	Q	D	K	S	G	F	I	E	E	D	E
						Domain EF						
Whiting pI 4.44	D	S	D	G	D	G	A	I	G	V	E	E
Hake pI 4.36	D	S	D	G	D	G	K	I	G	V	E	E
Rabbit pI 5.55	D	K	D	G	D	G	K	I	G	A	D	E
Frog pI 4.50	D	S	D	G	D	G	K	I	G	V	E	E
Frog pI 4.88	D	K	D	G	D	G	K	I	G	V	D	E
Carp pI 4.25	D	S	D	G	D	G	K	I	G	V	D	E

Data are taken from refs. 75, 110, 120.

has emerged from experiments measuring the Ca^{2+}/Mg^{2+} exchange rate. Two methods have been used: one which assumes that the exchange rate is dependent on the dissociation rate of magnesium (78). These studies show that the exchange rate is rather slow and that parvalbumins cannot be involved in a twitch mechanism; the second measures the rearrangement rate of the protein conformation upon substitution of Ca^{2+} in the Mg^{2+} bound form (79). It shows that the Ca^{2+}/Mg^{2+} exchange rate is not dependent on the magnesium dissociation rate (magnesium dissociation rate = $1\ s^{-1}$; rate of conformational rearrangement = $100\ s^{-1}$).

If parvalbumin was a protein with two independent sites, then the exchange rate would be dependent on magnesium dissociation rate. These last results are therefore in favour of a sequential binding of calcium to parvalbumin.

Another argument supporting the sequential binding stems from the homology between parvalbumins and calmodulin. In this sequential binding, calcium will bind first to domain CD, then to domain EF.

Physiological role of parvalbumins

Parvalbumins are typically muscular proteins. Among all tissues examined only brain, beside muscle, has been found to contain some. However, they are not present in all muscle types but associate with fast, nerve-impulse activated, skeletal muscle. They bind calcium and magnesium with rather high affinities and do not interact with any other sarcoplasmic molecule. In a resting cell (0.6 mM $MgCl_2$ (79), 0.1 μM Ca^{2+} (1), 150 mM KCl, pH 7.4) parvalbumins are under the form Pa-Mg_2, due to their high affinity for magnesium ions. When upon stimulation the intracellular calcium concentration rises from 0.1 μM to 10 μM, binding of calcium only occurs after a delay determined by the Mg^{2+}/Ca^{2+} exchange rate which is rather slow ($t_{1/2}$ ca. 10 ms (80)). Thus, parvalbumins cannot be involved in a triggering mechanism which requires rapid binding of the calcium ions. However, the Mg^{2+}/Ca^{2+} exchange rate is compatible with a relaxing role of parvalbumins in muscle fibers. During the contraction cycle, the function of parvalbumins can be summarized as follows: upon nervous stimulation, calcium ions flow from the terminal cisternae into the cytosol and its intracellular concentration increases. Troponin C then binds calcium, allowing the interaction between actin and myosin, thereby contraction. After the delay imposed by the Mg^{+}/Ca^{2+} exchange rate, calcium binds to parvalbumin (which has a higher affinity for calcium than the regulatory sites of troponin C) thereby inducing relaxation. Calcium is then removed from parvalbumins by the sarcoplasmic reticulum (81).

Parvalbumins thus appear as soluble relaxing factors. In conclusion, parvalbumins are very similar to calmodulin as far as the binding mechanism is concerned. However, modification of the affinity for calcium and magnesium has crucial consequences on the physiological role of the protein. The calcium binding sites on parvalbumin are called 'relaxing sites' as opposed to the triggering sites of calmodulin.

Troponin C: a calcium binding protein with relaxing and triggering sites

The regulation of vertebrate skeletal muscle contraction involves the binding of Ca^{2+} to the troponin-tropomyosin complex localized on the actin thin filaments. Troponin is composed of three subunits: troponin T, troponin I and troponin C, the Ca^{2+} binding subunit of the troponin-tropomyosin complex. The role of different proteins in the regulation of muscle contraction has been discussed in numerous reviews (82, 83, 84). TnC is present in skeletal as well as in cardiac muscle. However, it has not been found in smooth muscle where the contraction is not regulated by an actin but rather by a myosin-linked system (85).

