REVIEW ARTICLE

Interactive properties of calmodulin

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1. Introduction

External signals often lead to transient increases of the intracellular $[Ca^{2+}]$ (Berridge, 1987). Calmodulin (CaM) is the prime sensor of this signal and conveys it to multiple target enzymes, especially protein kinases and phosphatases. For a general survey of the properties of CaM, the reader is referred to Cheung (1980), Klee & Vanaman (1982), Manalan & Klee (1984) and Wang *et al.* (1985). This review deals more specifically with the cation binding properties of CaM in the absence and presence of its target enzymes. Although our understanding has considerably increased (Kilhoffer *et al.*, 1983; Cox *et al.*, 1984; Forsén *et al.*, 1986), some enigmas remain and, unfortunately, some basic and long-lasting controversies have not yet been solved.

2. Structural observations on the cation binding domains of CaM

The elucidation of the primary structure of CaM by Watterson *et al.* (1980) clearly showed that CaM contains four Ca^{2+} -binding domains with an E-helix–loop–F-helix repeat (Kretsinger, 1980). Nothing in the amino acid sequence of these domains permits speculation as to their affinity or selectivity. Close examination of Table 1 reveals two previously unnoticed particularities: (a) three out of four Ca^{2+} -binding loops contain one ‘acid pair’ [a definition coined by R. Reid of Ottawa (personal communication) to indicate that opposing Ca^{2+} ligands, i.e. pair-wise X and –X, or Y and –Y, or Z and –Z, are both free carboxylic side chains], and (b) all four domains contain in the N-terminal α -helix flanking the Ca^{2+} -binding loop a Glu residue in position –9 and a

Glu or Asp residue in position –6 (for numerotation see Table 1). Since these positions are situated on the same side of the helix, one predicts a high negative charge density at these particular loci of CaM and possibly cation binding activity. Two more loci of this type are present in CaM: one in the central α -helix (Glu₁₀–Asp₁₃) and one in the E-helix of the fourth Ca^{2+} -binding domain (Asp₇–Glu₁₀). These loci are remarkably well conserved in all calmodulins (Salvato *et al.*, 1986).

The elucidation of the crystal structure of CaM to 0.3 nm resolution (Babu *et al.*, 1985) revealed that the molecule looks like a dumb-bell with the globular N- and C-halves linked by a 2.0 nm-long α -helical stretch. In the crystal the two halves of CaM are remarkably similar; the corresponding α -carbon positions can be superimposed with an average difference of approx. 0.1 nm. Within each half, the two Ca^{2+} -binding sites are structured so that allostery in metal ion binding might occur. It should be emphasized that the tridimensional structure was determined on crystals grown at pH 5 and, as in the case of troponin C (Wang *et al.*, 1987), may not be fully relevant at neutral pH. Nevertheless, a solution study on the domain organization in CaM (Tsalkova & Privalov, 1985) leads to the conclusion that the two halves behave as single co-operative blocks but that there is no significant allosteric interaction between the two halves of the molecule.

3. Ca^{2+} binding to CaM

3.1. Direct binding and kinetic studies. In 1975, an equilibrium dialysis study of Ca^{2+} binding to skeletal muscle troponin C and the effects of Mg^{2+} established

Table 1. The four metal-binding domains of bovine brain CaM

The putative auxiliary sites are indicated by the bridged asterisks. The numbers in parentheses correspond to the positions in the complete sequence of CaM. K': trimethyllysine.

		E-helix	Loop	F-helix
Domain I	(7–42)	E-Q-I-A-E ⁻⁹ -F-K-E ⁻⁶ -A-F-S-L-F-D ^X -K ^Y -D ^Z -G-D ^{-Y} -G ^{-X} -T ^{-Z} -T ^{-Z} -K-E-L-G-T-V-M-R-S-L-G-Q-N		
Domain II	(43–79)	P-T-E-A-E-L-Q-D-M-I-M-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		
Domain III	(80–115)	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'		
Domain IV	(116–149)	L-T-D-E-E-V-D-E-M-I-R-E-A-D-I-D-G-D-G-Q-V-N-Y-E-E-F-V-Q-M-M-T-A-K		

Abbreviation used: CaM, calmodulin.

Fig. 1. Ca^{2+} binding to CaM

The experimental data are from Burger *et al.* (1984) at pH 7.0 and an ionic strength equal to 150 mM. The solid and dotted lines represent the theoretical curves obtained by iterative fitting according to the Langmuir and Adair equations [for the interactive data treatment, see Burger *et al.* (1984)], respectively. The dashed line is a simulated one using the stoichiometric constants of Table 2 assuming the model of paired co-operative sites with $K'_N = 20 K_N$, $K_C = 10 K_N$.

unambiguously the affinities of the different Ca^{2+} -binding sites and introduced the notion of Ca^{2+} -specific and $\text{Ca}^{2+} + \text{Mg}^{2+}$ mixed sites (Potter & Gergely, 1975). In the line of this classification, the metal-binding properties of some other muscle Ca^{2+} -binding proteins could easily be interpreted (for review, see Wnuk *et al.*, 1982). A wealth of kinetic, conformational and n.m.r. studies confirmed the direct binding data.

