Positive Cooperative Binding of Calcium to Bovine Brain Calmodulin†

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ABSTRACT: Equilibrium dialysis measurements of the binding of Ca^{2+} to calmodulin have confirmed the existence of four high affinity Ca^{2+}-binding sites (K_d between 3×10^{-6} and 2×10^{-5} M). In the presence of 3 mM Mg^{2+}, the dissociation constants for Ca^{2+} are increased two- to fourfold (K_d between 5×10^{-6} and 4×10^{-5} M). Positive cooperativity of Ca^{2+} binding was observed at low Ca^{2+} concentrations with Hill coefficients of 1.33 and 1.22 in the absence and presence of 3 mM Mg^{2+}, respectively. The positive cooperativity is compatible with the steepness of the Ca^{2+} dependence of the conformational transition associated with the binding of 2 mol of Ca^{2+}/mol of calmodulin. This conformational change, which affects the environment of the aromatic residues of calmodulin as measured by UV absorption and near-UV circular dichroism spectroscopy, is not the result of a monomer–dimer equilibrium mediated by Ca^{2+}. Binding of Ca^{2+} to calmodulin is believed to occur by a sequential mechanism generating at least four different conformers of the protein in its free and liganded states. Even though the major conformational change is almost complete upon binding of 2 mol of Ca^{2+}/mol of calmodulin, the activation of cyclic nucleotide phosphodiesterase measured in the presence of limiting concentrations of calmodulin suggests that a calmodulin Ca_{3-4}^{2+} complex is required for interaction of calmodulin with the enzyme. As expected, on the basis of the strong affinity of the enzyme for the calmodulin·Ca^{2+} complex ($K_d = 1–3 \times 10^{-9}$ M), the Ca^{2+} dependence of phosphodiesterase activation is highly cooperative and leads to a sharp threshold of Ca^{2+} concentration for control of enzyme activity.

Calmodulin, a heat-stable Ca^{2+}-binding protein, was discovered as an activator of cyclic nucleotide phosphodiesterase (Cheung, 1970), which is specific for the Ca^{2+}-dependent form of the enzyme (Kakiuchi & Yamasaki, 1970). In contrast to similar Ca^{2+}-binding proteins such as troponin C, parvalbumin, and intestinal Ca^{2+}-binding protein, which are tissue specific and are believed to have evolved for specific functions (Goodman et al., 1979; Kretsinger, 1980), calmodulin is a ubiquitous protein found in all tissues examined thus far. In the past few years the list of its functions has been increasing rapidly (for recent reviews of the literature see Wolff & Brostrom, 1979; Wang & Waisman, 1979; Cheung, 1980). Calmodulin is now believed to regulate the modulation of cellular processes by Ca^{2+} when the ion functions as a second messenger as postulated by Rasmussen et al. (1972). The Ca^{2+}-binding properties of the purified protein were first reported by Teo & Wang (1973) and have been examined by others (Lin et al., 1974; Watterson et al., 1976; Klee, 1977a; Wolff et al., 1977; Dedman et al., 1977; Jarrett & Kyte, 1979). Although all studies indicate the presence of four Ca^{2+} binding sites, the number of classes and the affinities of the sites vary under the different published experimental conditions. Since calmodulin is a multifunctional protein responsible for activating different enzyme systems, it is important to understand its behavior in the presence and absence of Ca^{2+}. Therefore, a detailed physical study of this protein was undertaken.

Materials and Methods

Aristar KCl and KOH, and Analar $MgCl_2$ and $CaCl_2$ were obtained from Gallard-Schlesinger Chemical Corp. Hepes[1] and EGTA were products of Sigma. $^{45}CaCl_2$ (19.4 Ci/g) was purchased from New England Nuclear. Other reagents and bovine brain calmodulin were as previously described (Klee, 1977a). Bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase (70–100 units/mg) was purified and quantitated as previously described (Klee et al., 1979). Calmodulin concentrations were measured spectrophotometrically at 277 and 259 nm with a Cary Model 118 spectrophotometer using an $\epsilon_{277nm} = 3300$ (corrected when needed for the hypochromic effect due to the presence of Ca^{2+} (Klee, 1977a) and an $\epsilon_{259nm} = 2550$.

Removal of Contaminating Metals. The buffer solution used for these studies was 0.01 M Hepes–KOH buffer, pH 7.5, containing 0.1 M KCl and freed of Ca^{2+} as described below (buffer A). Untreated solutions of 0.1 M KCl routinely contained 4×10^{-7} M or less Ca^{2+}. Various batches of Hepes were contaminated by Ca^{2+} to different extents (2×10^{-7} to 2×10^{-5} M in 0.01 M Hepes solutions). The buffer solutions were routinely treated with Chelex (Bio-Rad Laboratories). A stock solution (1 L) of 0.02 M Hepes, pH 7.5, containing 0.2 M KCl was passed through a column containing 60 g of Chelex in the K^+ form equilibrated with the same buffer. Alternatively, concentrated stock solutions of 0.05 M Hepes, pH 7.8, and of 1.0 M KCl were treated with Chelex batchwise. Ca^{2+}-free solutions (less than 2×10^{-7} M Ca^{2+}) were stored in polyethylene containers that had been boiled for 5 min and rinsed several times in deionized water (less than 10^{-7} M Ca^{2+}). Dialysis tubing (F-27 (Arthur Thomas)) was boiled once in 5% $NaHCO_3$ and twice in deionized water for 5 min each and then rinsed twice with deionized water. Cuvettes for spectrophotometric measurements were soaked for 4–6 h in a 1:1 mixture of ethanol and 1 M HCl, rinsed with deionized water, and rinsed 4 times with buffer A.