Calcium binding to skeletal TnC has been extensively studied by Potter and Gergely (86). The protein exhibits four calcium binding sites, two which have a high affinity for Ca^{2+}, but also bind Mg^{2+} ($K_{Ca} = 1.1\text{–}4.3\ 10^7\ M^{-1}$, $K_{Mg} = 1.8\ 10^3\ M^{-1}$) and two sites which bind only Ca^{2+} with a lower affinity ($K_{Ca} = 1.6\text{–}6.1\ 10^5\ M^{-1}$). Two additional sites for Mg^{2+} have been described ($K_{Mg} \cong 10^3\ M^{-1}$) (86) (Table 10). The primary structure of skeletal TnC (87, 88) is very similar to that of calmodulin and shows four homologous calcium binding domains numbered I, II, III, IV from the amino terminus. Using fluorescence spectroscopy (87, 90, 91, 92), NMR (93, 94) and circular dichroism (89, 95, 96)

Table 10. Ion binding to troponin C.

Ca^{2+} binding	Binding constants (M^{-1})			
	K_1	K_2	K_3	K_4
Skeletal TnC[a]	$4.3 \cdot 10^7$	$1.1 \cdot 10^7$	$6.1 \cdot 10^5$	$1.6 \cdot 10^5$
Cardiac TnC[b]	$2.8 \cdot 10^7$	$4.2 \cdot 10^7$	$2.5 \cdot 10^5$	–
Mg^{2+} binding	Binding constants (M^{-1})			
Skeletal TnC[a]	4 equivalent sites, $K = 1.8 \cdot 10^3$			
Cardiac TnC[b]	5 equivalent sites, $K = 1.0 \cdot 10^3$			

[a] From ref. 86; [b] From ref. 119.

the high affinity Ca^{2+} binding sites could be assigned to domains III and IV and the sites with lower affinity to domains I and II.

Recent studies suggest that domains I and II are able to bind monovalent ions such as Na^+ ($K_a \cong 10^2\ M^{-1}$) (97). This allows us to compare domains I and II of TnC to the Ca^{2+} binding domains present in calmodulin.

Cardiac TnC has only three Ca^{2+} binding sites, two with high affinity for Ca^{2+} which also bind Mg^{2+} in a competitive manner and one with lower affinity for Ca^{2+} (98, 99). The complete amino acid sequence of cardiac troponin C (CTnC) has been reported (100) and compared to that of its skeletal muscle counterpart. Although the two proteins are highly homologous, a peptide corresponding to site I in cardiac TnC shows a number of amino acid substitutions involving key metal-coordinating Asp and Glu residues. As a result of these substitutions, site I in cardiac TnC has lost its metal-binding capacity (100).

Therefore, the sites with high affinity in cardiac TnC are probably located in domains III and IV, and the site with lower affinity in domain II. Domains III and IV of TnC exhibit a high affinity for Ca^{2+}, bind Mg^{2+} in a competitive manner, but do not have any affinity for monovalent ions. They are therefore very similar to the Ca^{2+}/Mg^{2+} sites of parvalbumins (previous section). On the other hand, domains I and II in skeletal TnC and domain II of cardiac TnC show lower affinity for Ca^{2+}, but bind Ca^{2+}, Mg^{2+} and monovalent ions in a competitive manner. They closely resemble the Ca^{2+}-binding sites of calmodulin. Thus TnC contains two classes of sites: one class similar to relaxing sites of parvalbumins and the other equivalent to the triggering sites of calmodulin. Recently, Reid and Hodges (101, 102) have studied the calcium binding mechanism of TnC using synthetic peptides similar to domains of calmodulin. They conclude that Ca^{2+} binding to TnC follows a sequential pathway, with the sequence of binding being III → IV → II → I.

