Thermodynamic Analysis of Calcium and Magnesium Binding to Calmodulin†

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ABSTRACT: To elucidate some aspects still debated concerning the interaction of Ca^{2+} and Mg^{2+} with CaM, the thermodynamic binding parameters of Ca^{2+}−CaM and Mg^{2+}−CaM complexes were characterized by flow dialysis and isothermal microcalorimetry under different experimental conditions. In particular, the enthalpy and entropy changes associated with Ca^{2+} and Mg^{2+} binding to their sites were determined, allowing a better understanding of the mechanism underlying cation−CaM interactions. Ca^{2+}−CaM interaction follows an enthalpy−entropy compensation relationship, suggesting that CaM explores a subspace of isoenergetical conformations which is modified by Ca^{2+} binding. This Ca^{2+}-induced change in CaM dynamics is proposed to play a key role in CaM function, i.e. in its interaction with and/or activation of target proteins. Furthermore, data show that Mg^{2+} does not act as a direct competitor for Ca^{2+} binding on the four main Ca^{2+} binding sites, but rather as an allosteric effector. This implies that the four main Mg^{2+} binding sites are distinct from the EF-hand Ca^{2+} binding sites. Finally, Ca^{2+} is shown to interact with auxiliary binding sites on CaM. These weak affinity sites were thermodynamically characterized. The results presented here challenge the current accepted view of CaM ion binding.

Transient Ca^{2+} waves are induced in the cytosol of most eucaryotic cells by external stimuli (*1*). Transduction of the Ca^{2+} signal occurs through Ca^{2+} binding to proteins that exhibit Ca^{2+} affinities and binding kinetics compatible with the concentration and time range of the Ca^{2+} wave. More than 400 sequences of such Ca^{2+}-binding proteins are known. All these proteins are similar in their primary structure and probably have evolved from a common ancestor (*2*). Their evolutionary, functional, and structural Ca^{2+}-binding unit is the helix−loop−helix motif, commonly called the EF-hand domain (*3*−*5*). This motif is a linear sequence of about 36 amino acids, composed of a 12-residue Ca^{2+} -binding loop surrounded by two α-helices. The various Ca^{2+}-binding proteins differ in the number of these functional domains and their affinity for Ca^{2+}.

CaM,[1] a member of this superfamily, is found in the cytoplasm of all eucaryotic cells so far tested, in contrast to other Ca^{2+}-binding proteins which are restricted to some cell lineages (*6*). It appears as a prototype for the study of the role of Ca^{2+}-binding proteins in the cell (*7*, *8*). The protein consists of four EF-hand domains, and its primary structure was determined by Watterson et al. (*9*). In its crystal structure, CaM appeared as a dumbbell-shaped protein with two lobes linked by a central helix (*10*−*13*), which has been shown by NMR to be nonhelical in its central part and flexible in solution (*14*−*17*). Each lobe contains two coupled Ca^{2+}-binding sites.

Ca^{2+} binding is essential for CaM activation. Its binding induces a major reorganization of the protein, leading to the formation of a hydrophobic cavity which allows CaM to interact with its target enzymes (*12*, *16*−*21*). Various studies have shown that Ca^{2+} interaction with CaM is complex, involving coupling between the main Ca^{2+} binding sites (*22*, *23*) and probably auxiliary cationic sites, as previously suggested by Milos et al. (*24*, *25*) and confirmed by Lafitte et al. (*26*, *27*). Despite the number of studies, controversies concerning the mechanism of ion binding to CaM still remain. Namely, is CaM composed of two independent lobes with two cooperative sites in each (*28*, *29*) or composed of two coupled globular domains (*30*, *31*)? Several reports support this latter model (*32*, *33*), and a recent differential scanning calorimetric study showed that the electrostatic potential played an important role in the coupling between the two lobes (*34*).

Another controversy concerns CaM−Mg^{2+} interaction, whose understanding is of great interest since Mg^{2+} (although at high concentrations) has been suggested recently to contribute to the specificity of CaM in target activation (*35*). The parameters of CaM−Mg^{2+} interaction are still largely debated in the literature, in particular the number of sites, their affinity for Mg^{2+}, and the existence of a direct competition between Mg^{2+} and Ca^{2+} for their binding to CaM. The accepted model considers a direct competition of Ca^{2+} and Mg^{2+} on the four main Ca^{2+} binding sites (*35*, *36*), although it has been suggested that Mg^{2+} might be an allosteric effector of CaM (*24*−*27*).

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[1] Abbreviations: CaM, calmodulin; SynCaM, synthetic calmodulin.

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To gain further insight into these problems, we combined flow dialysis and isothermal microcalorimetry to study the binding of Ca^{2+} and Mg^{2+} to SynCaM, a synthetic hybrid of mammalian and plant CaM able to activate all the CaM-dependent enzymes (*37*). This synthetic calmodulin is used as a standard of comparison in our study. We have shown that the mean calcium affinity of SynCaM is lower than the mean calcium affinity of calmodulin from ram testes when measured under the exact same experimental conditions (J. Haiech, unpublished results; ref *38* and this paper). Quantitative comparison of affinity constants of different calmodulins is probably meaningless as few changes in the primary structure or the experimental conditions induce significant changes in the thermodynamic parameters. Affinity constants and enthalpy and entropy changes of Ca^{2+} and Mg^{2+} interactions with SynCaM were determined. Careful analysis of these parameters let us further pinpoint some aspects of cation−CaM interaction and clearly indicate that Ca^{2+} and Mg^{2+} do not directly compete in their binding to CaM.