Metal Determinations. Determination of divalent cations was performed with a Perkin-Elmer Model 603 atomic absorption spectrophotometer using a HG-2100 graphite furnace and high-density graphite rods (some graphite rods exhibited a large matrix effect and a low sensitivity in this buffer matrix and were not used). Standard calibration curves were fitted to either $Y = A(e^{-BX} - 1)$ above 1000 pg of Ca^{2+} per sample

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; CaM, calmodulin; cAMP, adenosine 3′,5′-monophosphate; CD, circular dichroism.

or $Y = AX + B$ below 1000 pg of Ca^{2+} per sample, where X is the absorbance reading, Y is picograms of Ca^{2+} present, and A and B are constants. The instrument response was tested before and after each experiment to ensure that there was no drift in absorbance arising from rod aging. Thermal settings were: dry at 105–110 °C for 30–40 s, char at 1100 °C for 20–30 s, and atomize at 2700 °C for 8 s. No significant matrix effect was observed between 0 and 800 pg of Ca^{2+} in buffer A with the rods used. Samples to be analyzed were diluted in buffer A prior to the determination. When 0.1 mM EGTA was present in the solutions, the samples were first diluted 100-fold with deionized water, and determinations and control curves were done on samples diluted with water.

Equilibrium Dialysis Experiments. Dialysis bags each contained 0.9 mL of 7×10^{-6} M to 1.5×10^{-5} M calmodulin in buffer A or 0.9 mL of buffer A (control) and were dialyzed at 0–4 °C against 20 vol of buffer A containing 80 mL (bed volume)/L of Chelex equilibrated in buffer A. The dialysis buffer and Chelex were changed every 12 h (once or twice) until the free Ca^{2+} concentration was less than 4×10^{-7} M. Each duplicate set of bags, one containing buffer A and the other calmodulin in buffer A, was transferred to polyethylene tubes, each containing 40 mL of buffer A made to 4×10^{-7} to 2.5×10^{-4} M Ca^{2+} and 0.4 to 1 μCi of $^{45}CaCl_2$. To ensure low Ca^{2+} concentrations, one or two experimental tubes contained only $^{45}CaCl_2$ and 1 to 5 mL of Chelex (as above) was used as a Ca^{2+} sink.[2] Dialysis was then carried out for 18–24 h at 25 °C (at 26 °C equilibrium of Ca^{2+} binding was reached after 6 h). Aliquots of 100 μL were then removed from the dialysates, the control bags, and the calmodulin samples. Radioactivity was measured in duplicate aliquots with a Beckman LS 355 scintillation counter, using aquasol as scintillation fluid. The specific activity of $^{45}Ca^{2+}$ was routinely determined in each dialysate by measuring the Ca^{2+} concentrations as described above. The specific activity of $^{45}Ca^{2+}$ in the sample or control bags was also checked occasionally and was not significantly different from that of the $^{45}Ca^{2+}$ in the dialysates. The identity of Ca^{2+} concentration monitored by radioactivity or direct Ca^{2+} determinations in the control bags and the dialysates was evidence that equilibrium had been reached and that no significant amount of Ca^{2+} was leaching from the dialysis bags. Protein concentration in the dialyzed samples was measured at the end of the experiment. When $MgCl_2$ was included in the dialysis buffer, Chelex was omitted from the dialysate tubes.

Circular Dichroism and UV-Difference Spectroscopic Analyses. Circular dichroic and UV-difference spectra were measured as previously described (Klee, 1977a) with the following changes. The cell light path was 1 cm, and the sensitivity was 0.02°/100% of the chart for circular dichroic measurements. The mean residue weight based on the sequence data of Watterson et al. (1980) was 113.2. The sample solutions (4–5 mg of calmodulin/mL) were prepared as described above for equilibrium dialysis experiments. The final Ca^{2+} concentration in the dialysis buffer was 4×10^{-6} M. Another set of determinations was done in the presence of 10^{-6} M EDTA in order to obtain a low free Ca^{2+} concentration. In the latter case the protein solutions were first dialyzed twice against 100 volumes of buffer A containing 1 mM EGTA and then twice against buffer A containing 10^{-6} M EDTA (the total Ca^{2+} concentration measured in the dialysis buffer was 8×10^{-7} M). Spectra were recorded after successive additions of 3–30-μL aliquots of a 75 mM $CaCl_2$ solution. The measurements were corrected for dilutions. Ca^{2+} concentrations were determined by atomic absorption on aliquots removed from the cuvette after each addition.