Physiological significance of the calcium-binding sites of TnC

Potter and Gergely have shown that the lower affinity calcium binding sites in TnC are involved in the Ca^{2+} regulation of actin-myosin interaction (86) and they assume that domains III and IV, which under physiological conditions are always occupied either by Mg^{2+} or Ca^{2+} are present to preserve the protein structure. However, domains III and IV may also be involved in the relaxing mechanism of muscle contraction according to the following scheme: during relaxation, the intracellular concentration of Mg^{2+} and Ca^{2+} (0.6 mM $MgCl_2$, 150 mM KCl, 0.1 μM $CaCl_2$) are such that domains III and IV are occupied by Mg^{2+}. Upon stimulation the intracellular calcium concentration rises to 10 μM and Ca^{2+} binds to the ion-free domains I and II of TnC allowing the interaction between actin and myosin and thereby contraction to take place. Meanwhile, Ca^{2+} exchanges with Mg^{2+} in domains III and IV, thereby inducing a conformational change (103). The latter may perhaps facilitate the removal of Ca^{2+} from the triggering sites I and II. However more experimental evidence is needed to give a complete description of the physiological role of domains III and IV in TnC.

Troponin C and calmodulin possess a number of similar properties, as might be expected from their common evolutionary origin and the similarity of their primary structures. However, the two proteins differ notably in their affinity for Ca^{2+} and Mg^{2+} and thus in the types of Ca^{2+}-binding sites present. TnC represents in fact a specialized form of calmodulin which was required for the Ca^{2+} control of the highly organized structure involved in skeletal muscle contraction.

Conclusion

The study of calcium-binding proteins along two lines, one evolutionary and the other one biophysical, led to the following conclusions.

To manage the Ca^{2+}-signal in eukaryotic cells, nature has designed a superfamily of calcium binding proteins which have all evolved by gene tandem duplications from a common 33- to 40-residues long precursor with one Ca^{2+}-binding domain. Calcium binds to all these proteins in a sequential way. Each calcium-binding site is able to bind Ca^{2+}, Mg^{2+} and monovalent ions (such as Na^{+} or K^{+}) in a competitive manner. However, these sites can be distinguished by their affinities for different ions. Based on these affinities, the calcium binding sites can be assigned to one of the following two classes.

1. *The triggering sites* which bind Ca^{2+}, Mg^{2+} and monovalent ions with affinity constants of ca. 10^7 M^{-1}, 10^4 M^{-1} and 10^2 M^{-1} respectively. Binding of Ca^{2+} to these sites occurs with diffusion-limited kinetics.

2. *The relaxing sites* which bind Ca^{2+}, Mg^{2+} and monovalent ions with affinity constants of 10^8 M^{-1}, 10^5 M^{-1} and ca. 10 M^{-1}. In a physiological medium, binding of Ca^{2+} to these sites is dependent on the Mg^{2+}/Ca^{2+} exchange rate.

The phylogenetic tree of calcium-binding proteins (104) shows that the four domain ancestor had properties similar to those of calmodulin, i.e., was composed of triggering sites. Proteins with triggering sites are involved in the on switch of all processes controlled by Ca^{2+} within the cell. These proteins interact with target proteins to propagate the conformational changes occurring upon Ca^{2+}-binding. Calmodulin which is very close to the ancestral protein, is multifunctional and can replace CaBP, such troponin C, in the triggering of cellular events in response to calcium influx.

The relaxing sites which appeared during evolution are present on more specialized proteins such as parvalbumins and TnC. They are involved in the off switch of a calcium pulse and represent a kinetic improvement of the relaxation phase in that they can rapidly scavenge Ca^{2+} ions from the Ca^{2+}-specific (or triggering) sites as soon as Mg^{2+} dissociates from the protein. Specialization from triggering to relaxing sites may be the result of certain amino acid substitutions in the twelve-residue calcium binding loop.

Additional evidence in favour of the above model is needed and may come in the future from the elucidation of the calcium binding mechanisms of other calcium binding proteins such as the glial S-100 protein or the intestinal calcium binding protein.