MATERIALS AND METHODS

Protein and Protein Concentration. Synthetic CaM, SynCaM, was produced and purified with previously described procedures (*37*, *39*). Protein purity was checked by SDS gel electrophoresis, high-performance capillary electrophoresis, and ESI mass spectrometry. Purity of the protein used in this study was ≥99%. Protein concentration was measured by amino acid analysis and by UV spectrophotometry with a molar extinction coefficient at 276.5 nm of 1560 M^{-1} cm^{-1} (*40*) or using the Bradford technique. For all experiments, ultrapure water (milli-Q apparatus, Millipore Inc.) and plasticware washed in 1 N HCl were used to minimize Ca^{2+} contamination. Ca^{2+} was removed from protein with the trichloroacetic acid treatment (*41*).

Flow Dialysis. Flow dialysis experiments were carried out according to Haiech et al. (*41*) at 25 °C in 50 mM Hepes buffer at pH 7.5. To the upper chamber of the dialysis cell which contains 1 mL of protein (concentration of $\sim 3 \times 10^{-5}$ M) was added 1 μL of $^{45}Ca^{2+}$ (about 1 μCi). The lower compartment was perfused by the buffer at the flow rate of 240 mL/h. One microliter aliquots with increasing $CaCl_2$ concentrations were added to the upper compartment. For each Ca^{2+} addition, five fractions of 1 mL each were collected. The last three fractions out of the five were counted with a liquid scintillator. A final addition of 1 M $CaCl_2$ ensures a complete chase of $^{45}Ca^{2+}$. In this way, for each Ca^{2+} addition, the amount of free Ca^{2+} can be determined. Each binding experiment gives a data set (n_i, x_i), in which x_i is the free concentration of ligand and n_i the average number of moles of ligand bound per mole of protein. Nonlinear fitting was carried out with Microsoft Excel software or with Microcal Origin software using the Adair−Klotz equation according to Haiech et al. (*41*)

$$\nu = (K_1x + 2K_1K_2x^2 + 3K_1K_2K_3x^3 + 4K_1K_2K_3K_4x^4)/(1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4) + 2[Kx/(1 + Kx)] \quad (1)$$

where ν is the number of calcium bound per calmodulin, K_1, K_2, K_3, and K_4 are the macroscopic constants describing the binding to the calcium specific sites (EF-hand sites), and K is the association binding constants for the auxiliary sites. From a previous report using mass spectrometry, we have shown that there were four high-affinity calcium sites (the so-called calcium specific sites), two medium-affinity sites (the so-called auxiliary sites), and four low-affinity sites that we neglect in this study (*26*, *27*). We assume that the auxiliary sites are equivalent and independent and that they are independent of the specific sites. In the same study, we have shown that there are four magnesium sites (two with affinities higher than those of the two others).

Isothermal Calorimetry. Binding of Ca^{2+} to the main sites of CaM and binding of Mg^{2+} were analyzed by isothermal titration microcalorimetry (ITC) using a Microcal MCS ITC instrument. Experiments were carried out at 25 °C in the same buffer in which flow dialysis experiments were carried out. The CaM concentration (in the 1.34 mL cell of the calorimeter) ranged from 0.02 to 0.2 mM, while Ca^{2+} and Mg^{2+} concentrations (in the syringe) varied from 3 to 20 mM. CaM was titrated up to a ligand/protein concentration ratio of about 10. The heat of dilution was measured by injecting the ligand(s) into a buffer solution without protein. The value obtained was subtracted from the heat of reaction to obtain the effective heat of binding. Data were analyzed using Microcal Origin software and were fitted with eq 2 to obtain the thermodynamic parameters for the interaction of Ca^{2+} with each Ca^{2+} binding site of CaM: enthalpy of binding (ΔH), association constant (K_a), and, consequently, free energy change (ΔG) and entropy change (ΔS).

$$\Delta H = [\Delta H_1K_1x + (\Delta H_1 + \Delta H_2)K_1K_2x^2 + (\Delta H_1 + \Delta H_2 + \Delta H_3)K_1K_2K_3x^3 + (\Delta H_1 + \Delta H_2 + \Delta H_3 + \Delta H_4)K_1K_2K_3K_4x^4]/(1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4) \quad (2)$$

Ca^{2+} binding to the auxiliary sites of SynCaM was measured with a Thermometric 2277 Thermal Activity Monitor apparatus, according to a previously described flow microcalorimetric method (*42*). The flow rate was 20 mL/h in two calorimeter circuits, and the sensitivity was 10 μW full-scale, with a background noise of 0.1 μW. Interactions were studied by binding various $CaCl_2$ concentrations to a fixed protein quantity (1 mL at about 2×10^{-5} M). The experimental heat quantities were corrected, when necessary, for dilution effects. The calculated enthalpy (ΔH) was expressed as a function of macroscopic association constants, K_i, acquired by flow dialysis (eq 3):

$$\Delta H = [\Delta H_1K_1x + (\Delta H_1 + \Delta H_2)K_1K_2x^2 + (\Delta H_1 + \Delta H_2 + \Delta H_3)K_1K_2K_3x^3 + (\Delta H_1 + \Delta H_2 + \Delta H_3 + \Delta H_4)K_1K_2K_3K_4x^4]/(1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4) + 2\Delta H[Kx/(1 + Kx)] \quad (3)$$

where ΔH_1, ΔH_2, ΔH_3, and ΔH_4 represent the enthalpy change associated with the binding of the first, second, third, and fourth Ca^{2+} to the protein, respectively, and x corresponds to the free Ca^{2+} concentration. ΔH represents the enthalpy change associated with the binding to the auxiliary