Unfortunately this was not the case with CaM. Many direct binding studies in buffers of sufficient ionic strength to eliminate electrostatic effects indicated that the shape of the Ca^{2+} -binding curve is close to the ideal Langmuir isotherm. For instance, in Fig. 1 we have fitted the experimental Ca^{2+} -saturation curve (Burger *et al.*, 1984) to two models: either four independent Ca^{2+} -binding sites of equal affinity, or four interacting Ca^{2+} -binding sites. The experimental accuracy did not allow us to exclude one of these two models. At low ionic strength the binding constants are about 10-fold higher due to the reinforcement of the electrostatic interaction between Ca^{2+} and CaM (Ogawa & Tanokura, 1984; Iida & Potter, 1986; Milos *et al.*, 1986).

However, from studies using $^{43}\text{Ca}^{2+}$ -n.m.r. (Andersson *et al.*, 1982; Teleman *et al.*, 1986) and stopped-flow fast kinetics (Martin *et al.*, 1985; Suko *et al.*, 1985) it appears that CaM has two pairs of sites: one pair in the *N*-terminal half of CaM has a k_{off} of 300–500 s^{-1} and the other (in the *C*-terminal half) a k_{off} of 10–40 s^{-1} .

Assuming a diffusion controlled on-rate constant of $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, these off-rate constants point to the existence of two low- (fast exchanging) and two high- (slowly exchanging) affinity pairs of sites with a 10–20-fold difference in the binding constants. These findings predict that upon raising $[\text{Ca}^{2+}]$, the ion first binds to the *C*-terminal half of CaM, which happens to contain the two Tyr residues, and later to domains I and II. The model is consistent with studies on the conformational changes of the Tyr residues (see section 3.3), but not with the direct binding studies mentioned above. The apparent discrepancy between the kinetic and equilibrium binding studies would be solved if the Ca^{2+} -on-rate constants also are different in the *N*- and *C*-terminal halves of CaM: with the above indicated k_{off} values and k_{on} equal to $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the *N*-terminal sites and $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the *C*-terminal sites, the calculated equilibrium constants are close to the experimentally observed ones.

The activation energy for dissociation of one Ca^{2+} ion (ΔG_a^*) amounts to 59 and 67 kJ/site for fast and slow binding respectively (Teleman *et al.*, 1986). With an equilibrium free energy change (ΔG^0) of –29 kJ/site (Milos *et al.*, 1986) the activation energies for association (ΔG_a^*) amount to 30 and 38 kJ/site for the *N*- and *C*-terminal pairs of sites respectively.

3.2. The model of paired co-operative sites. Recently, Wang (1985) proposed a theoretical explanation of the discrepancies between the above two lines of studies. He simulated Ca^{2+} -binding curves on the basis of a model assuming two pairs of binding sites of respectively high and low affinity. With properly chosen values of the site binding constants (k) for the two pairs, and assuming relatively strong positive co-operativity between the two sites in each pair, an overall binding isotherm with the appearance of a non-co-operative binding curve of four equivalent sites can be generated. The Wang model states that binding of the first Ca^{2+} to the *N*-terminal half is governed by the microscopic affinity constant k_N ($= k_I = k_{II}$, where the roman figures refer to the particular Ca^{2+} -binding domain as defined in Table 1) and binding of the second by k'_N , with $k'_N = 20 k_N$. The same statement can be made for the *C*-terminal sites with the understanding that $k_C = 10 k_N$. In this model, the relationship between the site binding constants and stoichiometric constants is given by the following set of equations:

$$\begin{aligned} K_1 &= 2k_N + 2k_C \\ K_1 K_2 &= k_N k'_N k_C + k_C k'_C \\ K_1 K_2 K_3 &= 2k_N k'_N k_C + 4k_N k_C k'_C \\ K_1 K_2 K_3 K_4 &= k_N k'_N k_C k'_C \end{aligned} \quad (1)$$

Table 2 shows the most plausible values for the four independent parameters k_N , k'_N , k_C and k'_C and for the corresponding stoichiometric Ca^{2+} -binding constants. Fig. 1 shows that a good fitting is indeed obtained between the model of Wang and the experimental data.

Minowa & Yagi (1984) have studied Ca^{2+} -binding to the tryptic fragments of CaM, which retain most of the ion-binding properties of the intact protein (Forsén *et al.*, 1986). A strong positive co-operativity was observed in the *C*-terminal half, but not in the *N*-terminal half of CaM. A 3-fold difference was found in the median affinity between the two halves. Based on these data alone, the model of paired co-operative Ca^{2+} -binding sites is less likely.