Ultracentrifugal Analyses. Sedimentation velocity and high-speed sedimentation equilibrium experiments (Yphantis, 1964) were performed using a Beckman Spinco Model E ultracentrifuge equipped with a photoelectric scanner (Schachman & Edelstein, 1966) and double sector, charcoal-filled epon centerpieces. The samples were scanned at 277 nm. Calmodulin solutions (1.0 mg/mL) were dialyzed against 40 volumes of buffer A containing 0.5 mM EDTA or 0.5 mM $CaCl_2$. The samples (400 μL) were spun at 56 000 rpm for 4 h at 25 °C to simultaneously deplete the meniscus, allow rapid equilibrium, and collect sedimentation velocity data. The speed was then lowered at 18 000 rpm and scans were taken at 12-h intervals. No time-dependent change in the concentration pattern along the cell was detected after 12 h, which was taken to indicate equilibrium. The buoyancy term $(\partial\rho/\partial C_2)_\mu$ (Banks et al., 1976) was determined by a magnetic densimeter (Kupke & Crouch, 1978). Two samples containing approximately 2 mL of 8 mg/mL calmodulin were dialyzed against the buffers used for the ultracentrifugation experiments. Three dilutions of each sample were made to generate a linear relationship between density and protein concentration and values for $(\partial\rho/\partial C_2)_\mu$ were determined from the slopes of the two curves.

Enzymatic Assays. Activation of Ca^{2+}-dependent, cAMP phosphodiesterase was measured as previously described (Klee, 1977a). Total Ca^{2+} concentration was measured by atomic absorption spectroscopy. Free Ca^{2+} concentration was calculated from the computer program of Perrin & Sayce (1967) and the constants of Sillen & Martell (1964) or measured with a Radiometer F-2112 Ca^{2+}-sensitive electrode. The Ca^{2+} concentration was varied by forming an exponential step gradient as follows: a 4-mL solution containing 0.01 M Hepes, pH 7.5, 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM dithioerythritol, 0.1 mM EGTA, 10^{-6} M [^{3}H]cAMP (specific activity $5–6 \times 10^5$ cpm/nmol), 10^{-7} M [^{14}C]AMP (12 000–15 000 cpm/mL), 0.1 mg of bovine serum albumin/mL, 1.4×10^{-9} M calmodulin, and 50 μM Ca^{2+} (measured by atomic absorption) was monitored continuously with the Ca^{2+} electrode. Duplicate aliquots of 200 μL were removed and replaced each time by an equal volume (400 μL) of the same solution containing 300 μM Ca^{2+}. Enzymatic activity was measured on duplicate 200-μL samples after addition of 2 μL (0.016 μg) of phosphodiesterase. Incubation time was 10 min at 30 °C.

Results

Bovine brain calmodulin contains four calcium binding sites as shown in Figure 1. This result is in agreement with previous reports in the literature for calmodulin from several sources (Lin et al., 1974; Watterson et al., 1976; Klee, 1977a; Wolff et al., 1977; Dedman et al., 1977). Unnoticed in earlier reports is the fact that the binding curve at low Ca^{2+} concentrations is indicative of positive cooperativity.[3] The deviation from the normal ligand-binding isotherm is amplified in the Scatchard plot of the data, which exhibits a pronounced downward curvature (see insert of Figure 1). The Hill coefficient obtained from the Scatchard plot was 1.33 (Dahlquist, 1978). The data shown in Figure 1 were similar

[2] When Chelex was present in the dialysis fluid, samples for Ca^{2+} determination were removed after sedimentation of the resin.

[3] The experimental errors in calcium binding determinations include errors in protein determinations (±5%), in dilution of the sample for Ca^{2+} determinations by atomic absorption analysis or by radioactivity (±5%), and in free Ca^{2+} determinations (±4%). The error for each determination never exceeded ±25% and averaged ±8%.

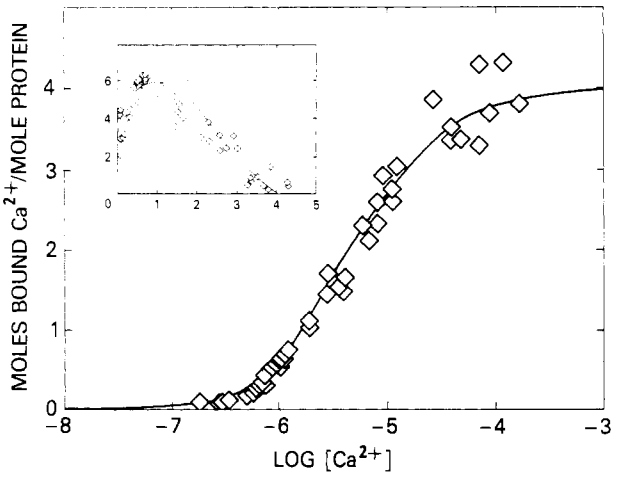

FIGURE 1: Binding of Ca^{2+} to calmodulin. The Ca^{2+} bound to calmodulin measured by equilibrium dialysis is shown as a function of the log of free molar Ca^{2+} concentration. The experimental points represent five different experiments using two different preparations of calmodulin. The line is a representation of eq 1 and 2 using the constants shown in Table I. The insert shows a Scatchard plot of the same data. The line is a transformation of the line in Figure 1 to the Scatchard representation.