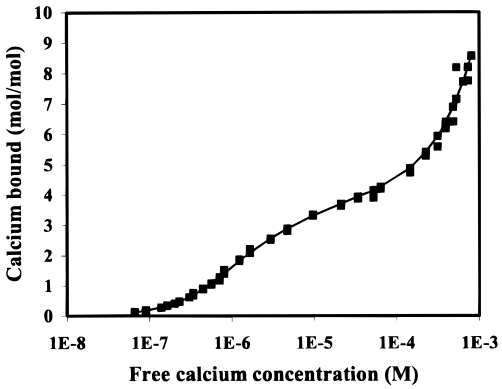

FIGURE 1: Calcium binding to SynCaM in 50 mM Hepes buffer at pH 7.5. The number of moles of calcium bound per mole of protein is expressed as a function of the free calcium concentration. Calcium binding experiments were perfromed using flow dialysis as described in Materials and Methods. Each experiment was performed at least three times with protein concentrations ranging from 10^{-5} to 5×10^{-5} M.

sites. Nonlinear fitting was carried out with Microsoft Excel software.

RESULTS AND DISCUSSION

Ca^{2+} Binding to SynCaM Studied with Flow Dialysis

In 50 mM Hepes buffer at pH 7.5, the binding isotherm is biphasic (Figure 1). The first part of the curve with the plateau at 4 mol of Ca^{2+} per mole of CaM corresponds to Ca^{2+} binding to the four main Ca^{2+}-binding sites (EF-hand sites). The second phase, which shows increased Ca^{2+} binding when the free Ca^{2+} concentration is above 10^{-4} M, suggests the presence of auxiliary Ca^{2+}-binding sites and corroborates previous observations (*25*−*27*). These auxiliary sites have Ca^{2+} affinities in the millimolar range. It was thought previously that this part of the curve corresponded to a nonspecific Ca^{2+} binding to the dialysis membrane (*30*). However, when the protein concentration was varied (between 10 and 100 μM calmodulin), the same curve was obtained pointing out that this second part of the binding isotherm is also relevant to Ca^{2+} binding to the protein.

For binding to the main Ca^{2+}-binding sites, our experimental data were fit using the Adair−Klotz equation as indicated in Materials and Methods. Values for the macroscopic association constants K_1, K_2, K_3, and K_4 were 1.85 $\times$ 10^6, 1.22 $\times$ 10^6, 4.4 $\times$ 10^5, and 1.0 $\times$ 10^5 M^{-1}, respectively. These values are in good agreement with those obtained previously for SynCaM (*23*, *30*). The mechanism of Ca^{2+} binding to CaM is complex, and several models (*23*, *31*) have been proposed to explain the various Ca^{2+} binding data. However, so far, only one model takes into account most, if not all, of the data present in the literature. This model is termed the sequential model (*30*, *31*, *41*, *43*), although it will be better described as a preferential pathway binding model. In this model, it is proposed that the calcium binding sites of calmodulin may exist under two conformations: a conformation where they exhibit a weak affinity for calcium (the low-affinity conformation) or a conformation where they exhibit a high affinity for calcium (the high-affinity conformation). The calcium affinity in this conformation is at least 100-fold higher than that in the low-affinity conformation (see the appendix of ref *41*).

In the apo form, only one Ca^{2+} binding site in CaM is able at first to bind Ca^{2+} with a high affinity (high-affinity conformation). Binding to the first site allows then the second site to undergo a conformational change (from the low-affinity conformation to the high-affinity conformation) and to bind Ca^{2+} with an affinity in the same range as the first one and so on. The binding sequence previously determined (*31*) is III → IV → I → II, where I−IV represent the Ca^{2+} binding sites, starting the numbering from the NH_2 terminus of the protein. The binding of calcium to calmodulin follows a preferential pathway. The first sequence, proposed in 1981 (II, I, III, and then IV), was based on the calmodulin sequence published by Watterson et al. (*9*) and on the number of carboxylic group present in each EF-hand site. Moreover, this sequence of calcium filling was corroborated by the terbium titration of calmodulin. Unfortunately, due to two deaminations, the sequence of calmodulin determined by Edman degradation was not the same as the one obtained by DNA sequencing, and it was shown that terbium does not behave like calcium when it binds to calmodulin (*44*). Using the true sequence of calmodulin, the rationale presented in the ref *41* is in agreement with the actual proposed sequence.

This sequential model is strongly corroborated by recent results using a different experimental approach (*32*, *33*, *45*). This sequential model implies there is a strong asymmetry of the molecule in the apo form (only site III has a high affinity for calcium) and there is a strong "positive cooperativity" between the sites of the same lobe and also between the two lobes of the proteins. More precisely, there are strong coupling factors between the different sites of the molecule.

Assuming the sequential model for Ca^{2+} binding, the association constants for Ca^{2+} binding to each specific EF-hand site can be determined. Indeed, under these conditions, the macroscopic binding constants determined above correspond to the association constants for each site when it is in the high-affinity conformation, and thus, the values of K_1 (1.85×10^6 M^{-1}), K_2 (1.22×10^6 M^{-1}), K_3 (4.4×10^5 M^{-1}), and K_4 (1.0×10^5 M^{-1}) represent the association constants for Ca^{2+} binding to the first (site 1), second (site 2), third (site 3), and fourth (site 4) sites, respectively, when those sites are in the high-affinity conformations.[2] Indeed, due to the preferential calcium binding pathway, in the binding polynomial (the denominator of the Adair−Klotz equation), the term K_1x does not refer to the calmodulin with one calcium (whatever the site where the calcium is bound) but to calmodulin with one calcium in site III (the first site to be occupied) and so on for the other terms of the binding polynomial.

