Sequential Conformational Changes in Calmodulin upon Binding of Calcium†

Danielle Burger, Jos A. Cox, Michelle Comte, and Eric A. Stein*

ABSTRACT: Conformational changes occurring in calmodulin upon Ca binding have been analyzed as a function of the degree of saturation of the protein by Ca^{2+} ions. The determination of Ca binding to calmodulin, carried out by equilibrium dialyses and by means of a Ca^{2+}-selective electrode, yields four stoichiometric constants, $K_1 = 1.16 \times 10^5\ M^{-1}$, $K_2 = 2.65 \times 10^5\ M^{-1}$, $K_3 = 8.33 \times 10^4\ M^{-1}$, and $K_4 = 1.91 \times 10^4\ M^{-1}$, when calculated by means of the Adair equation. However, the cooperative effects between the four sites are small, and a statistical study reveals that all four binding sites may be identical and independent with an intrinsic constant of $9.31 \times 10^4\ M^{-1}$. On the basis of these data, we have analyzed the circular dichroic changes at 279 and 222 nm as well as the appearance of hydrophobicity by means of a fluorescent hydrophobic probe; both were monitored as a function of free $[Ca^{2+}]$ or of the mean saturation of calmodulin by Ca^{2+}. The intrinsic tyrosine ellipticity change and the hydrophobic exposure are concomitant with the formation of the $CaM{\cdot}Ca_{n\geq 2}$ species. The far-UV circular dichroic change as a function of free Ca^{2+} and of the mean saturation indicates that each $CaM{\cdot}Ca_n$ species contributes to a different extent to the signal: half-maximal increase of the α-helix content occurs upon binding of the first Ca^{2+}, whereas the maximal value is reached upon binding of the third Ca^{2+}. Our previous studies have shown that only $CaM{\cdot}Ca_3$ and $CaM{\cdot}Ca_4$ interact with target enzymes; the present work indicates that the main structural event occurring in the step $CaM{\cdot}Ca_2 \rightarrow CaM{\cdot}Ca_3$ is the exposure of a hydrophobic plate at the target-accessible surface of calmodulin, which is stabilized by the formation of a highly amphiphatic and surface-seeking α-helix.

A number of enzymes are regulated in a Ca^{2+}-dependent manner by calmodulin [for review, see Klee & Vanaman (1982)]. We have shown that the CaM[1]–enzyme interaction and subsequent enzyme activation do not follow the mean Ca^{2+} saturation of CaM but occur when CaM is saturated with at least three Ca^{2+} (Cox et al., 1981, 1982; Malnoë et al., 1982; Burger et al., 1983). On the other hand, the ability of CaM to function as a ubiquitous Ca vector inside the cell must somehow be related to the conformational changes occurring upon binding of Ca^{2+} to the activator. Using hydrophobic fluorescent probes such as 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS),[1] LaPorte et al. (1980) and Tanaka & Hidaka (1980) have demonstrated the appearance of a hydrophobic region on the surface of CaM upon Ca binding. Since these hydrophobic compounds are able to inhibit CaM-dependent activation of target enzymes, the above authors concluded that the Ca^{2+}-induced hydrophobicity of CaM was a requirement for the CaM–enzyme interaction. The Ca^{2+}-dependent conformational changes in CaM have been studied mainly by near- and far-UV circular dichroism, tyrosine fluorescence, and NMR spectroscopy [for review, see Klee & Vanaman (1982) and Krebs (1981)]. However, few studies were carried out to establish the relationship of conformational changes with saturation of CaM by Ca^{2+}. Crouch & Klee (1980) stated that "the conformational transition that affects the environment of the aromatic residues follows closely the probability of having a calmodulin–Ca_2^{2+} complex. It is therefore assumed to correspond to the formation of this complex and not to that of the calmodulin·Ca_1^{2+} conformer(s)." In most other reports, the conformational changes in CaM and its interaction with target enzymes were not studied in the same conditions of pH and ionic strength, and therefore a general interpretation of the mode of action of CaM could not be proposed. In order to distinguish between the conformational changes which are only distantly related to the process of activation and those which directly lead to the formation of a target interacting domain, we have investigated the conformational changes in CaM in conditions identical with those of the Ca binding to CaM and those of the direct binding of CaM to target enzymes (Burger et al., 1983). The experimental approach involved near- and far-UV circular dichroism and hydrophobic titration of CaM with TNS, in all cases as a function of the saturation of CaM by Ca^{2+}.