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References

1. Marban, E., Rink, T. J., Tsien, R. W. and Tsien, R. Y., 1980. Nature (London) 286: 845–850.
2. Ebashi, S., Kodama, A. and Ebashi, F., 1968. J. Biochem. 64: 465–477.
3. Le Peuch, C. J., Demaille, J. G. and Pechère, J. F., 1978. Biochim. Biophys. Acta 537: 153–159.
4. Haiech, J., Derancourt, J., Pechère, J. F. and Demaille, J. G., 1979. Biochimie 61: 583–587.
5. Moews, P. C. and Kretsinger, R. H., 1975. J. Mol. Biol. 91: 201–208.
6. Cheung, W. Y., 1970. Biochem. Biophys. Res. Commun. 38: 533–538.
7. Kakiuchi, S. and Yamazaki, R., 1970. Biochem. Biophys. Res. Commun. 41: 1104–1107.
8. Wolff, D. J. and Bostrom, C. O., 1979. Adv. Cyclic Nucl. Res. 11: 27–88.
9. Cheung, W. Y., 1980. Science 207: 19–27.
10. Klee, C. B., Crouch, T. H. and Richman, P. G., 1980. Ann. Rev. Biochem. 49: 489–515.
11. Means, A. R. and Dedman, J. R., 1980. Nature 285: 73–77.
12. Klee, C. B., 1977. Biochemistry 16: 1017–1024.
13. Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakon, M. and Kosaki, G., 1981. FEBS Lett. 126: 203–207.
14. Autric, A., Ferraz, C., Kilhoffer, M. C., Cavadore, J. C. and Demaille, J. G., 1980. Biochim. Biophys. Acta 631: 139–147.
15. Levin, R. M. and Weiss, B., 1977. Mol. Pharmacol. 13: 690–697.
16. Jamieson, G. A., Jr. and Vanaman, T. C., 1979. Biochem. Biophys. Res. Commun. 90: 1048–1056.
17. Charbonneau, H. and Cormier, M. J., 1979. Biochem. Biophys. Res. Commun. 90: 1039–1047.
18. Sharma, R. K. and Wang, J. H., 1979. Adv. Cyclic Nucl. Res. 10: 187–198.
19. Le Peuch, C. J., Ferraz, C., Walsh, M. P., Demaille, J. G. and Fisher, E. H., 1979. Biochemistry 24: 5267–5273.