Table 2. Plausible binding and rate constants of Ca^{2+} interaction with CaM according to the model of Wang

Microscopic binding constants (M^{-1})*	Off-rate constants (s^{-1})†	Stoichiometric binding constants (M^{-1})
$k_N = 6.14 \times 10^3$	Approx. 16000	$K_1 = 1.35 \times 10^5 \ddagger (1.16 \times 10^5)$
$k'_N = 1.23 \times 10^5$	800	$K_2 = 5.76 \times 10^5 (2.65 \times 10^5)$
$k_C = 6.15 \times 10^4$	1600	$K_3 = 1.31 \times 10^4 (8.33 \times 10^4)$
$k'_C = 1.23 \times 10^6$	80	$K_4 = 5.62 \times 10^4 (1.90 \times 10^4)$

* Calculated by iterative curve fitting of the experimental binding data of Fig. 1 to the Adair equation (1923) with the constraints of the model of Wang, i.e. the stoichiometric constants are related to the site binding constants by eqn. (1) and the site binding constants are interrelated by $k'_N/k_N = k'_C/k_C = 20$; $k_C/k_N = 10$.

† Assuming an on-rate constant for Ca^{2+} binding of $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Eigen & Hammes, 1963).

‡ Calculated from the microscopic constants using eqn. (1). In parentheses are the corresponding experimental values of Burger *et al.* (1984) (model 2) for comparison.

As analysed in Table 2, the model of Wang predicts an off-rate constant of approx. 800 s^{-1} for dissociation of the first Ca^{2+} from the *N*-terminal half of CaM and of approx. 80 s^{-1} for dissociation of the first Ca^{2+} from the *C*-terminal half. Due to the positive co-operative interaction within pairs, the dissociation rate of the remaining Ca^{2+} is much faster than that of the first; the latter is thus rate-limiting (see also Forsén *et al.*, 1986). The experimentally determined rate constant for the *N*-terminal pair, as determined by $^{43}\text{Ca}^{2+}$ -n.m.r. (Teleman *et al.*, 1986), ^3H -n.m.r. (Ikura, 1986) and stopped-flow (Martin *et al.*, 1985) is in the same range as the predicted one, i.e. approx. 500 s^{-1} , thus validating the model of Wang for these sites. However, the experimental k_{off} for the high-affinity *C*-terminal pair is between 3 and 10 s^{-1} (Malencik *et al.*, 1981; Martin *et al.*, 1985; Suko *et al.*, 1985; Teleman *et al.*, 1986; Ikura, 1986), i.e. much lower than the one predicted by the model of Wang (1985).

In the light of the controversy between direct binding data and kinetic studies, it is of interest to look to the fundamental parameters of the binding process, i.e. the enthalpy and entropy changes. Enthalpy titration studies at 25°C were carried out by Tanokura & Yamada (1984) and by Milos *et al.* (1986). Though the former authors assumed two categories of enthalpy changes, the latter observed an identical and endothermic enthalpy change for each of the four Ca^{2+} -binding domains with $\Delta H^\circ = 4.9 \text{ kJ/site}$ and $\Delta S^\circ = 113 \text{ J/(K} \cdot \text{site)}$. At low ionic strength and below 30°C Ca^{2+} binding is exothermic with $\Delta H^\circ = -8 \text{ kJ/site}$ for all four sites (Forsén *et al.*, 1986). It should be noted that complete dehydration of Ca^{2+} is strongly endothermic ($\Delta H^\circ = 1600 \text{ kJ/Ca}^{2+}$), but that upon complex formation with six protein-oxygens nearly all the heat is recovered. Compared with this high value the rather subtle differences between different Ca^{2+} -binding proteins, i.e. from 4.9 (for CaM; Milos *et al.*, 1986) to -32 (for troponin C; Potter *et al.*, 1977), -37 (for parvalbumin; Moeschler *et al.*, 1980) and -118 (for α -lactalbumin; Schaer *et al.*, 1986) kJ/Ca^{2+} , must reflect local differences in the strength of the $\text{O}-\text{Ca}^{2+}$ bonds in their Ca^{2+} -binding domains. For the three intracellular proteins, and especially for CaM, the entropy gain due to water release from the free Ca^{2+} ion (i.e. 184 J/K) outweighs by far the entropy loss due to the conformational changes in the proteins.

3.3. Ca^{2+} -induced conformational studies. Proton n.m.r. studies allow to distinguish two phases (for review, see

Forsén *et al.*, 1986). Up to $2 \text{ Ca}^{2+}/\text{CaM}$ the ^1H -n.m.r. spectrum varies continuously from a metal-free type spectrum to the $2 \text{ Ca}^{2+}/\text{CaM}$ type spectrum, suggesting that only one conformational change takes place in the step $\text{CaM} \rightarrow \text{CaM} \cdot \text{Ca}_2$ and that during the titration no significant amounts of $\text{CaM} \cdot \text{Ca}_1$ are formed. Such a behaviour is predicted from the model of paired co-operative sites. During the second phase, comprising binding of the third and fourth Ca^{2+} , only one spectrum is observed. The few resonance shifts during this phase are continuous from 2 to 4 $\text{Ca}^{2+}/\text{CaM}$ (Klevit *et al.*, 1984) and do not allow statements about the existence or abundance of the $\text{CaM} \cdot \text{Ca}_3$ species.