Materials and Methods

$CaCl_2$ Titrisol standard and EGTA[1] were purchased from Merck (Darmstadt, West Germany); TNS[1] was from Fluka (Buchs, Switzerland), HEDTA[1] and TES[1] were from Sigma (St Louis, MO); all other chemicals were of analytical grade.

Bovine brain CaM was purified by affinity chromatography on phenyl-Sepharose according to the method described by Gopalakrishna and Anderson (1982) followed by hydroxylapatite chromatography (Wallace & Cheung, 1979). Protein concentration was determined as previously described (Cox et al., 1981).

All experiments were carried out at room temperature in 60 mM TES, pH 7.0, and 150 mM NaCl. Total Ca^{2+} concentrations were measured by atomic absorption with a Perkin-Elmer 330 atomic absorption spectrophotometer using Titrisol standards. Under our experimental conditions, the instrumental precision was above 90% at 2 μM total $[Ca^{2+}]$ and above 95% for values of total $[Ca^{2+}]$ of 10 μM or more.

For Ca-binding experiments, the buffers and CaM solutions were passed over Chelex-X-100 to reduce the contamination by Ca^{2+} and to remove bound Ca^{2+}. No Ca chelator was used. Ca binding to CaM was measured by equilibrium dialyses (500-μL samples in 100 mL of dialysate) for 48 h with three buffer changes or, for measurements above 10^{-5} M Ca^{2+}, by means of the Philips IS 561 Ca^{2+}-selective electrode stand-

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* Address correspondence to this author at P.O. Box 78 Jonction, 1211 Geneva 8, Switzerland.

[1] Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEDTA, *N'*-(2-hydroxyethyl)ethylenediamine-*N,N',N'*-triacetic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; TNS, 6-(4-toluidino)-2-naphthalenesulfonic acid; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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ardized with Ca^{2+} Titrisol solutions in the above-mentioned buffer. In the latter experiments, the total Ca^{2+} concentration was controlled after each increment. The statistical distribution of the four CaM·Ca_n species as a function of free [Ca^{2+}] or of the mean saturation was calculated as previously described (Cox et al., 1981).

In all the experiments dealing with conformational changes as a function of free [Ca^{2+}], free Ca^{2+} concentrations were controlled by means of EGTA (0.2 mM or 1.0 mM) or HEDTA (1.0 mM). The choice of the chelator was determined by estimation of its buffering capacity as explained by Durham (1983). The total Ca^{2+} concentration was controlled in each sample after the optical measurements. Free Ca^{2+} concentrations were calculated as previously detailed (Burger et al., 1982), taking into account the Ca^{2+}-buffering capacity of CaM.

Far-UV circular dichroism (CD)[1] measurements at varying concentrations of free Ca^{2+} were performed in a 0.1-cm quartz cuvette and recorded at 222 nm on a Jasco J-20A spectropolarimeter with constant N_2 flushing. The appearance of hydrophobicity as a function of free [Ca^{2+}] was followed by changes of TNS fluorescence (McClure & Edelman, 1966) upon its binding to CaM; a Baird Atomic FC 100 spectrofluorometer was used with the excitation wavelength set at 328 nm, where fluorescence changes were maximal, and optimum emission at 446 nm.

Stoichiometric titration experiments of CaM with Ca^{2+} were carried out in the absence of any Ca^{2+} chelator and starting with metal-free CaM obtained by means of the procedure of Haiech et al. (1981). The initial Ca contamination was less than 0.03 Ca/mol of CaM. To a 750 μM solution of CaM were added increasing amounts of Ca^{2+} in a negligibly small volume. After each increment, the ellipticity was measured at 222 nm in a 0.01-cm quartz cuvette or, in separate experiments, at 279 nm in a 1-cm cuvette. After each measurement, an aliquot of solution was withdrawn for the determination of total Ca^{2+}. In these particular experiments the amount of free [Ca^{2+}] was negligible as compared to the amount bound to the protein, and the CD signals are directly comparable to the mean saturation.