20. Wang, J. H. and Desai, R., 1976. Biochem. Biophys. Res. Commun. 72: 926–932.
21. Wang, J. H. and Desai, R., 1977. J. Biol. Chem. 252: 4175–4184.
22. Klee, C. B. and Krinks, M. H., 1978. Biochemistry 17: 120–126.
23. LaPorte, D. C. and Storm, D. R., 1978. J. Biol. Chem. 253: 3374–3377.
24. Sharma, R. K., Wirch, E. and Wang, J. H., 1978. J. Biol. Chem. 253: 3575–3580.
25. Sharma, R. K. and Wirch, E., 1979. Biochem. Biophys. Res. Commun. 91: 338–344.
26. Wallace, R. W., Lynch, T. J., Tallant, E. A. and Cheung, W. Y., 1978. Arch. Biochem. Biophys. 187: 328–334.
27. Wallace, R. W., Lynch, T. J., Tallant, E. A. and Cheung, W. Y., 1979. J. Biol. Chem. 254: 377–382.
28. Van Eldik, L. J. and Watterson, D. M., 1981. J. Biol. Chem. 256: 4205–4210.
29. Wallace, R. W. and Cheung, W. Y., 1979. J. Biol. Chem. 254: 6564–6571.
30. Chafouleas, J. G., Dedman, J. R., Munjaal, R. P. and Means, A. R., 1979. J. Biol. Chem. 254: 10262–10267.
31. Grand, R. J. A. and Perry, S. V., 1979. Biochem. J. 183: 285–295.
32. Watterson, D. M., Sharief, F. and Vanaman, T. C., 1980. J. Biol. Chem. 255: 962–975.
33. Fletcher, J. E., Spector, A. A. and Ashbrook, J. D., 1970. Biochemistry 9: 4580–4587.
34. Lin, Y. M., Liu, Y. P. and Cheung, W. Y., 1974. J. Biol. Chem. 249: 4943–4954.
35. Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F. and Vanaman, T. C., 1976. J. Biol. Chem. 251: 4501–4513.
36. Wolff, D. J., Poirier, P. G., Bostrom, C. O. and Bostrom, M. A., 1977. J. Biol. Chem. 252: 4108–4117.
37. Jarett, H. W. and Kyte, J., 1979. J. Biol. Chem. 254: 8237–8244.
38. Dedman, J. R., Potter, J. D., Jakson, R. L., Johnson, J. D. and Means, A. R., 1977. J. Biol. Chem. 252: 8415–8422.
39. Crouch, T. H. and Klee, C. B., 1980. Biochemistry 19: 3692–3698.
40. Martin, R. B. and Richardson, F. S., 1979. Quater. Rev. Biophys. 12: 181–209.
41. Wallace, R. W., Tallant, E. A., Dokter, M. E. and Cheung, W. Y., 1982. J. Biol. Chem. 257: 1845–1854.
42. Kilhoffer, M. C., Demaille, J. G. and Gérard, D., 1980. FEBS Lett. 116: 269–272.
43. Kilhoffer, M. C., Gérard, D. and Demaille, J. G., 1980. FEBS Lett. 120: 99–103.
44. Wang, C. A. and Aquaron, R. R., 1980. In: Calcium binding proteins: structure and function (Siegel et al., eds) pp. 251–252, Elsevier North Holland, Amsterdam.
45. Krebs, J. and Carafoli, E., 1982. Eur. J. Biochem. 124: 619–627.
46. Kilhoffer, M. C., Demaille, J. G. and Gérard, D., 1981. Biochemistry 20: 4407–4414.
47. Drabikowski, W., Kuznicki, J. and Grabarek, Z., 1977. Biochim. Biophys. Acta 485: 124–133.
48. Jarett, H. W. and Penniston, J. T., 1977. Biochem. Biophys. Res. Commun. 77: 1210–1216.