Table I: Constants Characterizing the Binding of Ca^{2+} to Calmodulin[a]

	$-Mg^{2+}$	$+Mg^{2+}$ [a]
n_1	1.7 (3×10^5)[b]	3.04 (1.9×10^5)
n_2	4.8 (8.6×10^5)	3.4 (2.1×10^5)
n_3	0.667 (1.2×10^5)	0.667 (0.4×10^5)
n_4	0.25 (0.45×10^5)	0.25 (0.26×10^5)
k [c]	1.8×10^5	0.62×10^5

[a] $[Mg^{2+}]$ was 3 mM. [b] The numbers in parentheses are the macroscopic binding constants K_1, K_2, K_3, and K_4. [c] k is the apparent intrinsic binding constant (see text).

for two different preparations of calmodulin, using different methods to measure Ca^{2+} concentrations as described under Materials and Methods. To eliminate the possibility of a slowly exchangeable Ca^{2+}, which would not be detected by measurement of $^{45}Ca^{2+}$, direct Ca^{2+} determinations by atomic absorption spectroscopy were also carried out on the calmodulin·Ca^{2+} complexes. The shape of the binding curve was identical over a twofold concentration range of protein.[4] The Ca^{2+}-binding curves (Figure 1) are represented by (Adair, 1925)

$$R = \frac{[Ca^{2+}]\, dF/d[Ca^{2+}]}{F} \quad (1)$$

where R represents the number of Ca^{2+} bound per calmodulin and F describes the sum of all states of bound Ca^{2+} with respect to free $[Ca^{2+}]$. The function F is given as follows:

$$F = 1 + K_1[Ca^{2+}] + K_1K_2[Ca^{2+}]^2 + K_1K_2K_3[Ca^{2+}]^3 + K_1K_2K_3K_4[Ca^{2+}]^4 \quad (2)$$

The macroscopic association constants K_i can be represented as $K_i = n_i k$, where k is an intrinsic association constant and the parameters, n, are used to describe different association constants for different sites. For four intrinsically equal independent binding sites, $n_1 = 4$, $n_2 = 6/4$, $n_3 = 4/6$, and $n_4 = 1/4$. Deviation from these values can be used to describe the observed cooperativity. The solid lines in Figure 1 represent the observed binding isotherm with the values of n and k given in Table I. In the presence of 3 mM Mg^{2+}, as reported by Wolff et al. (1977), the affinity of calmodulin for Ca^{2+} is decreased. The apparent intrinsic binding constant is decreased by a factor of 3 (Table I). The larger effect of Mg^{2+} observed by Wolff et al. (1977) may be due to the low ionic strength used in their experiments. It is not clear from our data if calmodulin binds 3 or 4 mol of Ca^{2+}/mol in the presence of Mg^{2+}. The Scatchard plot of the Ca^{2+} binding data in the presence of 3 mM Mg^{2+} was qualitatively similar to that shown in the insert of Figure 1. The cooperative interaction was present but diminished (Hill coefficient, 1.22), and the difference in affinity between the two first Ca^{2+} binding sites to be occupied was less pronounced.

[4] These data are not shown in the manuscript but were available to referees as supplementary figures.

Table II: Physical Characteristics of Calmodulin[a]

	EGTA	$CaCl_2$
mol weight	18 700 ± 200	18 700 ± 300
second virial coeff (L·mol/g²)	$(6.16 \pm 0.22) \times 10^{-6}$	$(3.55 \pm 0.49) \times 10^{-6}$
$(\partial\rho/\partial C_2)_\mu$ [b]	0.284	0.289
V' (mL/g)	0.712	0.707
sedimentation coeff, $s_{20,w}$ (S)	1.83 ± 0.08	1.87 ± 0.08

[a] See Materials and Methods for conditions of study. [b] In 0.1 KCl, 0.01 M Hepes, pH 7.5, 0.5 mM EGTA, or 0.5 mM $CaCl_2$, 25 °C.

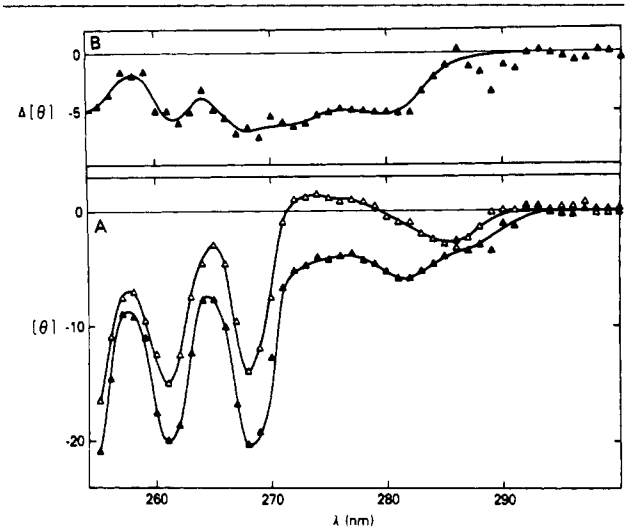

FIGURE 2: Effect of KCl (0.1 M) on the near-UV circular dichroic spectrum of calmodulin. (A) The CD spectrum of calmodulin 3.5–5 mg/mL) was measured in 0.01 M Hepes–KOH buffer, pH 7.5, containing 10^{-6} M EDTA (△) and after addition of KCl to a final concentration of 0.1 M (▲). The data are expressed as mean residue ellipticity [θ]. (B) The difference in ellipticities (± KCl) is shown at the top of the figure.