Two other conformational studies do *not* support the model of Wang, which predicts that $\text{CaM} \cdot \text{Ca}_1$ and $\text{CaM} \cdot \text{Ca}_3$ are relatively non-abundant species. (a) The change in the α -helical content occurs in two steps (Burger *et al.*, 1984); half-maximal increase of the far-u.v. ellipticity occurs upon binding of the first Ca^{2+} , whereas the maximal value is reached upon binding of the third Ca^{2+} . (b) The increase in volume of CaM upon binding of Ca^{2+} is not monotonic but suggests a symmetry of pairs (Kupke & Dorrier, 1986); in addition to the continuous volume increase due to dehydration of Ca^{2+} and of the charged groups in the binding loop, an extra volume change occurs in steps 2 and 4 as a result of conformational changes in CaM. The data of Burger *et al.* (1984) and of Kupke & Dorrier (1986) thus indicate that $\text{CaM} \cdot \text{Ca}_1$ and $\text{CaM} \cdot \text{Ca}_3$ are relatively abundant species during the titration of CaM by Ca^{2+} .

3.4. Ca^{2+} - H^+ antagonism in CaM. Proton antagonism in Ca^{2+} binding to CaM was suggested from the data of Haiech *et al.* (1981) and was definitely established by Iida & Potter (1986). By measuring proton release upon binding of Ca^{2+} at different pH values, Milos *et al.* (1986) observed that the proton- Ca^{2+} antagonism is strictly identical for the four Ca^{2+} -binding sites from pH 5.0 to 7.5. Maximal proton release as a function of pH follows a classical Langmuir isotherm with a $\text{p}K_i$ of 6.17 and a plateau value of $3 \text{ H}^+/\text{CaM}$. The most plausible ligand having such an ionization constant is a pair of carboxyl groups involved in a close carboxyl-carboxylate interaction (Sawyer & James, 1982). In Ca^{2+} -free CaM, three pairs of two carboxyl groups would be forced in a restricting, near-neighbour arrangement, leading to anomalous high $\text{p}K_i$ values. Ca^{2+} binding disrupts this interaction and forces one or both carboxyl groups in

Fig. 2. Possible location of the sites of Ca^{2+} - H^+ antagonism in CaM

The central α -helix (residues 75-90) of CaM is depicted. The three carboxyl-carboxylate bridges, Asp_{78} - Glu_{82} , Asp_{80} - Glu_{83} and Glu_{84} - Glu_{87} , in metal-free CaM (left) are disrupted by Ca^{2+} and four salt bridges, Asp_{78} - Arg_{74} , Asp_{80} - Lys_{77} , Glu_{82} - Arg_{86} and Glu_{87} - Arg_{90} , are formed instead (right). Possible twisting of the central α -helix is not depicted.

each pair to participate in other bonds, leading to a lowering of the affinity for protons ($\text{pK}_a < 4.0$). Actually we have two hypotheses about the location of the three carboxyl-carboxylate bridges in metal-free CaM. (a) In the light of a recent report on structure stabilization in the central α -helix of Ca^{2+} -binding proteins (Sundaralingam *et al.*, 1985), we proposed a model as presented in Fig. 2. (b) The three ionic geometries responsible for Ca^{2+} - H^+ antagonism may be situated closer to the Ca^{2+} -binding sites. As mentioned in section 2, all domains except III contain an 'acid pair' in one of the co-ordinating positions. In the absence of Ca^{2+} these three pairs could be engaged in a carboxyl-carboxylate bridge, thus stabilizing the metal-free form of CaM.

4. Mg^{2+} binding to CaM

Mg^{2+} interacts with metal-free CaM with a dissociation constant in the millimolar range (Haiech *et al.*, 1981; Ogawa & Tanokura, 1984; Iida & Potter, 1986). The conformational studies (Richman & Klee, 1979; Seamon, 1980; Kilhoffer *et al.*, 1981) unambiguously established that the Mg^{2+} -loaded configuration is far from the Ca^{2+} -saturated conformation, thus suggesting, in contrast with the situation in parvalbumin (Cox *et al.*, 1979) and troponin C (Potter & Gergely, 1975), that the binding sites are different. However, the interpretation of the Ca^{2+} - Mg^{2+} antagonism was less straightforward. Three well-documented studies (Haiech *et al.*, 1981; Ogawa & Tanokura, 1984; Iida & Potter, 1986) indicate that there is straight competition of Ca^{2+} and Mg^{2+} for the same four sites. A recent $^{25}\text{Mg}^{2+}$ -n.m.r. study at very low ionic strength (Tsai *et al.*, 1987) revealed two classes of Mg^{2+} binding sites with Ca^{2+} competition occurring at both of them; the reported dissociation constants for Mg^{2+} are