49. Seamon, K. B., 1980. Biochemistry 19: 207–215.
50. Ho, H. C., Desai, R. and Wang, J. H., 1975. FEBS Lett. 50: 374–377.
51. Richman, P. G., 1978. Biochemistry 17: 3001–3005.
52. Richman, P. G. and Klee, C. B., 1978. J. Biol. Chem. 253: 6323–6326.
53. Richman, P. G. and Klee, C. B., 1978. Biochemistry 17: 928–935.
54. Richman, P. G. and Klee, C. B., 1979. J. Biol. Chem. 254: 5372–5376.
55. Walsh, M. P. and Stevens, F. C., 1977. Biochemistry 16: 2742–2749.
56. Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H. and Sharma, R. K., 1981. Proc. Natl. Acad. Sci. USA 78: 871–874.
57. Haiech, J. and Demaille, J. G., 1981. Metabolic Interconversion of Enzymes (Holzer, ed.), Proc. in Life Sciences, pp. 303–313, Springer Verlag, Berlin.
58. Cox, J. A., Malnoë, A. and Stein, E. A., 1981. J. Biol. Chem. 256: 3218–3222.
59. Malnoë, A., Cox, J. A. and Stein, E. A., 1982. Biochim. Biophys. Acta 714: 84–92.
60. Blumenthal, D. K. and Stull, J. T., 1980. Biochemistry 19: 5608–5614.
61. Johnson, J. D., Holroyde, M. J., Crouch, T. H.. Solaro, R. J. and Potter, J. D., 1981. J. Biol. Chem. 256: 12194–12198.
62. Kuznicki, J., Grabareck, Z., Brzeska, H., Drabikowski, W. and Cohen, P., 1981. FEBS Lett. 130: 141–145.
63. Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fush, Y. and Nagata, T., 1981. Proc. Natl. Ac. Sci. USA 78: 4354–4357.
64. Head, J. F., Masure, R. and Kaminer, B., 1982. FEBS Lett. 137: 71–74.
65. Klevit, R. E., Levine, B. A. and Williams, R. J. P., 1980. FEBS Lett. 123: 25–29.
66. LaPorte, D. C., Wierman, B. M. and Storm, D. R., 1980. Biochemistry 19: 3814–3819.
67. Grand, R. J. A., Levine, B. A. and Perry, S. V., 1982. Biochem. J. 203: 61–68.
68. Pechère, J. F., Capony, J. P. and Demaille, J. G., 1973. Syst. Zool. 22: 533–548.
69. Blum, H. E., Lehky, P., Kohler, L., Stein, E. and Fisher, E. H., 1977. J. Biol. Chem. 252: 2834–2838.
70. Baron, G., Demaille, J. G. and Dutruge, E., 1975. FEBS Lett. 56: 156–160.
71. Moews, P. C. and Kretsinger, R. H., 1975. J. Mol. Biol. 91: 229–232.
72. Sowadski, J., Cornick, G. and Kretsinger, R. H., 1978. J. Mol. Biol. 124: 123–132.
73. Horrocks, W., De W., Jr. and Sudnick, D. R., 1979. J. Am. Chem. Soc. 101: 334–340.
74. Cavé, A., Parello, J., Drakenberg, T., Thulin, E. and Lindman, B., 1979. FEBS Lett. 100: 148–152.
75. Haiech, J., Derancourt, J., Pechère, J. F. and Demaille, J. G., 1979. Biochemistry 13: 2752–2758.
76. Lee, L. and Sykes, B. D., 1981. Biochemistry 20: 1156–1162.
77. Permyakov, E. A., Yarmolenko, V. V., Emelyanenko, V. I., Burstein, E. A., Classet, J. and Gerday, C., 1980. Eur. J. Biochem. 109: 307–315.