Results

Calcium Binding to Calmodulin. In spite of the numerous Ca-binding studies on CaM reported to date, there were two reasons for reevaluating these binding parameters: (1) given the strong influence of the ionic strength on interactions between Ca^{2+} and this very acidic protein (Haiech et al., 1981), comparison of Ca binding to CaM and its conformational changes is not valid unless the eperimental conditions are identical or at least very similar; (2) the first and fourth binding constants were poorly evaluated in our previous Ca-binding study [Figure 1 of Cox et al. (1981)], as exclusively equilibrium dialyses were used and too few measurements were carried out in the range of these constants. In the present study, the binding of the fourth Ca^{2+} was followed with a Ca^{2+}-selective electrode, and multiple experiments were carried out in the two extreme zones of the curve. The experimental data (Figure 1) were analyzed with a nonlinear least-squares curve fitting procedure using a MINUIT routine (Wnuk et al., 1979) according to two different models: (1) The first model is described by the Langmuir equation assuming identical and independent sites. In our particular case, the equation is as follows:

$$\nu = \frac{4K'[Ca^{2+}]}{1 + K'[Ca^{2+}]} \quad (1)$$

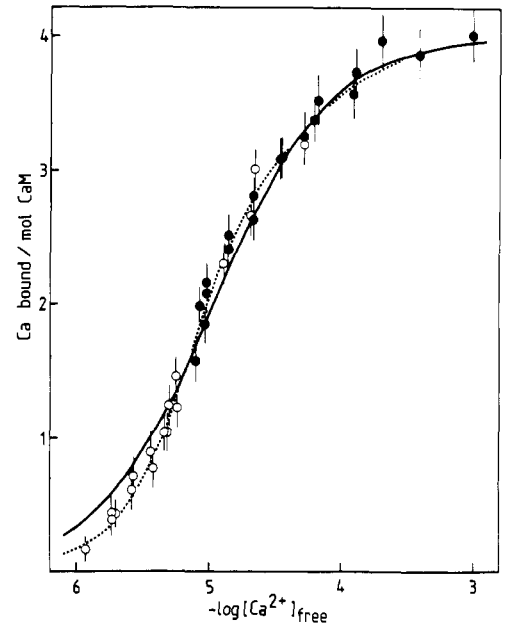

FIGURE 1: Ca binding to calmodulin at pH 7.0 and ionic strength equal to 150 mM. (○) From equilibrium dialyses; (●) from potentiometric titration with the Ca^{2+}-sensitive electrode. The solid line represents the theoretical curve obtained with the constants evaluated by an iterative computer program assuming four identical and independent sites (Langmuir equation); the dotted line is the theoretical curve assuming four interacting sites (Adair equation). The vertical bars represent the maximal possible errors on each point as evaluated from four different studies on Ca-binding proteins performed in our laboratory [see Wnuk et al. (1982)]; these error values were used for the calculation of χ^2 in the text.

where ν is the amount of Ca^{2+} bound per mole of protein and K' is the intrinsic binding constant of the four sites, which is identical with the value of the site binding constants in this model; (2) The second model of different *or* interacting sites is described by the Adair equation:

$$\nu = (K_1[Ca^{2+}] + 2K_1K_2[Ca^{2+}]^2 + \ldots + 4K_1K_2\ldots K_4[Ca^{2+}]^4)/(1 + K_1[Ca^{2+}] + K_1K_2[Ca^{2+}]^2 + \ldots + K_1K_2\ldots K_4[Ca^{2+}]^4) \quad (2)$$

where K_1, K_2, etc., are the stoichiometric (or macroscopic) binding constants of the first, second, etc., site. It is obvious that the second model will offer a better fit to the experimental data, since four instead of one parameters can be adjusted, thus providing more flexibility.

This is indeed shown in Figure 1, where the solid line represents the best-fitting theoretical curve using eq 1 with $K' = 9.31 \times 10^4$ M^{-1} and the broken line the best fit using eq 2 with the following stoichiometric binding constants: $K_1 = 1.16 \times 10^5$ M^{-1}, $K_2 = 2.65 \times 10^5$ M^{-1}, $K_3 = 8.33 \times 10^4$ M^{-1}, and $K_4 = 1.91 \times 10^4$ M^{-1}. The quality of fitting of the 36 experimental points of Figure 1 to either eq 1 or eq 2 was tested by means of a statistical analysis. By use of the experimental error values shown in Figure 1, the χ^2 value is equal to 42.5 for the model with four identical and independent sites and 16.0 for the model with four interacting sites. Both these values are lower than those for the 95% confidence interval, i.e., 49.8 for the first model with 35 deg of freedom and 46.2 for the second model with 32 deg of freedom. It can therefore be concluded that on statistical grounds neither of the two models can be disregarded. If we assume that the experimental error is half of that indicated by the error bars in Figure 1, the χ^2 values would be 170 and 64, respectively, indicating that the experimental data differ significantly from the theoretically determined ones in the case of the simplest model but not in the case of the model with four interacting sites.