Thermodynamic Analysis of Ca^{2+} and Mg^{2+} Binding to SynCaM

The heat effect generated by Ca^{2+} binding to CaM and measured by microcalorimetry takes into account the two main mechanisms involved in the interaction of Ca^{2+} with Ca^{2+}-binding proteins:

[2] Throughout this paper, sites 1−4 refer to the sites occupied first, second, third, and fourth during Ca^{2+} binding and should not be mistaken with sites I−IV which refer to the location of the site in the protein structure.

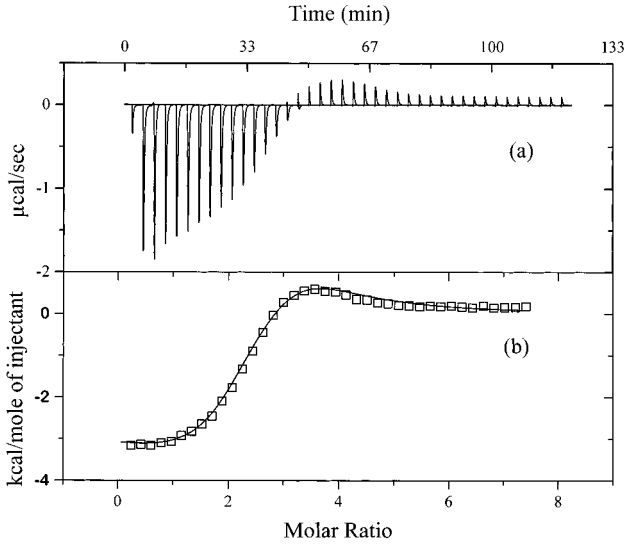

FIGURE 2: Typical ITC data curves of CaM−Ca^{2+} interaction at 25 °C in 50 mM Hepes at pH 7.5. (a) Titration of 1.34 mL of SynCaM (5.1×10^{-5} M) with 4 mM $CaCl_2$ with 40 injections of 3 μL each. (b) Binding isotherm derived from panel a.

(i) Ca^{2+} binding itself, involving mainly cation desolvation (*46*), is characterized by $\Delta H_{binding}$ (ΔH_{bind}) and $\Delta S_{binding}$ (ΔS_{bind}). It is known from X-ray studies that the seven oxygen atoms involved in Ca^{2+} ligation in the Ca^{2+} binding site are arranged in a pentagonal bipyramidal array (*4*, *47*). In solution, the mean number of water molecules surrounding Ca^{2+} is seven (*4*, *48*). Thus, one can assume that the geometry of Ca^{2+} coordination in water or in the protein is identical, implying that ΔH_{bind} is almost zero.

(ii) The conformational modification of SynCaM associated with Ca^{2+} binding is characterized by $\Delta H_{conformation}$ (ΔH_{conf}) and $\Delta S_{conformation}$ (ΔS_{conf}).

Thus, the observed ΔH (ΔH_{obs}) and ΔS (ΔS_{obs}) can be written as follows: $\Delta H_{obs} = \Delta H_{bind} + \Delta H_{conf}$ and $\Delta S_{obs} = \Delta S_{bind} + \Delta S_{conf}$.

In various systems and namely upon Ca^{2+} binding to proteins, a linear relationship between ΔH and $T\Delta S$ has been observed (*46*). Such a linear relationship, termed enthalpy−entropy compensation (*49*), has been extensively investigated (*50*, *51*) and suggests that the protein explores a set of isoenergetical conformations (*50*). In the case of enthalpy−entropy compensation and taking into account $\Delta H_{bind} = 0$ as indicated above, the observed entropy ΔS_{obs} takes the following form: $\Delta S_{obs} = k\Delta H_{obs} + \Delta S_{intercept}$. In this case, $\Delta S_{intercept}$ stands for ΔS_{obs} when $\Delta H_{obs} = 0$ and corresponds to the entropy of Ca^{2+} desolvation (*46*).

Ca^{2+} Binding. A typical experimental ITC curve for Ca^{2+} binding to SynCaM is shown in Figure 2. Data were fitted according to eq 2 defined in Materials and Methods, with the stoichiometry of Ca^{2+} binding fixed to 4 (Adair−Klotz equation with a binding polynomial of degree 4), as determined by flow dialysis and mass spectrometry. Values for the enthalpy changes (ΔH_1, ΔH_2, ΔH_3, and ΔH_4) and Ca^{2+} association constants (K_1, K_2, K_3, and K_4) for binding of the first, second, third, and fourth Ca^{2+}, respectively, are thus obtained. Now, assuming a sequential Ca^{2+} binding mechanism, these values of enthalpy changes and association binding constants (K_a) will correspond to the enthalpy changes and association binding constants associated with

Table 1: Thermodynamic Parameters of Calcium Binding to SynCaM Main and Auxiliary Sites[a]

	K_a[b] (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
site 1	1.85×10^6	-13.4 ± 0.4	-35.7 ± 1.7	75 ± 4
site 2	1.22×10^6	-12.5 ± 1.0	-34.7 ± 0.7	75 ± 11
site 3	4.4×10^5	3.5 ± 1.3	-32.2 ± 0.6	120 ± 3
site 4	1.0×10^5	6.3 ± 0.8	-28.5 ± 1.4	117 ± 12
auxiliary sites	650	24 ± 3	-16 ± 2	135 ± 14

[a] Values correspond to the mean of three to five experiments. [b] Values indicated correspond to those determined by flow dialysis.

Table 2: Thermodynamic Parameters for Calcium Binding to SynCaM in the Presence of 5 mM Magnesium[a]

	K_a (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
site 1	1.52×10^6	-15.5 ± 0.1	-35.3 ± 1.0	66 ± 1
site 2	0.63×10^6	-22.2 ± 0.2	-33.1 ± 0.4	37 ± 2
site 3	2.2×10^5	-1.5 ± 0.1	-30.5 ± 0.3	97 ± 1
site 4	0.13×10^5	-11.3 ± 0.2	-23.6 ± 0.2	41 ± 1

[a] Values correspond to the mean of three to five experiments.