0.5 mM in the *N*-terminal and 3.3 mM in the *C*-terminal half of CaM. However, other authors did not observe such pronounced competition effects at millimolar $[\text{Mg}^{2+}]$ (Potter *et al.*, 1983; Cox *et al.*, 1981; Burger *et al.*, 1984). Recently we used a protein-expensive but sensitive method to probe directly the binding of Mg^{2+} to CaM (Milos *et al.*, 1986). The study was further complemented with microcalorimetric titrations, which have the advantage over other binding methods of measuring accurately the binding process even at low protein and very high ligand concentrations. The combination of the two methods revealed that up to six Mg^{2+} ions bind to metal-free CaM with identical affinity; the K_D value equals 7 mM at physiological ionic strength (Milos *et al.*, 1986).

The Mg^{2+} - H^+ antagonism, studied by monitoring proton release upon Mg^{2+} binding to metal-free CaM (Milos *et al.*, 1986), is qualitatively very different from the Ca^{2+} - H^+ antagonism, which incites us to assume that the six Mg^{2+} -binding sites are different from the four Ca^{2+} -binding sites. Likely candidates for the six Mg^{2+} -binding sites are proposed in Table 1 (for rationale, see Milos *et al.*, 1986). The α -carbon atoms of the two amino acid residues are on the same side of the E helices, and the centres of the γ - or δ -carboxyl groups can easily be positioned within 0.22 nm distance of the centre of a Mg^{2+} ion, each of the carboxyl groups co-ordinating from opposite sides the metal ion in the bidentate mode. Interestingly, these putative Mg^{2+} -binding amino acid residues are very well conserved in all calmodulins (Salvato *et al.*, 1986).

The rationale for the tentative localization of the Mg^{2+} -binding sites in the *N*-terminal α -helix of each EF-hand domain stems not only from the arguments developed above, but also from the Ca^{2+} - Mg^{2+} antagonism which has been examined by direct binding methods and by microcalorimetry (Milos *et al.*, 1986). All our data together lead us to propose a scheme of negative free energy coupling between the two types of sites: the Ca^{2+} and Mg^{2+} sites are *not* identical; Mg^{2+} is antagonistic to Ca^{2+} at each site and decreases the Ca^{2+} affinity by a factor of 4. Complementarily, Ca^{2+} decreases the affinity for Mg^{2+} by a factor of 4. Fig. 3 dissects the driving forces involved in the interaction of Ca^{2+} and Mg^{2+} with CaM and provides further proof of the relationship between the Ca^{2+} and Mg^{2+} binding processes.

5. Capital and auxiliary ion binding sites in CaM

The hypothesis of the existence of two classes of sites for divalent cations, as we inferred from the Ca^{2+} - Mg^{2+} antagonism, received support from recent studies on the effects of non-physiological cations such as Zn^{2+} (Baudier *et al.*, 1983; Andersson *et al.*, 1982) and the cytotoxic cations Al^{3+} (Siegel & Haug, 1983) and Pb^{2+} (Fullmer *et al.*, 1985) on CaM. For instance, Zn^{2+} and Al^{3+} bind to CaM without producing the conformational changes which occur when Ca^{2+} binds. An indirect, but highly revealing, study on capital and auxiliary sites as been done by Mills & Johnson (1985). They used the fluorescence of felodipine as a means of monitoring the formation of an allosterically potentiated conformer of CaM. For felodipine binding to occur, the Ca^{2+} -binding sites (capital or C sites) *must* be occupied; for formation of the allosteric conformer, the auxiliary sites (A sites) *also must* be occupied. La^{3+} , Tb^{3+} , Pb^{2+} and Cd^{2+} are capable of inducing the allosteric conformer in the

Fig. 3. Summary of the relative standard free energy, enthalpy and entropy of the Ca^{2+} -, Mg^{2+} -, Zn^{2+} -, $\text{Ca}^{2+} + \text{Mg}^{2+}$ - and $\text{Ca}^{2+} + \text{Zn}^{2+}$ -saturated forms of CaM

The metal-free form was taken as reference. The scale and sign of the entropy changes are drawn so that they reflect their contribution to the driving forces of the equilibria.

presence and absence of Ca^{2+} : according to the model they bind to both the C and the A sites. Zn^{2+} , Mn^{2+} and H^+ also are capable of forming the conformer, but only in the presence of Ca^{2+} : this means that Zn^{2+} and Mn^{2+} have a preference for the A but not for the C sites. Recently Johnson and coworkers (personal communication) have shown that, as Zn^{2+} , Mg^{2+} induces (half-maximal effect at 7 mM) the allosteric conformation in the presence of 1 mM- Ca^{2+} . Cu^{2+} and Hg^{2+} bind to the A sites only. However, they do not promote the formation of the allosteric conformer; instead they antagonize the effect of the potentiating metals such as La^{3+} and Zn^{2+} . Fig. 4 schematically depicts the topography of the two types of ion-binding sites of CaM and their specificity towards the different cations mentioned above.