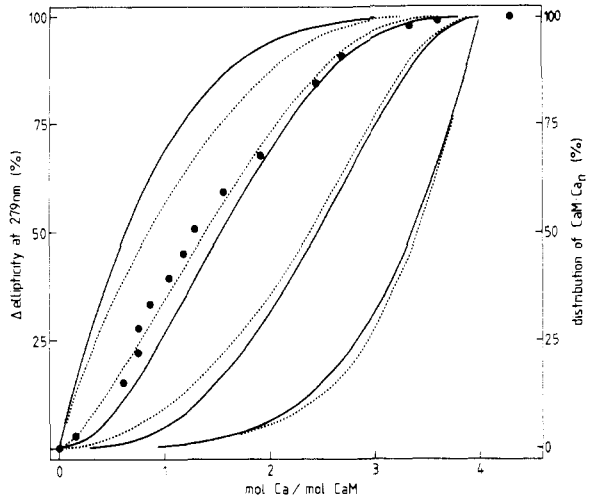

FIGURE 2: Near-UV circular dichroism as a function of the mean saturation in a stoichiometric titration of 750 μM metal-free CaM with increments of Ca^{2+}; the total [Ca^{2+}] was determined after each addition. The signals are taken as 0% and 100% at the start (less than 0.03 Ca^{2+} per CaM) and end (6 Ca^{2+} per CaM) of the experiment. The solid lines represent the appearance of the CaM·Ca$_n$ species as a function of the mean saturation by using the model of four identical and independent sites; the dotted lines refer to the model of four interacting sites (see Figure 1).

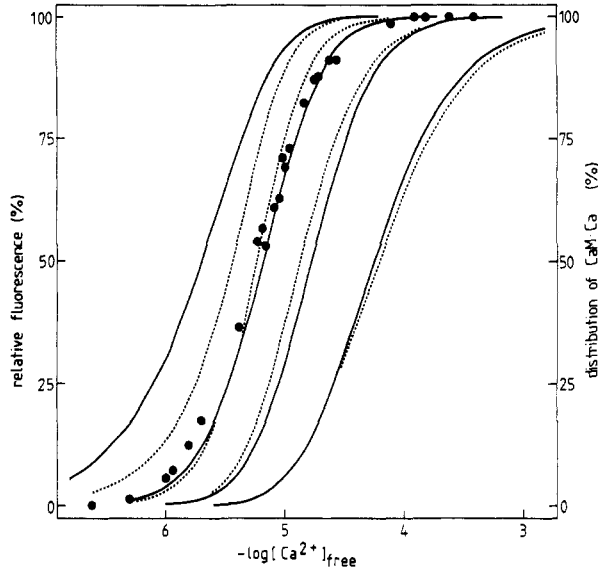

FIGURE 3: TNS fluorescence titration of calmodulin as a function of free [Ca^{2+}]. 1.5 μM TNS and 60 μM CaM were incubated for 2 h with variable concentrations of free Ca^{2+} (in the presence of Ca chelator) before the measurements. The signal is taken at 0% in 1 mM EGTA and 100% in 2.5 mM $CaCl_2$. The solid lines represent the appearance of the CaM·Ca$_n$ species as a function of free [Ca^{2+}] by using the model of four independent sites; the dotted lines refer to the model of interacting sites (see Figure 1).

In the section dealing with the conformational changes as studied by circular dichroism and interaction with hydrophobic probes, we have compared these changes with the statistical distribution curves of the CaM·Ca$_n$ species calculated with the parameters resulting from both models.

Conformational Transition of Tyr Monitored during Stoichiometric Titration of CaM with Ca^{2+}. CaM contains two Tyr residues at positions 99 and 139, which constitute sensitive indicators of conformational changes in the protein. The intrinsic near-UV fluorescence signal is enhanced 2.3-fold and the negative ellipticity at 279 nm 2-fold upon binding of Ca^{2+} to CaM. We have chosen near-UV ellipticity rather than fluorescence measurements on account of the multiple side effects occurring in the latter due to the fact that the emission and excitation wavelengths are too close to each other and the fluorescence yield of Tyr is low. The near-UV circular dichroism was monitored starting with a 750 μM Ca-free CaM solution and then after increments of Ca^{2+}. Under these conditions and up to a ratio of 3.5 Ca^{2+} per CaM, all added Ca^{2+} can be considered as being bound to the protein. Figure 2 shows the Tyr CD signal together with the distribution of the CaM:Ca$_n$ species as a function of the mean saturation. The experimental data coincide with the curve describing the appearance of the CaM species containing at least two Ca^{2+} in any of its binding domains. Interestingly, monitoring the ellipticity of Tyr and of Phe during the stoichiometric titrations of ca. 300 μM CaM by Ca^{2+}, Crouch & Klee (1980) also produced curves which are nearly superimposable on those of the appearance of CaM·Ca$_{n\geq 2}$ of Figure 2. The sigmoidicity of the spectral change in the data of Crouch and Klee and in our Figure 2, as well as the steepness of the curve, is not a priori an indication of positive cooperativity in Ca binding since the theoretical curve of the appearance of CaM·Ca$_{n\geq 2}$ is even more sigmoidal and steeper in the model assuming identical and noninteracting sites.