Ca^{2+} binding to each individual Ca^{2+} binding site of CaM. Data obtained indicate that association constants for binding to sites 1−3 obtained using ITC [$(4.8 \pm 1.1) \times 10^6$, $(1.1 \pm 0.29) \times 10^6$, and $(1.9 \pm 0.98) \times 10^5$ M^{-1}, respectively] were in very good agreement with those measured by flow dialysis [$(1.85 \pm 1.2) \times 10^6$, $(1.22 \pm 0.8) \times 10^6$, and $(4.4 \pm 0.6) \times 10^5$ M^{-1}, respectively], whereas for the fourth site, Ca^{2+} affinity was found to be about 30 times lower [$K_4 = (0.3 \pm 1.4) \times 10^4$ M^{-1}] than that measured by flow dialysis [$K_4 = (1.0 \pm 1) \times 10^5$ M^{-1}]. This discrepancy can be explained by the presence of the auxiliary Ca^{2+} binding sites described above, which may interfere during the saturation of the last main Ca^{2+} binding site, but which cannot be taken into account using our ITC experimental conditions due to their low Ca^{2+} affinity (see discussion in a following paragraph). Thus, to obtain more accurate enthalpy changes, K_a values were fixed (taking those obtained by flow dialysis) and only the association enthalpy parameter ΔH was fitted. ΔH values are given in Table 1. Gibbs free energy changes ($\Delta G = -RT \ln K_a$) and entropy changes ($\Delta S = \Delta H/T - \Delta G/T$) at 298 K were then calculated from these ΔH and K_a values. Results (Table 1) indicate that entropy of Ca^{2+} binding is highly positive and largely dominates Ca^{2+}−CaM interaction in the four EF-hand sites. To discriminate between the two aspects of the interaction, Ca^{2+} binding per se and CaM conformational changes, we measured the thermodynamic parameters of Ca^{2+}−CaM complex formation under different experimental conditions: CaM alone (Table 1), CaM in the presence of 5 mM $MgCl_2$ (Table 2), and CaM in the presence of 150 mM KCl (Figure 3). Plots of enthalpy changes versus entropy changes are linear under all the conditions tested (Figure 3), corresponding to the enthalpy−entropy compensation described above. In general, such entropy−enthalpy compensation passes through the origin. In our case, this is not true, and we conclude that we have two effects (see above): the conformational changes of the protein associated with the binding of calcium ions and the binding of the calcium ion per se, which involves the desolvation of the ion and the coordination of the ion in the protein site.

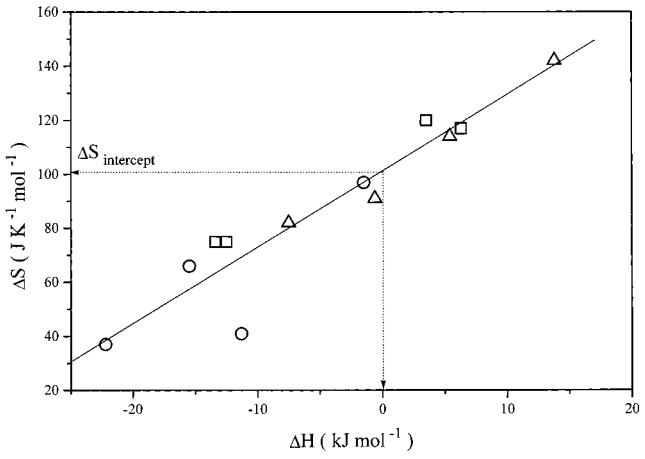

FIGURE 3: Relationship between ΔH and ΔS for calcium binding to the EF-hand calcium-binding sites of CaM. ΔH and ΔS were measured in 50 mM Hepes at pH 7.5 (□), 50 mM Hepes at pH 7.5 containing 5 mM $MgCl_2$ (○), and 50 mM Hepes at pH 7.5 containing 150 mM KCl (△). Data were taken from Tables 1 and 2 for experiments in 50 mM Hepes at pH 7.5 and 50 mM Hepes at pH 7.5 and 5 mM $MgCl_2$, respectively. A least-squares fit was performed for each set of data.

The first phenomenon is attributed to the enthalpy−entropy compensation effect. It is important to note that each specific site occupied by calcium is going to induce specific conformational changes. On the other hand, the desolvation phenomenon (number of water molecules removed from the hydration shell of the ion) and the geometry of coordination of the calcium ion in the four main sites (EF-hand sites) are supposed to be similar. Therefore, the binding per se of the calcium ion to the different sites is going to induce an offset of the linear relationship between entropy and enthalpy. Such an offset is characterized by the ΔS_{bind} (mainly the removal of water molecules from the calcium ion) and the ΔH_{bind} (mainly due to the difference in the geometry of coordination of the calcium ion in water and in the protein). ΔS_{bind} and ΔH_{bind} are independent of the calcium sites of calmodulin if the number of water molecules involved in the coordination of calcium is the same whatever the site and if the geometry of coordination of calcium in each site is similar. In the case of the four EF-hand sites of calmodulin, such previous conditions are satisfied. However, we cannot determine ΔS_{bind} and ΔH_{bind} as we have one equation with two unknowns. Therefore, it is necessary to know either ΔS_{bind} or ΔH_{bind}. If we assume that the geometry of coordination of the calcium ion is similar in water and in the protein (therefore, $\Delta H_{bind} = 0$), then we are able to derive a value for ΔS_{bind} that is equal to $\Delta S_{intercept}$ (see below). On the other hand, if we are confident in the value of entropy desolvation of calcium ion given in handbooks, it is possible to derive ΔH_{bind} and to obtain some information on the difference between the geometry of coordination of calcium in water and in the molecule.