A recent study on the interaction of Zn^{2+} with CaM in the absence and presence of Ca^{2+} and/or Mg^{2+} (Milos, M., Comte, M., Schaer, J.-J. & Cox, J. A., unpublished work) indicates that Zn^{2+} does not compete with Ca^{2+} for the C sites, but does so with Mg^{2+} at the A sites. There are six Zn^{2+} -binding sites and their occupancy decreases the affinity of the four C sites for Ca^{2+} by virtue of negative free energy coupling (with the basic thermodynamic parameters depicted in Fig. 3). Interestingly,

in S-100 protein also the Zn^{2+} - and Ca^{2+} -binding sites are different (Baudier *et al.*, 1986; Leung *et al.*, 1987) and in S-100b there is evidence of positive free energy coupling between the two types of sites (Baudier *et al.*, 1986).

As for the function of the A sites, their occupancy in the resting cell (empty C sites) induces subtle conformational changes which could abolish certain hydrophobic interactions of CaM (Milos *et al.*, 1987). In the presence of Ca^{2+} (filled C sites), occupancy of the A sites seems to modify the mode of interaction of CaM with drugs and enzymes (Mills & Johnson, 1985).

6. Ca^{2+} binding to CaM in the presence of a target enzyme or peptide

6.1. The model of linked functions and equation of Huang. Ca^{2+} is needed for the activation by CaM of its target enzymes, and quantitative studies on cyclic nucleotide phosphodiesterase (Cox *et al.*, 1981; Huang *et al.*, 1981), cerebellar adenylate cyclase (Malnoe *et al.*, 1982), erythrocyte $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase (Cox *et al.*, 1982), skeletal muscle phosphorylase b kinase (Burger *et al.*, 1983), multifunctional protein kinase (Pifl *et al.*, 1984) and smooth and skeletal muscle myosin light chain kinase (Blumenthal & Stull, 1980) indicate that binding

Fig. 4. Schematic representation of the capital (A) and auxiliary (C) ion-binding sites in CaM and their specificity towards different di- and trivalent cations

The thick line indicates the direction of the polypeptide sequence from N- to C-terminal end.

of three to four Ca^{2+} is required to activate these enzymes. Direct binding studies of CaM to synaptosomal membranes (Malnoë *et al.*, 1982) and to phosphorylase b kinase (Burger *et al.*, 1983) also indicate that the high-affinity complex (K_D approx. 1 nM) is only formed if at least three Ca^{2+} bind to CaM. CaM-binding and enzyme activation are different processes and may not display the same Ca^{2+} dependency, especially when the enzyme itself also interacts with Ca^{2+} . In the absence of Ca^{2+} , the interaction of CaM with the enzyme displays a low affinity (K_D above μM), but no reliable estimate of this affinity has been published. Wang *et al.* (1980) were the first to propose a scheme of energy coupling in the interaction between enzyme, CaM and Ca^{2+} :

In this scheme there are nine independent parameters: the four binding constants of Ca^{2+} to CaM, the four constants that describe binding of Ca^{2+} to enzyme-bound CaM, and one independent binding constant (arbitrarily we will use K_e) which links the two pathways. Huang & King (1985) have derived a general equation which defines the activation (or binding) of an enzyme by CaM and Ca^{2+} with the restrictive condition that in the experiments all CaM is in the free form, i.e. $[\text{CaM}]_T \gg [\text{enzyme}]_T$:

$$\frac{E \cdot \text{CaM}}{E_T} = \frac{\text{CaM}_F \phi_1}{K_e \phi_2 + \text{CaM}_F \phi_1} \quad (2)$$

with:

$$\phi_1 = 1 + K_1'[\text{Ca}^{2+}] + K_1'K_2'[\text{Ca}^{2+}]^2 + K_1'K_2'K_3'[\text{Ca}^{2+}]^3 + K_1'K_2'K_3'K_4'[\text{Ca}^{2+}]^4$$

and:

$$\phi_2 = 1 + K_1[\text{Ca}^{2+}] + K_1K_2[\text{Ca}^{2+}]^2 + K_1K_2K_3[\text{Ca}^{2+}]^3 + K_1K_2K_3K_4[\text{Ca}^{2+}]^4$$