TNS Fluorescence Titration of CaM. In order to investigate the appearance of hydrophobicity in CaM as a function of free [Ca^{2+}] with the fluorescent probe TNS, the experiments must be carried out under conditions where the concentration of TNS–CaM complex is negligible as compared to total CaM and does not perturb the equilibrium between the different CaM·Ca$_n$ species. At saturating concentration of Ca^{2+} (2.5 mM), TNS binds to CaM with an apparent K_{diss} of 34 μM (not shown). Since the fluorescence measurements detailed below are carried out with 1.5 μM TNS and 60 μM CaM, the maximal [CaM–TNS] represents less than 1% of total [CaM]. TNS fluorescence at low free [Ca^{2+}] (1 mM EGTA) represents 5% of the signal obtained at saturating Ca^{2+} concentration. As shown in Figure 3, the TNS fluorescence titration curve of CaM coincides with that showing the appearance of the CaM species containing at least two Ca^{2+}. Statistically, the coincidence is equally good whether the model with identical and independent sites or the one with interacting sites is considered. Hence, the hydrophobicity induced by the binding of two Ca^{2+} ions to CaM is sufficient for the interaction of TNS with CaM. The same results are obtained in the presence of 5.0 mM Mg^{2+}, which indicates that Mg^{2+} affects neither the binding of Ca^{2+} to CaM (Cox et al., 1981; Potter et al., 1981) nor the conformational changes induced by this binding.

Circular Dichroism Titration of Ca Binding to CaM. CaM saturated with Ca^{2+} exhibits 16% more negative ellipticity at 222 nm than Ca-depleted CaM, in agreement with previous reports (Dedman et al., 1977; Klee, 1977; Walsh et al., 1978). The Ca^{2+}-dependent titration curve (Figure 4A) shows a midpoint at 5.75 μM Ca^{2+} and correlates neither with the appearance of any of the CaM·Ca$_n$ species nor with the mean Ca^{2+} saturation curve. Apparently each species contributes to the signal, but to a different extent. Therefore, we assume that the Ca^{2+}-dependent CD change at any free [Ca^{2+}], Δs, is the sum of the particular contributions of each CaM·Ca$_n$ species to the signal and can be described by the following equation:

$$\Delta' s = \Delta S_1(\text{CaM·Ca}_1) + \Delta S_2(\text{CaM·Ca}_2) + \Delta S_3(\text{CaM·Ca}_3) + \Delta S_4(\text{CaM·Ca}_4) \quad (3)$$

where the CaM·Ca$_n$ species are expressed as a percentage of total CaM; $\Delta S_i = S_i - S_0$ (i varying from 1 to 4), with S_0 and S_i being the CD intensity coefficients of the species bearing

