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Calcium Binding to Calmodulin and Its Globular Domains*

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Sara Linse, Anna Helmersson, and Sture Forsén

From Physical Chemistry 2, Lund University, Chemical Centre, S-221 00 Lund, Sweden

The macroscopic Ca^{2+}-binding constants of bovine calmodulin have been determined from titrations with Ca^{2+} in the presence of the chromophoric chelator 5,5′-Br_2BAPTA in 0, 10, 25, 50, 100, and 150 mM KCl. Identical experiments have also been performed for tryptic fragments comprising the N-terminal and C-terminal domains of calmodulin. These measurements indicate that the separated globular domains retain the Ca^{2+} binding properties that they have in the intact molecule. The Ca^{2+} affinity is 6-fold higher for the C-terminal domain than for the N-terminal domain. The salt effect on the free energy of binding two Ca^{2+} ions is 20 and 21 kJ·mol^{-1} for the N- and C-terminal domain, respectively, comparing 0 and 150 mM KCl. Positive cooperativity of Ca^{2+} binding is observed within each globular domain at all ionic strengths. No interaction is observed between the globular domains. In the N-terminal domain, the cooperativity amounts to 3 kJ·mol^{-1} at low ionic strength and ≥10 kJ·mol^{-1} at 0.15 M KCl. For the C-terminal domain, the corresponding figures are 9 ± 2 kJ·mol^{-1} and ≥10 kJ·mol^{-1}. Two-dimensional ^{1}H NMR studies of the fragments show that potassium binding does not alter the protein conformation.

Calmodulin is a regulatory Ca^{2+}-binding protein which transmits a transiently increased intracellular Ca^{2+} concentration to an activation of specific enzymes. This is possible because of substantial rearrangement of the calmodulin molecule on Ca^{2+} binding. The crystal form of Ca^{2+}-loaded calmodulin (Babu *et al.*, 1985, 1988; Kretsinger and Weissman, 1986) resembles a dumbbell with two globular domains separated by a central helix. Small angle x-ray scattering studies (Heidorn and Trewhella, 1988) indicate that calmodulin in solution also folds into two separate globular lobes, which on average are closer together than in the crystal state, owing to flexibility in the interconnecting helix. ^{1}H NMR studies aiming at a high resolution structure of calmodulin in solution have been initiated (Ikura *et al.*, 1990).

Calmodulin contains four relatively high affinity Ca^{2+} sites (K_a = 10^6–10^7 M^{-1} at low ionic strength) which have a helix-loop-helix conformation, so-called EF-hands (Kretsinger and Nockolds, 1973). Each globular domain contains two such sites, which enables cooperative calcium binding. The sites in the C-terminal domain have higher Ca^{2+} affinity than those in the N-terminal domain (Thulin *et al.*, 1984; Wang *et al.*, 1984; Martin *et al.*, 1985). Because of its biological importance as a regulator of cellular functions, calmodulin has been the focus of numerous biophysical studies (for a review see Forsén *et al.*, 1986).

Several studies of the Ca^{2+}-binding properties of calmodulin, at different conditions as regards choice of buffer and ionic strength, have been reported to date (see for example Dedman *et al.*, 1977; Jarret and Kyte, 1979; Crouch and Klee, 1980; Cox *et al.*, 1981; Haiech *et al.*, 1981; Keller *et al.*, 1982; Yoshida *et al.*, 1983; Ogawa and Tanokura, 1984; Burger *et al.*, 1984; Iida and Potter, 1986; for recent reviews see Forsén *et al.*, 1986 and Cox *et al.*, 1988). Some of these studies are apparently contradictory and report either four noninteracting sites with identical or different Ca^{2+} affinities or positive cooperativity between at least two sites. Part of the apparent controversy is likely to be due to the different methods of analyzing the primary data (Forsén *et al.*, 1986). In addition, binding data obtained for a system with two slightly different pairs of sites with cooperativity within each pair often closely resembles the data for a system with four equivalent and noninteracting sites (Wang, 1985). Therefore, any method adopted for determining Ca^{2+}-binding constants of calmodulin must provide accurate measurements of the free Ca^{2+} concentration or a parameter related to this quantity. To take maximum advantage of the accuracy of the chosen method, least squares fitting should be done *directly* to the *measured parameter*.

In the present work, we show that titrations in which calmodulin competes for calcium with a chromophoric chelator (5,5′-Br_2BAPTA)[1] can distinguish between cooperative and independent binding. The absorbance of the chelator as a function of total Ca^{2+} concentration reveals two consecutive processes in calmodulin, each involving cooperative binding of two Ca^{2+} ions. Studies of tryptic fragments comprising the N- and C-terminal domains help to identify these two processes and indicate that in the intact protein there is no cooperativity between the globular domains.