The data described above imply that binding of the first Ca^{2+} to calmodulin is accompanied by a conformational change that facilitates binding of the second Ca^{2+} atom. Since upon binding Ca^{2+} calmodulin is known to undergo conformational changes that affect the environment of its tyrosyl residues (Wang et al., 1975; Klee, 1977a; Wolff et al., 1977; Dedman et al., 1977; Richman & Klee, 1978; Richman, 1978; Seamon, 1979), we followed the effect of increasing Ca^{2+} concentration on the near-UV absorption and circular dichroic spectra of calmodulin. Under the conditions of these experiments (2–5 mg of protein/mL), the ratio of Ca^{2+} to calmodulin concentrations is representative of the Ca^{2+} bound/mol of protein up to 4 mol/mol. A large CD change between 250 and 290 nm has been reported by Wolff et al. (1977). Under the low ionic strength conditions used by these authors, the tyrosyl residues in the absence of Ca^{2+} showed a positive

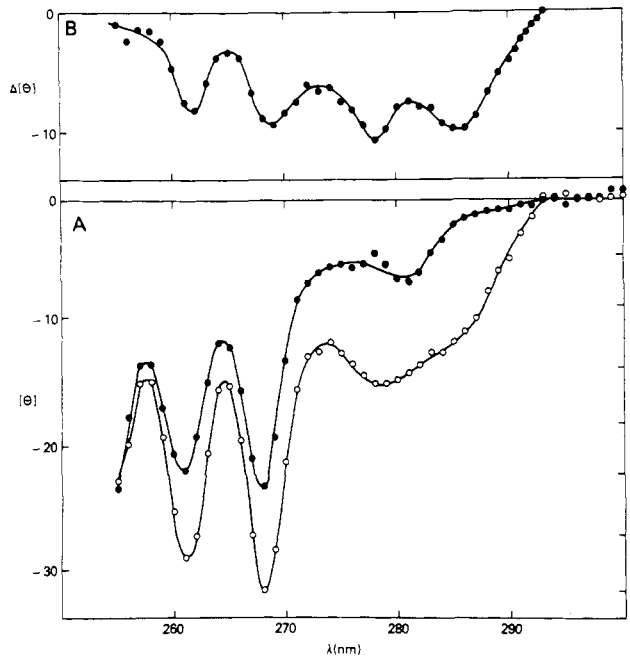

FIGURE 3: Effect of Ca^{2+} on the near-UV circular dichroic spectrum of calmodulin. (A) The CD spectrum of the protein (3.5–5 mg/mL) was measured in 0.01 M Hepes–KOH buffer, pH 7.5, containing 0.1 M KCl and 10^{-6} M EDTA (●) and after addition of $CaCl_2$ to a final concentration of 1.3×10^{-3} M (○). The data are expressed as mean residue ellipticity [θ]. (B) The calculated CD difference spectrum is shown at the top of the figure.

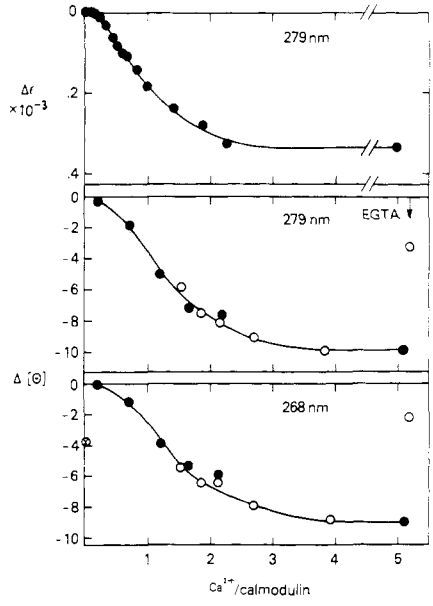

FIGURE 4: Changes in extinction coefficient at 279 nm and ellipticity at 279 and 268 nm as a function of bound Ca^{2+}. The open and closed circles for the CD data represent two different experiments. The closed circles were performed in the presence of 10^{-6} M EDTA and the open circles represent data obtained in the absence of chelator.

ellipticity. As shown in Figure 2A, addition of 0.1 M KCl in the absence of Ca^{2+} resulted in a reproducible negative ellipticity between 280 and 250 nm. The CD difference spectrum induced by 0.1 MKCl (Figure 2B) failed to reveal the fine structure of the tyrosyl and phenylalanyl residues. Binding of calcium to calmodulin in the presence of 0.1 M KCl induces an additional large negative increase of ellipticity (Figure 3A). The CD difference spectrum is shown at the top of the figure and indicates that Ca^{2+} affects the environment of both tyrosyl (279 and 286 nm) and phenylalanyl residues (268 and 261 nm) (Kay et al., 1974).

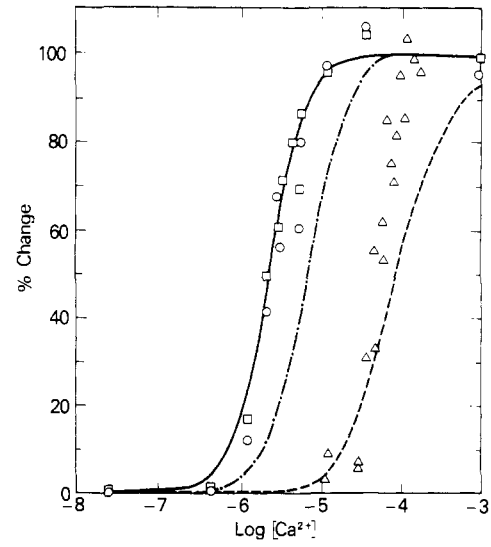

FIGURE 5: Calcium concentration dependence of near-UV circular dichroic changes and ability to stimulate phosphodiesterase. The changes in ellipticity at 268 (□) and 279 nm (○) and activation of phosphodiesterase (△) measured as described under Materials and Methods are expressed in percent of total change. The curves[5] indicate the percent of calmodulin in the fully liganded form in the presence of 3 mM Mg^{2+} (---), the percent of calmodulin with at least two Ca^{2+} bound/mol in the absence of Mg^{2+} (—), and with at least two Ca^{2+} bound/mol in the presence of 3 mM Mg^{2+} (·---·) using eq 1 and 2 with the binding constants shown in Table I. Additional figures were available for the reviewers.