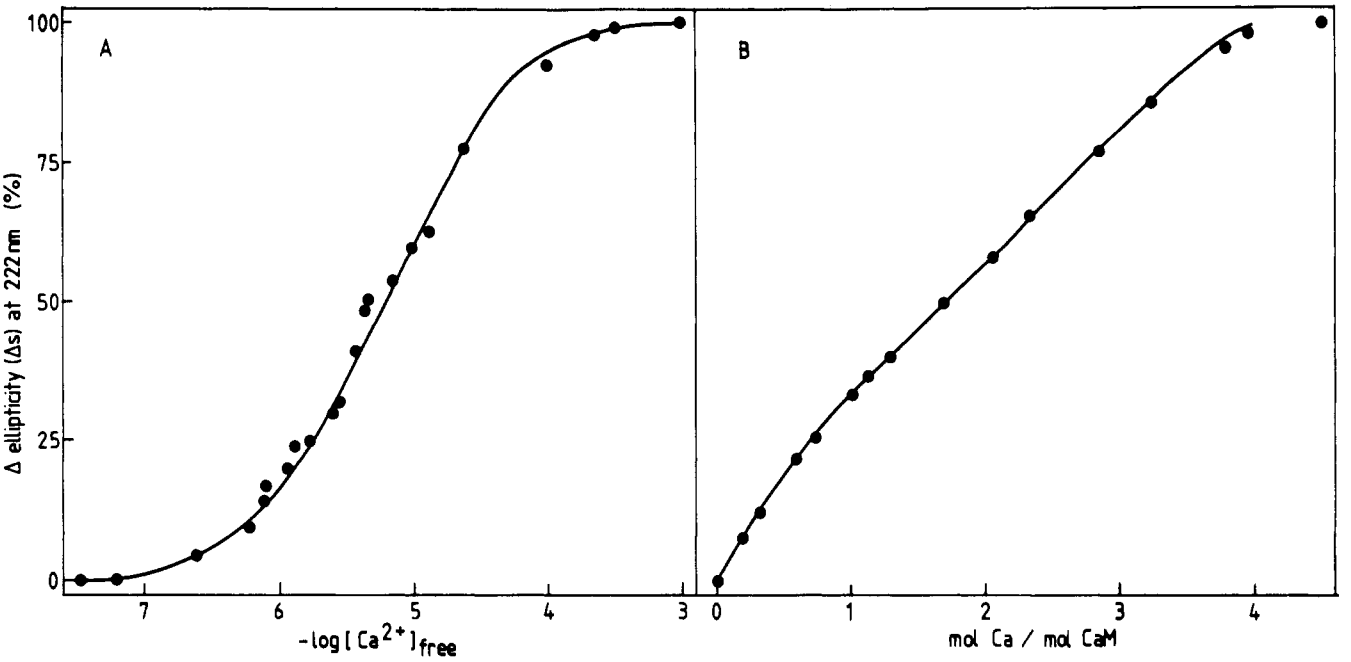

FIGURE 4: Far-UV circular dichroism of calmodulin. (A) Titration as a function of free [Ca^{2+}]. Experiments were carried out with samples containing 30 μM CaM in Ca chelator containing solutions. Data are expressed in percentage of signal change, taken as 0% in the presence of 1 mM EGTA and 100% in 2.5 mM free [Ca^{2+}]. The curve is calculated from eq 3 with $\Delta S_1 = 0.55$, $\Delta S_2 = 0.50$, $\Delta S_3 = 0.90$, and $\Delta S_4 = 1.00$. (B) Titration as a function of the mean saturation. The experimental procedure is similar to that of Figure 2. The curve is calculated from eq 3 with $\Delta S_1 = 0.45$, $\Delta S_2 = 0.45$, $\Delta S_3 = 0.9$, and $\Delta S_4 = 1.00$.

Table I: Stoichiometric (Macroscopic) Association Constants of Calmodulin (M^{-1})

experimental conditions					
60 mM TES, pH 7.0, 150 mM NaCl:[a]	model 1	3.73×10^5	1.40×10^5	6.21×10^4	2.33×10^4
	model 2	1.16×10^5	2.65×10^5	8.33×10^4	1.91×10^4
10 mM HEPES, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$[b]		1.9×10^5	2.1×10^5	4.0×10^4	2.6×10^4
10 mM MOPS, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$[c]		1.9×10^5	2.8×10^5	4.5×10^4	1.2×10^4
10 mM Tris, pH 7.55, 200 mM KCl[d]		2.7×10^5	3.0×10^5	4.5×10^4	1.7×10^3
10 mM Tris, pH 7.0, 49 mM $(NH_4)_2SO_4$[e]		5.0×10^5	1.7×10^5	5.9×10^4	5.0×10^3
10 mM imidazole, pH 7.0, 100 mM NaCl[f]		5.3×10^5	2.0×10^5	8.9×10^4	3.3×10^4
1 mM HEPES, pH 6.5, 100 mM KCl, 0.1 mM EGTA[g]		1.7×10^6	6.7×10^5	2.9×10^5	1.1×10^5

[a] This study. Model 1 corresponds to identical and independent sites and model 2 to interacting sites (see Results). [b] Crouch & Klee (1980). [c] Keller et al. (1982). [d] Haiech et al. (1981). [e] Cox et al. (1981). [f] Jarrett & Kyte (1979). [g] Dedman et al. (1977).