To apply the above equation, the concentration of CaM_F , i.e. of all the forms of CaM not bound to the enzyme, has to be known. Therefore experiments without separation of the two equilibrated phases such as optical methods (Malencik & Anderson, 1986) will not allow the determination of all the parameters of Scheme 1, unless very low concentrations ($< 10^{-10}$ M) of enzyme are used. Similarly, equilibrium dialysis experiments in which CaM is excluded from the dialysate (Olwin *et al.*, 1984; Maulet & Cox, 1983) do not allow to know CaM_F at each free Ca^{2+} concentration. To separate the Ca^{2+} binding to enzyme-bound CaM from that to free CaM, we introduced a method of equilibrium gel filtration (Burger *et al.*, 1983). The enzyme migrates and equilibrates in a column containing an appropriate Sephadex type. The column is pre-equilibrated with a fixed concentration of radiolabelled CaM and $[\text{Ca}^{2+}]$ using an EGTA buffer. The amount of enzyme-bound CaM and, in turn, of Ca^{2+} bound to this enzyme-bound CaM, can be determined by subtraction (Fig. 5). In this way, the isotherm of Ca^{2+} binding to bound CaM can adequately be constructed and subsequently the four K' values estimated by iterative fitting to the Adair equation. The determination of K_e also can be done by equilibrium gel filtration at very low Ca^{2+} levels or, provided the enzyme concentration in the assays is in the same range as the value of $1/K_e$, by optical methods and/or by microcalorimetry.

The study of the free energy coupling in the interactions between Ca^{2+} , CaM and rabbit muscle phosphorylase b kinase by this method (Burger *et al.*, 1983) was hampered

Scheme 1

Fig. 5. Typical elution profile of an equilibrium gel filtration experiment of myosin light chain kinase

Gel filtration at 4 °C on a Sephadex G-75 column (60 cm $\times$ 0.9 cm) equilibrated in 60 mM-Tes/NaOH, pH 7.0, 150 mM-NaCl, 1 mM-MgCl₂, 15 mM-mercaptoethanol containing 0.9 μ M-CaM (+17 nM-[³H]CaM) and 0.91 mM free Ca²⁺ (controlled by means of an EGTA buffer) supplemented with 10 kBq of ⁴⁵CaCl₂/ml. The concentration of bound Ca²⁺ and CaM was determined in each of the enzyme-containing fractions after subtraction of the corresponding mean concentrations in the fractions just before and after the enzyme-containing peak, and reported per mol of enzyme.

Table 3. Calcium-binding constants of CaM in the absence (K) or presence (K') of two selected target enzymes and affinity constants of metal-free (K_e) or Ca²⁺-saturated (K_s) CaM for the enzymes

Constant	Value (M ⁻¹)		
	No target	+ Phosphorylase b kinase*	+ Myosin light chain kinase†
K or K'			
1	1.16×10^5	1.16×10^5	1.16×10^5
2	2.65×10^5	2.65×10^5	2.65×10^5
3	8.33×10^4	1.05×10^9	2.49×10^8
4	1.90×10^4	1.90×10^4	1.03×10^5
K_e	—	3.33×10^4	1.85×10^5
K_s	—	6.35×10^8	3.00×10^9

* From Burger *et al.* (1983) and Cox *et al.* (1987).

† From Mamar-Bachi & Cox (1987).

by the fact that this enzyme contains also many intrinsic Ca²⁺-binding sites due to the ever-bound δ subunit (Shenolikar *et al.*, 1979; Burger *et al.*, 1982). Nevertheless, using model constructions on experimental data, it could

Fig. 6. Binding of Ca²⁺ to myosin light chain kinase (MLCK)-bound CaM

The experimental data were obtained as described in Fig. 5. The solid line represents the best fitting to the Adair equation with the constants reported in Table 3.

be established that the Ca²⁺-binding isotherm of enzyme-bound CaM (governed by the constants K'_n) displays strong positive co-operativity in the first three binding steps ($K'_3 = 10^5 \times K_3$) followed by a strong levelling off at three Ca²⁺ per CaM and a very slow rise up to four Ca²⁺ per CaM thereafter (Fig. 7 of Burger *et al.*, 1983). Table 3 lists the values of all the independent binding parameters for this enzyme (taken from Cox *et al.*, 1987). Recently, we also studied by equilibrium filtration the energy coupling involved in the interactions between Ca²⁺, CaM and smooth muscle myosin light chain kinase (Mamar-Bachi & Cox, 1987) which has the advantage over phosphorylase b kinase of not binding Ca²⁺ directly. With this enzyme the Ca²⁺-binding isotherm of enzyme-bound CaM could be determined with good precision (Fig. 6); the K' constants which best fit the experimental data to the Adair equation are listed in Table 3. The K_e value, measured at $[Ca^{2+}] = 10^{-7}$ M, amounts to 1.85×10^5 M⁻¹. The fact that the complex between metal-free CaM and the enzyme is not active has interesting physiological consequences (for discussion, see Mamar-Bachi and Cox, 1987).