When the change in ellipticity at 279 and 268 nm and the absorption at 279 nm were studied as a function of Ca^{2+} bound/mol of calmodulin, the conformational changes were almost completed upon binding of 2 mol of Ca^{2+}/mol of protein (Figure 4). Previous work had also indicated that the UV absorbance difference spectra and the far-UV circular dichroic spectra were completed upon binding of 2 mol of Ca^{2+}/mol of calmodulin (Klee, 1977a; Dedman et al., 1977; Yagi et al., 1978). In addition, the sigmoidicity of the Ca^{2+}-dependent spectral changes (Figure 4) and the steepness of the curve showing the Ca^{2+} dependence of the spectral changes (Figure 5) are compatible with the positive cooperativity shown directly by the Ca^{2+}-binding data (Figure 1). As shown in Figure 5, 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). An initial transition upon the binding of the first Ca^{2+} might be undetectable by this method because of the relative values of binding constants K_1 and K_2 or because of the lack of sensitivity of the method to the first conformational change. Binding of Ca^{2+} to the third and fourth sites results in no additional changes in the environment of the tyrosyl or phenylalanyl residues that are detectable by near-UV circular dichroic or UV-difference spectroscopy. This result, however,

[5] The probability of calmodulin with at least two sites occupied on the basis of eq 2 is

$$P = \frac{K_1K_2L^2\,(1 + K_3L + K_3K_4L^2)}{1 + K_1L + K_1K_2L^2 + K_1K_2K_3L^3 + K_1K_2K_3K_4L^4}$$

where L = free [Ca^{2+}] and

$$P = \frac{K_1K_2K_3K_4L^4}{1 + K_1L + K_1K_2L^2 + K_1K_2K_3L^3 + K_1K_2K_3K_4L^4}$$

for fully liganded calmodulin.

does not rule out the existence of an additional conformational transition, and, indeed, such a change has been detected by more sensitive techniques (McCubbin et al., 1979; Seamon, 1980).

Since a Ca^{2+}-dependent conformational change has been postulated to be responsible for the ability of calmodulin to activate enzymes under its control, (Liu & Cheung, 1976), we tested whether the binding of 2 mol of Ca^{2+}/mol of calmodulin was sufficient to activate phosphodiesterase. As shown in Figure 5, when activation of phosphodiesterase was measured at a limiting calmodulin concentration (10^{-9} M) and therefore was directly proportional to the concentration of active calmodulin molecules, the binding of 2 mol of Ca^{2+} was insufficient to ensure activation (Figure 5). The conformational change around the aromatic residues is therefore completed prior to conversion to a physiologically active species (Figure 5). Furthermore, when the Ca^{2+}-concentration dependence of phosphodiesterase activation is compared to the relative concentration of fully liganded calmodulin ($CaM \cdot Ca_4^{2+}$), the two curves do not coincide. The activation curve shows a strongly cooperative behavior, which can be explained by the strong interaction of calmodulin with phosphodiesterase. Since Ca^{2+} binding of calmodulin may be different from Ca^{2+} binding to the calmodulin–phosphodiesterase complex, a quantitative interpretation of the noncoincidence of the enzyme activation and Ca^{2+}-binding curve is not yet possible.

The conformational change induced by Ca^{2+} binding to calmodulin could represent a dimer–monomer transition, since this protein has been reported to behave abnormally under gel filtration experiments. Ultracentrifugation studies were carried out both in the presence and absence of Ca^{2+}. The data from three scans taken 12 h apart were used for the molecular weight determinations.[4] These data were fitted to the relationship (Van Holde, 1975; Knott & Reece, 1977)

$$C = C_H \exp[AM(R^2 - R_H^2)/(1 + BC)] \quad (3)$$

where C is the observed calmodulin concentration at the radial distance R, C_H is the calmodulin concentration at a fixed radial distance R_H, M is the molecular weight, B is the second virial coefficient, and A is given by

$$A = \frac{(\partial\rho/\partial C_2)\omega^2}{2RT}$$

where $(\partial\rho/\partial C_2)_\mu$ is the change in solution density with respect to calmodulin concentration under isopotential conditions (Banks et al., 1976), ω is the rotation rate in radiants/second, R is the gas constant, and T is the absolute temperature. Both in the presence and absence of Ca^{2+}, the molecular weight of calmodulin was 18 700. However, the second virial coefficient B was $3.55 \pm 0.49 \times 10^{-6}$ L·mol/g^2 in the presence of Ca^{2+} and $6.16 \pm 0.22 \times 10^{-6}$ L·mol/g^2 in the absence of Ca^{2+}. The most likely reason for this difference in the nonideal term is the binding of Ca^{2+} and the resultant loss of net negative charge on the protein. The results indicate that calmodulin exists mostly as a monomer both in the presence and absence of Ca^{2+}.