Once the offset has been removed, we obtain a linear entropy−enthalpy relationship that passes through the origin. This indicates, according to the physical meaning of linear thermodynamic relationships (*50*), that in the different ion−CaM complexes which may form under the experimental conditions tested, the protein maintains its conformations between different isoenergetical subspaces. During ion binding, CaM changes from one thermodynamic state to another, but in each of the states, it explores different substates of equal energy. Following the ideas of Weber (*52*, *53*), calcium ion binding will lead calmodulin to internally reorganize and upon this reorganization to exchange strong bonding interactions with weak ones. This type of reorganization will allow some part of the molecule to acquire or to lose some flexibility.

Assuming that $\Delta H_{binding} = 0$ (see above for discussion), the entropy change associated with Ca^{2+} binding per se can be easily determined. Its value (termed $\Delta S_{intercept}$; see the beginning of the paragraph), determined from Figure 3, is 101 ± 2 J K^{-1} mol^{-1}. This entropy release upon Ca^{2+} binding arises primarily from the release of bound water molecules hydrating the free Ca^{2+} (*46*). Knowing that only one of the seven water molecules remains bound to Ca^{2+} inside each binding sites (*13*), we can conclude that $\Delta S_{intercept}$ corresponds to the release of six water molecules from Ca^{2+}, and thus, the entropic contribution for the release of one water molecule is 16.8 ± 0.3 J K^{-1} mol^{-1}. Thus, Ca^{2+} desolvation upon binding is mainly responsible for this large entropic effect observed when the cation binds to SynCaM. The entropic change linked to the conformational modifications of CaM (ΔS_{conf}) can be calculated for each binding step by subtracting 101 J K^{-1} mol^{-1} from the entropy values listed in Table 1. A value of -26 J K^{-1} mol^{-1} was obtained for Ca^{2+} binding to sites 1 and 2, suggesting a rigidification of the molecule upon Ca^{2+} binding to the COOH lobe, and values of 19 and 6 J K^{-1} mol^{-1} were obtained for Ca^{2+} binding sites 3 and 4, respectively. This goes along with the observation that Ca^{2+} binding induces a local rigidification around the Ca^{2+}-binding site concomitant with a change in the flexibility of the remaining part of the protein (*54*).

On the other hand, as ΔH_{bind} is assumed to be close to zero, ΔH_{obs} corresponds to the enthalpy change associated with conformational changes. Enthalpy changes are exothermic, and thus favorable, when Ca^{2+} binds to CaM sites 1 and 2, whereas they are endothermic, and thus unfavorable, for binding to sites 3 and 4.

When the overall calcium binding mechanism is considered, binding of the first and second Ca^{2+} ions is enthalpically and entropically driven, whereas binding of the third and fourth Ca^{2+} ions is driven only by entropy. This indicates an asymmetry in CaM structure between the COOH lobe (containing sites 1 and 2) and the NH_2 lobe (containing sites 3 and 4) and goes along with the asymmetry in the electrostatic potentials of the two lobes (*55*) and the asymmetry between the flexibility of the two lobes (*16*, *34*).

Mg^{2+} Binding. The effect of Mg^{2+} on Ca^{2+} binding is still controversial. The accepted model considers a direct competition between Ca^{2+} and Mg^{2+} for the four main Ca^{2+} binding sites (*36*, *41*), but it has been suggested that Mg^{2+} might be an allosteric effector rather than a direct competitor of Ca^{2+} for its binding sites (*24*, *25*). More recently, evidence was obtained by mass spectrometry for the presence on CaM of a pair of high-affinity Mg^{2+}-binding sites and a pair of low-affinity sites (*26*). The presence of these sites was confirmed by NMR data which in addition point out that these sites were located within the Ca^{2+} binding structures of calmodulin (*35*). Using the same ITC experimental conditions as above, binding of Mg^{2+} to CaM and

Table 3: Thermodynamic Parameters of Magnesium Binding to SynCaM[a]

	K_a (M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J K^{-1} mol^{-1})
site 1	2.3×10^5	5.4 ± 0.8	-30.6 ± 4.1	121 ± 9
site 2	6.9×10^4	4.2 ± 1.5	-27.6 ± 4.6	107 ± 6
site 3	2.0×10^3	16.3 ± 8.4	-18.8 ± 2.9	118 ± 5
site 4	1.0×10^3	10.5 ± 5.4	-17.1 ± 1.2	93 ± 4

[a] Values correspond to the mean of three to five experiments.

binding of Ca^{2+} to CaM in the presence of 5 mM Mg^{2+} were investigated (Tables 3 and 2, respectively). Our results are consistent with the presence of two classes of Mg^{2+} binding sites on CaM: a first class, including two sites with association constants in the range of 10^5 M^{-1} and enthalpy changes of about 5 kJ mol^{-1}, and a second class, including two other sites with lower affinities (K_a of about 10^3 M^{-1}) and enthalpy changes in the range of 10−20 kJ mol^{-1} (Table 3). Comparisons of the thermodynamic parameters listed in Tables 1−3 are not consistent with a direct Ca^{2+} versus Mg^{2+} competition. Mg^{2+} decreases the Ca^{2+} association constants for the four EF-hand Ca^{2+} binding sites (Tables 1 and 2), but taking into account the respective affinities of Ca^{2+} and Mg^{2+} for CaM (Tables 1 and 3) and the concentration of the two cations, the affinity loss is too weak to fit with a direct competition at least for sites 1−3. The affinity loss on these sites may rather be attributed to an allosteric effect induced by Mg^{2+} binding to sites that differ from the main Ca^{2+} binding sites.