78. Robertson, S. P., Johnson, J. D. and Potter, J. D., 1981. Biophys. J. 34: 559–569.
79. Gupta, R. K. and Moore, R. D., 1980. J. Biol. Chem. 255: 3987–3993.
80. Birdsall, W. Y., Levine, B. A., Williams, R. J. P., Demaille, J. G., Haiech, J. and Pechère, J. F., 1979. Biochimie 61: 741–750.
81. Gerday, C. and Gillis, J. M., 1976. J. Physiol. 258: 96–97 P.
82. Chapman, R. A., 1979. Prog. Biophys. Mol. Biol. 35: 1–52.
83. Ebashi, S., 1980. Proc. Roy. Soc. London B 207: 259–286.
84. Perry, S. V., Grand, R. J. A., Nairn, A. C., Vanaman, T. C. and Wall, C. M., 1979. Biochem. Soc. Trans. 7: 619–622.
85. Adelstein, R. S. and Eisenberg, E., 1980. Ann. Rev. Biochem. 49: 921–956.
86. Potter, J. D. and Gergely, J., 1975. J. Biol. Chem. 250: 4628–4633.
87. Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. and Jackman, N., 1973. FEBS Lett. 36: 268–272.
88. Wilkinson, J. M., 1976. FEBS Lett. 70: 254–256.
89. Johnson, J. D. and Potter, J. D., 1978. J. Biol. Chem. 253: 3775–3777.
90. Iio, T. and Kondo, H., 1981. J. Biochemistry (Tokyo) 90: 163–177.
91. Leavis, P. C., Nagy, B., Lehrer, S. S., Bialkowska, M. and Gergely, J., 1980. Arch. Biochem. Biophys. 200: 17–21.
92. Wang, C. L., Leavis, P. C., Horrocks, W., De W., Jr. and Gergely, J., 1981. Biochemistry 20: 2439–2444.
93. Seamon, K. B., Hartshorne, D. J. and Bothner-By, A. A., 1977. Biochemistry 16: 4039–4046.
94. Levine, B. A., Mercola, D., Coffman, D. and Thornton, J. M., 1977. J. Mol. Biol. 115: 743–760.
95. Hincke, M. T., McCubbin, W. D. and Kay, C. M., 1978. Can. J. Biochem. 56: 384–395.
96. Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z. and Drabikowski, W., 1978. J. Biol. Chem. 253: 5452–5459.
97. Delville, A., Grandjean, J., Lazlo, P., Gerday, C., Grabarek, Z. and Drabikowski, W., 1980. Eur. J. Biochem. 105: 289–295.
98. Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L. and Means, A. R., 1977. In: Calcium Binding Proteins (Wasserman, R. H. et al., Eds.) pp. 239–250, Elsevier, New-York.
99. Leavis, P. C. and Kraft, E. L., 1978. Arch. Biochem. Biophys. 186: 411–415.
100. Van Eerd, J. P. and Kawasaki, Y., 1972. Biochem. Biophys. Res. Commun 47: 859–865.
101. Reid, R. E. and Hodges, R. S., 1980. J. Theor. Biol. 84: 401–444.
102. Reid, R. E., Gariepy, J., Saund, A. K. and Hodges, R. S., 1981. J. Biol. Chem. 256: 2742–2751.
103. Levine, B. A., Thornton, J. M., Fernandes, R., Kelly, C. M. and Mercola, D., 1978. Biochim. Biophys. Acta 535: 11–24.
104. Benzonana, G., Canopy, J. P. and Pechère, J. F., 1972. Biochim. Biophys. Acta 278: 110–116.
105. Laouari, D., Pavlovitch, J. H., Deceneux, G. and Balsan, S., 1980. FEBS Lett. 111: 285–289.
106. Huang, W. Y., Cohn, D. V., Hamilton, J. W., Fullmer, C. and Wasserman, R. H., 1975. J. Biol. Chem. 250: 7647–7655.
107. Fullmer, C. S. and Wasserman, R. H., 1981. J. Biol. Chem. 256: 5669–5674.
108. Gysin, R., Moore, B. W., Proffit, R., Devel, T. F., Cadwell, K. and Glaser, L., 1980. J. Biol. Chem. 256: 1515–1600.
109. Mac Manus, J. P., 1980. Biochim. Biophys. Acta 621: 296–304.
110. Goodman, M., Pechère, J. F., Haiech, J. and Demaille, J. G., 1979. J. Mol. Evol. 13: 331–352.
111. Grand, R. J. A. and Perry, S. V., 1978. FEBS Lett. 92: 137–142.
112. Vanaman, T. C. and Sharief, F., 1979. Fed. Proc. 38: 788.
113. Toda, H., Yazawa, M., Kondo, K., Honma, T., Narita, K. and Yagi, K., 1981. J. Biochem. 90: 1493–1505.
114. Yazawa, M., Yagi, K., Toda, H., Kondo, K., Narita, K., Yamazaki, R., Sobue, K., Kakiuchi, S., Nagao, S. and Nozawa, Y., 1981. Biochem. Biophys. Res. Commun. 99: 1051–1057.
115. Takagi, T., Nemoto, T., Konishi, K., Yazawa, M. and Yagi, K., 1980. Biochem. Biophys. Res. Commun. 96: 377–387.
116. Haiech, J., Klee, C. B. and Demaille, J. G., 1981. Biochemistry 20: 3890–3897.
117. Keller, C. H., Olwin, B. B., LaPorte, D. C. and Storm, D. R., 1982. Biochemistry 21: 156–162.
118. Thiry, P., Vandermeers, A., Vandermeers-Piret, M. C., Rathé, J. and Christophe, J., 1980. Eur. J. Biochem. 103: 409–414.
119. Holroyde, M. J., Robertson, S. P., Johnson, J. D., Solaro, R. J. and Potter, J. D., 1980. J. Biol. Chem. 255: 11688–11693.
120. Jauregui-Adell, J., Pechère, J. F., Briand, G., Richet, C. and Demaille, J. G., 1982. Eur. J. Biochem. 123: 337–345.

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