zero or one to four Ca^{2+}, respectively. Since the total ellipticity change can be attributed to the reaction CaM + $4Ca^{2+} \rightarrow$ CaM·Ca_4, the coefficient of the CaM·Ca_4 species, ΔS_4, is equal to 1. The iterative computer analysis of the experimental data of Figure 4A according to eq 3 using the species distribution curves for the model of four identical and noninteracting sites yields the following values: $\Delta S_1 = 0.55$, $\Delta S_2 = 0.50$, and $\Delta S_3 = 0.90$. These results suggest that binding of the first Ca^{2+} increases the negative ellipticity at 222 nm to half of the maximal value. Upon binding of the second Ca^{2+}, *no* ellipticity increase occurs. When the third Ca^{2+} binds to CaM, the ellipticity reaches its maximal value and does not change very much upon binding of the fourth Ca^{2+}.

The variation of ΔS was also monitored as a function of the mean saturation in a stoichiometric titration experiment under conditions very close to those of the near-UV ellipticity measurements (Figure 4B). The iterative computer evaluation of the coefficients from the experimental data of Figure 4B yielded the following values: $\Delta S_1 = 0.45$, $\Delta S_2 = 0.45$, and $\Delta S_3 = 0.90$, i.e., very close to those evaluated in Figure 4A in a completely different experimental setup. This confirms that the α-helical increase occurs only when the first Ca^{2+} and third Ca^{2+} bind to the protein.

Discussion

Since the analysis of the conformational changes presented in this study is based on the constants obtained from the direct Ca-binding experiments and since discrepancies about the latter are present in literature, we compared our binding parameters with those reported to date, in order to assess the accuracy of our conclusions. Table I summarizes the binding studies which have been carried out at comparable ionic strength. All data have been expressed in terms of macroscopic constants. Except for the earliest study by Dedman et al. (1977) and for important variations in the value of the fourth constant, there is good agreement between the different binding studies. The small variations may stem from differences in pH and in the iterative procedure used for the determination of the binding constants. Indeed, the slight cooperativity in the binding of the first two Ca^{2+}, documented in three studies (Table I), seems to result more from the data treatment than from real allostery as meant by Monod et al. (1965). The slightly better fit obtained with the Adair equation as compared to that resulting from the Langmuir equation does *not* compensate for the more rigorous constraint of the latter model. In the instance prevailing in this study and also in those of Crouch & Klee (1980), Keller et al. (1982), and Haiech et al. (1981), the simplest model of identical and independent sites is more appropriate. The conformational data presented in this report, especially the ellipticity at 222 nm as a function of free [Ca^{2+}] and of the mean saturation, also favor the model with independent sites rather than the allosteric model; the

analysis according to the latter model would yield an unlikely high ΔS_1 value of 1.8–2.0, i.e., a higher helical content than can exist in fully saturated calmodulin.

Kilhoffer et al. (1983) and Wallace et al. (1982) have claimed from structural studies that Ca binding to CaM is sequential and ordered; i.e., the first Ca^{2+} would always bind to one well-defined Ca-binding domain in each of the protein molecules; when more Ca^{2+} is provided, a second well-defined domain would pick up Ca^{2+}, etc. However, our data indicate that CaM has four identical (model 1) or nearly identical (model 2) intrinsic binding constants. Four other laboratories reached very similar conclusions. Therefore, according to the rules governing the relationship between the intrinsic and site binding constants (Klotz & Hunston, 1979), it is not conceivable that the Ca-binding domains of CaM are filled in an ordered way.