Moreover, the enthalpy−entropy compensation observed under the different experimental conditions (Figure 3) also argues against a direct competition between Ca^{2+} and Mg^{2+}. Indeed, if direct competition were to occur, addition of Ca^{2+} to Mg^{2+}-loaded protein would induce a Ca^{2+}−Mg^{2+} exchange. The number of water molecules involved in the exchange would then be less than six, and the enthalpy and entropy changes corresponding to Ca^{2+} binding per se, in the presence of Mg^{2+}, would be under the experimental curve. Now, Figure 3 shows that Mg^{2+} did not greatly affect the thermodynamic parameters associated with Ca^{2+} binding to CaM. In addition, it is noteworthy that Mg^{2+} binding to CaM does not lead to a linear relationship between ΔH and ΔS.

Thermodynamic Analysis of Ca^{2+} Binding to Auxiliary Sites

Flow dialysis experiments (Figure 1) strongly suggest the presence of auxiliary Ca^{2+} binding sites in addition to the four EF-hand sites. However, such sites cannot readily be titrated by the technique of flow dialysis, since their Ca^{2+} affinity is fairly weak. ITC also failed in such measurement because Ca^{2+} concentrations that are needed to saturate these sites are extremely high and not compatible with this method under our experimental conditions. Therefore, isothermal flow microcalorimetry was used to study Ca^{2+} binding to these weak affinity sites. Binding constants and thermodynamic parameters (ΔH, ΔG, and ΔS) of these sites were determined (Table 1), assuming that under our experimental conditions only the two auxiliary binding sites of moderate affinity for Ca^{2+} (class 2; *26*) could be titrated. The physiological relevance of these weak affinity Ca^{2+} binding sites has not yet been established. However, one can speculate that these sites may bind other cations, such as Mg^{2+} or Zn^{2+}, which thus may modulate CaM activity. The importance of Mg^{2+} in CaM−target peptide interaction has recently been addressed (*35*).

CONCLUSION

In this study, a detailed analysis of the thermodynamic parameters associated with Ca^{2+} and Mg^{2+} binding to specific sites on CaM was performed. Due to enthalpy−entropy compensation during Ca^{2+} binding, the thermodynamic parameters of Ca^{2+} binding per se and of the conformational change associated with Ca^{2+} binding to the main Ca^{2+}-binding sites could be evaluated. Binding per se implies desolvation of Ca^{2+}, where water molecules around the ion are replaced by the ligands in the Ca^{2+} binding sites. Previous studies suggested that CaM was asymmetric (*16*, *34*, *55*). This asymmetry also holds for the mechanism underlying Ca^{2+} binding. Indeed Ca^{2+} binding is enthalpically and entropically driven for binding to sites III and IV and entropically driven for binding to sites I and II.

Several studies have shown that Mg^{2+} binds to CaM. The thermodynamic study presented here strongly supports non-competition between Ca^{2+} and Mg^{2+} in their binding to the main Ca^{2+} binding sites. Mg^{2+} more likely binds to the auxiliary cation-binding sites present on CaM. Some of these may be close to the EF-hand Ca^{2+}-binding sites as indicated by the recent NMR study using ^1H- and ^{15}N-labeled CaM (*35*) but do not completely overlap with them. Such a satellite site of an EF-hand site has already been described in parvalbumin (*56*). In such a way, Mg^{2+} could act as an allosteric effector of the protein. It may share this activity with other cations found intracellulary, such as Zn^{2+} and Mn^{2+}, which would therefore participate in the fine-tuning of CaM activity, required for optimal cell functioning. Precise knowledge of ion binding to CaM is of paramount importance if one has to unravel the structure−function relationship of this multifunctional protein.

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REFERENCES

1. Berridge, M. J. (1993) *Nature 361*, 315−325.
2. Nakayama, S., Moncrief, N. D., and Kretsinger, R. H. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 473−507.
3. Kretsinger, R. H. (1979) *Adv. Cyclic Nucleotide Res. 11*, 2−26.
4. Falke, J. J., Drake, S. K., Hazard, A. L., and Peersen, O. (1994) *Q. Rev. Biophys. 3*, 219−290.
5. Kawasaki, H., and Kretsinger, R. H. (1994) *Protein Profile 1*, 343−517.
6. Nakayama, S., and Kretsinger, R. H. (1993) *J. Mol. Evol. 36*, 458−476.
7. Williams, R. J. P. (1992) *Cell Calcium 13*, 355−362.
8. Vogel, J. H. (1994) *Biochem. Cell Biol. 72*, 357−376.
9. Watterson, D. M., Sharief, F., and Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962−975.
10. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) *Nature 315*, 37−40.
11. Kretsinger, R. H., Rudnick, S. E., and Weissman, L. J. (1986) *J. Inorg. Biochem. 28*, 289−302.