Discussion

Several studies of the binding of Ca^{2+} to calmodulin have demonstrated the existence of 4 Ca^{2+}-binding sites/mol of protein (Lin et al., 1974; Watterson et al., 1976; Wolff et al., 1977). These results are consistent with four Ca^{2+}-binding domains postulated by Vanaman et al. (1977) on the basis of amino acid sequence data (Watterson et al., 1980; Dedman et al., 1978; Grand & Perry, 1978) by analogy with the structure of the Ca^{2+}-binding loops of parvalbumin (Kretsinger & Barry, 1975; Kretsinger, 1980). The measured affinities of these sites for Ca^{2+} vary according to the different conditions used in different laboratories. Most laboratories find two classes of Ca^{2+}-binding sites with association constants near 10^5 and 10^6 M^{-1} (Teo & Wang, 1973; Lin et al., 1974; Watterson et al., 1976; Klee, 1977a; Wolff et al., 1977). The anomalously large values reported by Wolff et al. (1977) are probably due to the low ionic strength used by these authors. Dedman et al. (1977) reported the presence of a single class of independent sites. The observation that most spectral changes are complete when an average of 2 mol of Ca^{2+} is bound per mol of calmodulin (Klee, 1977a; Dedman et al., 1977; Wolff et al., 1977; and this paper) also indicates the existence of two classes of sites, if it is assumed that the spectral changes are dependent on specific sites being occupied. We describe here a positive cooperativity of Ca^{2+} binding, at very low Ca^{2+} concentrations, which has not previously been observed. This cooperative interaction explains the steepness of Ca^{2+} dependence of the structural changes (Figure 5), as well as the sigmoidicity observed when spectral changes were followed as a function of Ca^{2+} occupancy. In order to account for the sigmoidicity, it appears necessary to assume that the spectral changes accompany Ca^{2+} binding at the second site. Recent nuclear magnetic resonance studies of Seamon (1980) clearly indicate that calmodulin undergoes a large conformational change upon binding 2 mol of Ca^{2+} and provide evidence for at least three conformational states of the protein: metal free, Ca_2^{2+} bound, and Ca_{3-4}^{2+} bound. Similar conclusions were also reached by McCubbin et al. (1979) and Walsh et al. (1979), who studied the Ca^{2+} dependence of the near-UV circular dichroic spectra of nitrated calmodulin. If, as indicated above, a positive cooperativity exists between the two first sites to be occupied by Ca^{2+}, a fourth conformation of the protein can be postulated, namely, $CaM \cdot Ca_3^{2+}$. Tyrosine-138, which is located in the fourth Ca^{2+}-binding loop, is responsible for the tyrosine spectral changes observed upon binding of two Ca^{2+} (Richman & Klee, 1979; Seamon, 1980). Phenylalanine spectral changes are also observed upon binding of 2 Ca^{2+}/mol. According to the model proposed by Kretsinger & Barry (1975), Phe-65 is in the same relative position in the second Ca^{2+}-binding loop as Tyr-138 is in the fourth. It is therefore possible that the absorption as well as the CD changes are due to similar changes in the microenvironment of Phe-65 and Tyr-138 upon binding of Ca^{2+} at two equivalent, independent, high-affinity Ca^{2+}-binding sites. Positive cooperativity may be the result of the displacement of an equilibrium between two conformational states of the protein upon binding of one Ca^{2+} to either site (Koshland et al., 1966). Alternatively, binding of the first Ca^{2+} is obligatorily at a specific site. In either case, the data are indicative of a sequential binding of pairs of Ca^{2+} to calmodulin. Studies on isolated proteolytic fragments of calmodulin such as those carried out by Drabikowski et al. (1977) and Walsh et al. (1977) may shed further light on the identity of those sites which are sequentially occupied by Ca^{2+}.

We cannot yet distinguish between the two models for the cooperative binding of Ca^{2+}. We have ruled out monomer–polymer equilibria as a source of this positively cooperative interaction. Ultracentrifugation studies failed to reveal significant differences in either the molecular weight or sedimentation coefficient of calmodulin in the presence and absence of Ca^{2+}. However, there is a difference in the second virial coefficient, B, in the presence and absence of Ca^{2+}. If one assumes that this difference is due only to changes in the Donnan term of this coefficient (Crouch, 1977), only four

negative charges are neutralized upon binding 4 mol of Ca^{2+}. This could indicate the release of a monovalent ion such as K^+ upon binding of a Ca^{2+} ion. The lack of difference in buoyancy terms in the presence or absence of Ca^{2+} indicates little or no change in the preferential interaction of the protein with the solvent. This is also compatible with K^+ displacement by Ca^{2+}, since the cations have similar molecular weights and the preferential interaction on a gram per gram basis would not change. The effect of ionic strength on the circular dichroic spectrum (Figure 2) and the effect of NaCl on the UV spectra (Richman & Klee, 1979) are also indicative of monovalent cation binding to calmodulin. It is possible that both monovalent and divalent cations such as Mg^{2+} (Richman & Klee, 1979) interact nonspecifically with negative charges of the protein and thus modify its Ca^{2+}-binding affinity without interacting with the specific Ca^{2+}-binding sites. Thus, because of effects produced by cations other than Ca^{2+}, the conditions under which calcium binding studies are performed become critical.

Binding of Ca^{2+} at two sites is sufficient to modify the calmodulin structure but not to activate cyclic nucleotide phosphodiesterase. Binding at the third or third and fourth sites is required. In the presence of phosphodiesterase, the binding of the last 2 mol of Ca^{2+} is greatly enhanced and becomes highly cooperative (as shown by the steepness of the Ca^{2+}-dependent activation curve in Figure 5) as expected on the basis of mass action. It remains to be determined whether other enzymes under calmodulin control require different degrees of Ca^{2+} occupancy.