By use of ^{43}Ca NMR, a convenient probe for the direct evaluation of the site binding constants (Andersson et al., 1982a,b) claimed that calmodulin contains two "low-affinity" sites with identical site binding constants of 9×10^4 M^{-1} and two "high-affinity" sites with site binding constants about 40-fold higher. The same group (Forsén et al., 1980; Andersson et al., 1983) reported that ^{113}Cd NMR confirms the existence of two high-affinity and two low-affinity sites and that positive cooperativity exists between the high-affinity sites. Moreover, ^{113}Cd NMR on proteolytic fragments of calmodulin allowed Andersson et al. (1983) to propose domains III and IV as the high-affinity Ca-binding sites in intact calmodulin. Unfortunately, ^{43}Ca measurements were done in distilled water and those of ^{113}Cd in a 10 mM Tris buffer. At such low ionic strength, an important electrostatic force certainly contributes to the interaction between Ca^{2+} and calmodulin, and the estimated binding constants do not correspond to the equilibrium binding constants (Tanford, 1961). For instance, in the presence of 10 mM Tris-HCl, pH 7.55, Haiech et al. (1981) found for the four stoichiometric association constants values as high as 7.7×10^6, 7.1×10^6, 1.6×10^6, and 7.7×10^5 M^{-1}, respectively. These values are apparent constants *not* directly related to the on- and off-rate constants. Therefore, the conclusions of the ^{43}Ca NMR studies should be restricted to water and not used in experiments carried out at physiological ionic strength. For the same reason, the ^{113}Cd NMR data are inadequate at physiological salt concentrations; furthermore, it is doubtful whether Cd binding to calmodulin follows a pattern similar to that of Ca binding, since metal ion competition shows that the ratio K_{Ca}/K_{Cd} is quite different for the two strong cation binding sites (Forsén et al., 1980). It is presently impossible to reconcile the divalent metal NMR data, which lead to a coherent and consistent picture of the interaction of calmodulin with metal ions and hydrophobic drugs in the absence of counterions, and the data obtained by direct equilibrium binding studies at physiological ionic strength, which are reproducible in different laboratories (Table I) and consistent with the activation mechanisms of different enzymes [for review, see Cox et al. (1984)]. This suggests only that the Ca-binding pattern of calmodulin, known to be supported by overall structural changes in the protein, is qualitatively different whether or not the charge groups are adequately shielded by counterions. In this respect, it is significant that the two ^{113}Cd signals are considerably broader in the presence of 40 mM TES (Sudmeier et al., 1980) than in water (Andersson et al., 1983), indicating that the two strong binding sites have a much higher affinity in water than in the presence of salt.

This study shows that structural changes in CaM, in contrast to Ca binding, are sequential and ordered because they follow the appearance of one $CaM{\cdot}Ca_n$ species rather than the mean saturation. Binding of the second Ca^{2+} changes the environment of the Tyr and Phe residues and confers simultaneously hydrophobicity to part of the surface of CaM, sufficient for the interaction of the protein with the hydrophobic probe TNS; however, it does not allow the activator to interact with target enzymes (Malnoë et al., 1982; Burger et al., 1983). Therefore, one may wonder which conformational events, other than hydrophobicity, are required for the CaM–target interaction. The studies of Blumenthal & Stull (1982) on the thermodynamic parameters in the activation of myosin light chain kinase by CaM have shown that, although hydrophobicity is needed, it is not sufficient for the formation of the CaM–enzyme complex. The latter authors explain their results by means of the two-step model proposed by Ross & Subramanian (1981) for protein–protein interactions: the first step occurs when two hydrophobic domains get close to one another and yield a hydrophobically associated complex which is not stable; the second step involves short-range interactions such as hydrogen bonds, salt linkages, and van der Waals interactions, which become possible due to the juxtaposition of appropriate amino acid residues. According to this model, the presence of a hydrophobic plate at the surface of CaM is the first requirement for the interaction. Since target enzymes do not interact with the $CaM{\cdot}Ca_2$ species, we suggest that in the latter species the hydrophobic region is accessible only to small probes such as TNS but not yet to the much larger physiological targets. This region becomes accessible upon the binding of the third Ca^{2+} and allows the formation of the active complex CaM–target enzyme. Our results indicate that a gain in ellipticity to near maximal value occurs in the step $CaM{\cdot}Ca_2 \rightarrow CaM{\cdot}Ca_3$, which is instrumental is transforming CaM into a biologically active species. Interestingly, recent studies of Gariépy & Hodges (1983) and Reid et al. (1983) on troponin C have shown that the main structural event, which occurs in a 90–123-residue fragment (corresponding to fragment 80–113 of the CaM sequence) upon interaction with phenothiazines or with Ca^{2+}, is a gain in the α-helical content of the N-terminal region of this peptide (residues 83–92 in the sequence of CaM). Furthermore, these authors showed that the drug binding site is restricted to this N-terminal helix. Hence, the gain in ellipticity which we observed upon binding of the third Ca^{2+} might well be attributed to an increase in the α-helical content of the 83–92 sequence of CaM. Formation of this highly amphiphatic and surface-seeking (Cox et al., 1984) α-helix may expose and stabilize the hydrophobic region formed in CaM upon binding of the second Ca^{2+} ion. Subsequently, according to the model of Ross & Subramanian (1980), this hydrophobic region interacts with the one on the surface of the target enzymes (Gopalakrishna & Anderson, 1983), thus allowing short-range interactions between CaM and its target.

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