12. Babu, Y. S., Bugg, C. E., and Cook, W. J. (1988) *J. Mol. Biol. 204*, 191−204.
13. Chattopadhyaya, R., Meador, W., Means, A., and Quiocho, F. (1992) *J. Mol. Biol. 228*, 117−119.
14. Ikura, M., Spera, S., Barbato, G., Kay, L. E., Krinks, M., and Bax, A. (1991) *Biochemistry 30*, 9216−9228.
15. Barbato, G., Ikura, M., Kay, L. E., Pastor, R. W., and Bax, A. (1992) *Biochemistry 31*, 5269−5278.
16. Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C. B., and Bax., A. (1995) *Nat. Struct. Biol. 2*, 768−776.
17. Zhang, M., Tanaka, T., and Ikura, M. (1995) *Nat. Struct. Biol. 2*, 758−767.
18. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) *Science 256*, 632−638.
19. Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) *Science 257*, 1251−1255.
20. Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) *Science 262*, 1718−1721.
21. Finn, B. E., Evenas, J., Drakenberg, T., Waltho, J. P., Thulin, E., and Forsen, S. (1995) *Nat. Struct. Biol. 2*, 777−783.
22. Klee, C. B., Newton, D. L., Ni, W. C., and Haiech, J. (1986) *Ciba Found. Symp. 122*, 162−182.
23. Haiech, J., Kilhoffer, M. C., Craig, T. A., Lukas, T. J., Wilson, E., Guerra-Santos, L., and Watterson, D. M. (1989) in *Calcium Binding Protein in Normal and Transformed Cells* (Pochet, R., Lawson, D. E. M., and Heizmann, C. W., Eds.) pp 43−56, Plenum, London.
24. Milos, M., Schaer, J. J., Comte, M., and Cox, J. A. (1986) *Biochemistry 25*, 6279−6287.
25. Milos, M., Comte, M., Schaer, J. J., and Cox, J. A. (1989) *J. Inorg. Biochem. 36*, 11−25.
26. Lafitte, D., Capony, J. P., Grassy, G., Haiech, J., and Calas, B. (1995) *Biochemistry 34*, 13825−13832.
27. Lafitte, D., Capony, J. P., Grassy, G., Haiech, J., and Calas, B. (1995) *J. Mass Spectrom. Rapid Commun. Mass Spectrom.*, S192−S196.
28. Wang, C. L. (1985) *Biochem. Biophys. Res. Commun. 130* (1), 426−430.
29. Forsén, S., Vogel, H. J., and Drakenberg, T. (1986) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) pp 113−157, Academic Press, New York.
30. Kilhoffer, M. C., Roberts, D. M., Adibi, A. O., Watterson, D. M., and Haiech, J. (1988) *J. Biol. Chem. 263*, 17023−17029.
31. Kilhoffer, M. C., Kubina, M., Travers, F., and Haiech, J. (1992) *Biochemistry 31*, 8098−8106.
32. Pedigo, S., and Shea, S. (1995) *Biochemistry 34*, 1179−1196.
33. Shea, M. A. Verhoeven, A. S., and Pedigo, S. (1996) *Biochemistry 35*, 2943−2957.
34. Protasevich, I., Ranjbar, B., Lobachov, V., Makarov, A., Gilli, R., Briand, C., Lafitte, D., and Haiech, J. (1997) *Biochemistry 36*, 2017−2034.
35. Ohki, S., Ikura, M., and Zhang, M. (1997) *Biochemistry 36*, 4309−4316.
36. Tsai, M. D., Drakenberg, T., Thulin, E., and Forsen, S. (1987) *Biochemistry 26*, 3635−3643.
37. Craig, T. A., Watterson, D. M., Prendergast, F. G., Haiech, J., and Roberts, D. M. (1987) *J. Biol. Chem. 262*, 3278−3284.
38. Kilhoffer, M. C., Haiech, J., and Demaille, J. G. (1983) *Mol. Cell. Biochem. 51*, 33−54.
39. Roberts, D. M., Crea, R., Malecha, M., Alvarado-Urbina, G., Chiarello, R. H., and Watterson, D. M. (1985) *Biochemistry 24*, 5090−5098.
40. Kilhoffer, M. C., Roberts, D. M., Adibi, A., Watterson, D. M., and Haiech, J. (1989) *Biochemistry 28*, 6086−6092.
41. Haiech, J., Klee, C. B., and Demaille, J. G. (1981) *Biochemistry 20*, 3890−3897.
42. Gilli, R. M., Sari, J. C., Sica, L. M., and Briand, C. M. (1988) *Biochim. Biophys. Acta 964*, 53−60.
43. Haiech, J., Kilhoffer, M. C., Lukas, T. J., Craig, T. A., Roberts, D. M., and Watterson, D. M. (1991) *J. Biol. Chem. 266*, 3427−3431.
44. Wang, C. L., Aquaron, R. R., Leavis, P. C., and Gergely, J. (1982) *Eur. J. Biochem. 124*, 7−12.
45. Pedigo, S., and Shea, S. (1995) *Biochemistry 34*, 10676−10689.
46. Kuroki, R., Nitta, K., and Yutani, K. (1992) *J. Biol. Chem. 267*, 24297−24301.
47. Strynadka, N. C., and James, M. N. (1989) *Annu. Rev. Biochem. 58*, 951−998.
48. Sussman, F., and Weinstein, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7880−7884.
49. Benzinger, T. H. (1971) *Nature 229*, 100−102.
50. Rhodes, W. (1991) *J. Phys. Chem. 95*, 1024−1025.
51. Grunwald, E., and Steel, C. (1995) *J. Am. Chem. Soc. 117*, 5687−5692.
52. Weber, G. (1993) *J. Phys. Chem. 97*, 7108−7115.
53. Weber, G. (1995) *J. Phys. Chem. 99*, 1052−1059.
54. Chabbert, M., Kilhoffer, M. C., Watterson, D. M., Haiech, J., and Lami, H. (1989) *Biochemistry 28*, 6093−6098.
55. Weber, P. C., Lukas, T. J., Craig, T. A., Wilson, E., King, M. M., Kwiatkowski, A. P., and Watterson, D. M. (1989) *Proteins 6*, 70−85.
56. Declerq, J. P., Tinant, B., Parello, J., and Rambaud J. (1991) *J. Mol. Biol. 220* (4), 1017−1039.

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