6.2. Nature of the free energy coupling in the interactions between Ca²⁺, CaM and its target. The apparent homotropic co-operativity in Ca²⁺-binding to the CaM-enzyme complex is due to the stabilization of the complex from its low- to its high-affinity form in the third Ca²⁺-binding step (Fig. 6). The increased affinity of CaM for Ca²⁺ is at least partly due to the strong decrease of the off-rate constants. Indeed, in stopped-flow studies of Ca²⁺ release from CaM in the presence of the non-physiological targets trifluoperazine (Martin *et al.*, 1985), phenoxybenzamine and melittin (Suko *et al.*, 1986), the off-rate constant of Ca²⁺ from one N-terminal site is almost unaffected whereas at the remaining three sites the constant is decreased by a factor of 10. It is possible, but not proven, that the on-rate constants at the C-terminal sites also change due to the presence of the target.

What kind of forces, i.e. hydrophobic, electrostatic, van der Waals' interactions or hydrogen bonds, are responsible for the observed increase of the Ca²⁺ affinity?

Microcalorimetry is well suited for this purpose, since the relative contributions of enthalpy and entropy changes to the overall free energy change of a reaction are determined by the nature of the force of interaction (Ross & Subramanian, 1981). With a natural target enzyme such a microcalorimetric investigation is not yet feasible for technical reasons. Therefore we recently used the model peptide melittin instead of an enzyme (Milos *et al.*, 1987). Melittin is the first (Comte *et al.*, 1983) and most intensively (Maulet & Cox, 1983; Steiner *et al.*, 1986; Seeholzer *et al.*, 1986) studied peptide that forms with CaM complexes that resemble those formed with intact target enzymes. Although the melittin model has some drawbacks, the homotropic co-operativity in Ca^{2+} -binding to the CaM-melittin complex resembles the one described for myosin light chain kinase (Section 6.1). The enthalpy titration of Ca^{2+} binding to the complex shows that bindings of the third and fourth Ca^{2+} are 2.5 kJ/site more endothermic than in the absence of melittin. In other words, although the presence of melittin increases the affinity of CaM for Ca^{2+} , Ca^{2+} binding becomes more endothermic and the positive free energy coupling in the CaM- Ca^{2+} -melittin system is mostly driven by hydrophobic associations, i.e. pronounced dehydration of non-polar groups in the Ca^{2+} -binding sites and in the two surface-accessible hydrophobic domains of CaM.

7. Conclusions

In spite of a great number of investigations of nearly all the possible aspects of the metal-binding properties of CaM, the behaviour of the protein remains enigmatic. For its interaction with Ca^{2+} two mutually exclusive binding models have been proposed, each of them with its experimental support. The first model, of four nearly independent binding sites with very similar affinities, is favoured by direct binding and microcalorimetric studies and studies on Ca^{2+} - Mg^{2+} and Ca^{2+} - H^+ antagonism. The second model is much more sophisticated, with two pairs of sites with pronounced positive co-operativity within each pair and a marked difference in affinity between the pairs. Kinetic data and some conformational studies favour the latter model. At this time, it is difficult to solve the controversy about the two binding models. Probes directly involved in Ca^{2+} chelation and specific for each of the four Ca^{2+} -binding domains may become available thanks to increased resolution of n.m.r. spectra. Crystals of CaM with different contents of Ca^{2+} can constitute an alternative tool for this purpose.

Not only Ca^{2+} binding, but also its modulation by physiological ions, is intriguing. There is indeed strong evidence that CaM contains six auxiliary ion-binding sites and that the latter modulate the affinity of the Ca^{2+} -binding sites. Under non-pathological conditions, the auxiliary sites are specific for Mg^{2+} and partly occupied by the latter ion. Cytotoxic cations also bind to the auxiliary sites. Progress on the definite location of the latter, as well as of the proton-binding sites involved in Ca^{2+} - H^+ antagonism, is expected to come very rapidly from the study of the ion binding properties of genetically engineered CaMs, which can be constructed at will (Roberts *et al.*, 1986; Putkey *et al.*, 1986; Craig *et al.*, 1987), for probing some of the above reported hypotheses.

The Ca^{2+} -binding properties of CaM are markedly influenced by the presence of the target enzyme with an increase of the binding constants and the appearance of

quite strong positive co-operativity. In two enzyme systems, the third Ca^{2+} -binding constant was subject to the most dramatic change. The increase in the affinity constants of CaM for Ca^{2+} and the changing affinity of the enzyme for CaM have been integrated in a coherent system of free energy coupling and could be analysed quantitatively for the case of phosphorylase b kinase and for myosin light chain kinase with an equation first proposed by Huang and collaborators. It can be anticipated that, although all the CaM-regulated enzymes are tuned in a similar way by Ca^{2+} and CaM, the variations on the theme are as numerous as in Ravel's Bolero and create the complexity of living cells.

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