The multifunctional character of calmodulin may be related both to its complex, cooperative interactions with Ca^{2+} and to its posttranslational modification leading to the presence of a trimethyllysyl residue. The extreme conservation of virtually its entire sequence throughout evolution indicates that every part of its primary structure is important for one or another of its multiple functions. Indeed, analogous proteins such as troponin C from various sources have been found to be lacking at least some of the calmodulin functions such as the ability to stimulate phosphodiesterase or erythrocyte (Ca^{2+} + Mg^{2+})-ATPase (Stevens et al., 1976; Klee, 1977b; Gopinath & Vincenzi, 1977). Other functions have been preserved at least partially (Cohen et al., 1979). Thus, the interaction of calmodulin with any one of the several proteins under its control may involve one or another part of its structure and one or another state of Ca^{2+} occupancy (Klee et al., 1980).

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Role of Hydroxyl Radicals in the Iron–Ethylenediaminetetraacetic Acid Mediated Stimulation of Microsomal Oxidation of Ethanol†

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ABSTRACT: The microsomal oxidation of ethanol or 1-butanol was increased by ferrous ammonium sulfate–ethylenediaminetetraacetic acid (1:2) (Fe–EDTA) (3.4–50 μM). The increase was blocked by hydroxyl radical scavenging agents such as dimethyl sulfoxide or mannitol. The activities of aminopyrine demethylase or aniline hydroxylase were not affected by Fe–EDTA. The accumulation of H_2O_2 was decreased in the presence of Fe–EDTA, consistent with an increased utilization of H_2O_2. Other investigators have shown that Fe–EDTA increases the formation of hydroxyl radicals in systems where superoxide radicals are generated. The stimulation by Fe–EDTA appears to represent a pathway involving hydroxyl radicals rather than catalase because (1) stimulation occurred in the presence of azide, which inhibits catalase, (2) stimulation occurred in the presence of 1-butanol, which is not an effective substrate for catalase, and (3) stimulation was blocked by hydroxyl radical scavenging agents, which do not affect catalase-mediated oxidation of ethanol. A possible role for contaminating iron in the H_2O or buffers could be ruled out since similar results were obtained with or without chelex-100 treatment of these solutions. The stimulatory effect by Fe–EDTA required microsomal electron transfer with NADPH, and H_2O_2 could not replace the NADPH-generating system. In the absence of microsomes or catalase, Fe–EDTA also stimulated the coupled oxidation of ethanol during the oxidation of xanthine by xanthine oxidase. These results suggest that during microsomal electron transfer, conditions may be appropriate for a Fenton type or a modified Haber–Weiss type of reaction to occur, leading to the production of hydroxyl radicals.

Recent experiments have implicated a role for hydroxyl radicals (·OH)[1] in the microsomal system for oxidizing primary aliphatic alcohols. Several ·OH scavengers were effective competitive inhibitors of microsomal oxidation of ethanol and 1-butanol (Cederbaum et al., 1977, 1978, 1979). These agents did not inhibit microsomal electron transfer, microsomal drug metabolism, or H_2O_2 plus catalase dependent oxidation of ethanol (Cederbaum et al., 1978). In addition, microsomes metabolized several ·OH scavengers during electron transfer: ethylene was generated from methional or from 2-keto-4-thiomethylbutyric acid, and methane was generated from Me_2SO (Cohen & Cederbaum, 1979, 1980). Hydrocarbon gas production was inhibited by competing ·OH scavengers such as ethanol and 1-butanol (Cohen & Cederbaum, 1979, 1980). These experiments indicated that microsomes generated ·OH, and that the oxidation of alcohols reflected the interaction of the alcohols with ·OH to form the corresponding aldehydes.

By what mechanisms can microsomes generate ·OH? Two H_2O_2-dependent pathways that are often invoked as sources of ·OH in biological systems are the ferrous ion catalyzed decomposition of H_2O_2 [the Fenton reaction (Walling, 1975)]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$

or the reaction between the superoxide radical ($O_2^{-}\cdot$) and H_2O_2 [the Haber–Weiss reaction (Haber & Weiss, 1934)]:

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + \cdot OH + OH^- \quad (2)$$

Microsomes generate both H_2O_2 and $O_2^{-}\cdot$ during NADPH oxidation (Aust et al., 1972; Prough & Masters, 1973; Dybing et al., 1976; Strobel & Coon, 1971; Thurman et al., 1972; Hildebrandt & Roots, 1975; Nordblom & Coon, 1977) and thus have the potential for generating ·OH by either reaction 1 or reaction 2. In our experiments with microsomes, a role for H_2O_2 as a precursor for ·OH was evident from the observation that (1) the addition of azide to inhibit catalase and thereby allow H_2O_2 to accumulate increased the oxidation of 1-butanol as well as the metabolism of ·OH scavengers to hydrocarbon gases (Cederbaum et al., 1978; Cohen & Cederbaum, 1979, 1980) and (2) the addition of H_2O_2 in the presence of azide increased the NADPH-dependent oxidation of ethanol and 1-butanol (Cederbaum et al., 1978). However,

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[1] Abbreviations used: ·OH, hydroxyl radical; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Fe–EDTA, ferrous ammonium sulfate and EDTA in a 1:2 molar ratio; SOD, superoxide dismutase.

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