EXPERIMENTAL PROCEDURES

Materials—Bovine testes calmodulin and its tryptic fragments were prepared as previously described (Andersson *et al.*, 1983; Vogel *et al.*, 1983), and the purity was checked by sodium dodecyl sulfate-gel electrophoresis, agarose gel electrophoresis, and ^{1}H NMR. The residual Ca^{2+} content was determined by atomic absorption spectroscopy or by comparing the difference between the fitted values of AMAX and AMIN (*cf.* below) with those of the same chelator solution containing 250 μM EDTA instead of protein. 5,5′-Br_2BAPTA (Tsien, 1980) was from Molecular Probes (Eugene, Oregon). All other chemicals were of highest purity commercially available.

^{1}H NMR—^{1}H NMR spectra were obtained at 500.13 MHz on a GE-OMEGA 500 spectrometer, at 27 °C and pH 7.5 in H_2O.

Ca^{2+}-binding Constants—Each protein (20–30 μM) was titrated with Ca^{2+} in the presence of the tetrapotassium salt of 5,5′-

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[1] The abbreviations used are: 5,5′-Br_2BAPTA, 5,5′-dibromo-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; TR_1C, tryptic fragment comprising residues 1–74 of bovine calmodulin; TR_2C, tryptic fragment comprising residues 78–148 of bovine calmodulin; NH, amide proton; $C^{\alpha}H$, proton bound to α-carbon.

Br_2BAPTA (25–30 μM) and different concentrations of KCl, in 2 mM Tris/HCl buffer at pH 7.5 and 25 °C as previously described (Linse *et al.*, 1987, 1988). Macroscopic Ca^{2+}-binding constants were obtained from least squares fits directly to the data, *i.e.* to the absorbance at 263 nm as a function of total Ca^{2+} concentration. The analysis was based entirely on concentration. Two macroscopic binding constants (K_1 and K_2) were used in the case of each fragment and four (K_1, K_2, K_3, and K_4) in the case of intact calmodulin. Fixed parameters in the fit were KDQ = the Ca^{2+} dissociation constant of the chelator, CQ_i the chelator concentration at each titration point i, $CATOT_i$ = the total Ca^{2+} concentration at point i, including initial and added Ca^{2+}, and CP_i = protein concentration (by weight of lyophilized protein) at point i. CQ_i, CP_i, and $CATOT_i$ include corrections for the dilutions caused by Ca^{2+} additions. Variable parameters in the fit were K_1 through K_4, AMAX, AMIN, and F. AMAX and AMIN are the absorbances one would measure for the initial solution if it was completely Ca^{2+}-free or contained saturating amounts of Ca^{2+}, respectively. F is a correction factor that accounts for the fact that the protein concentration obtained by weight is not correct owing to the presence of residual water in the lyophilized protein. In our experience, no currently used method for protein concentration determination provides values that are more accurate than the experimental Ca^{2+} titration data itself (in the case of strong Ca^{2+} binding). This validates the use of F as an adjustable parameter (*cf.* below). For each set of variable parameters, the Newton-Raphson method was used to solve for the free Ca^{2+} concentration, Y, at each titration point, i, from the following equation

$$CATOT_i - Y - \frac{Y \cdot CQ_i}{Y + KDQ} - \frac{F \cdot CP_i \cdot \sum_{k=1}^{N} \left(k Y^k \cdot \prod_{j=1}^{k} K_j \right)}{1 + \sum_{k=1}^{N} \left(Y^k \cdot \prod_{j=1}^{k} K_j \right)} = 0$$

where $N = 4$ in the case of intact calmodulin and $N = 2$ for each fragment. In this equation, the last and next to last terms equal the protein-bound calcium and chelator-bound calcium, respectively. The absorbance of 5,5′-Br_2BAPTA at 263 nm decreases when it binds Ca^{2+}, and the absorbance at point i can then be calculated as

$$Abs_{calculated,i} =$$

$$[AMAX - (AMAX - AMIN)\,Y/(Y + KDQ)]CQ_i/CQ_1$$

where CQ_1 is the initial chelator concentration. Thus, the changes in absorbance at 263 nm were assumed to arise from the chelator only. (It was experimentally verified that the changes in A_{263} on calcium addition to 30 μM calmodulin were neglible as compared to 30 μM chelator.) The error square sum, ESS, was obtained by summing over all points in the titration

$$ESS = \Sigma(Abs_{calculated,i} - Abs_{measured,i})^2$$

The variable parameters were iterated in a separate procedure until an optimal fit (minimum ESS) was found. This procedure is based on numerical evaluation of the first and second derivative of ESS with respect to each parameter. The uncertainties in the final values of the parameters were estimated as previously described (Linse *et al.*, 1991) and do not include errors in the Ca^{2+} affinities for the chelator, which have been reported for 0, 50, 10, and 150 mM KCl (Linse *et al.*, 1991). Here determined values are KDQ = $1.8 \cdot 10^{-7}$ M at 10 mM KCl, and KDQ = $4.4 \cdot 10^{-7}$ M at 25 mM KCl. To make sure our results are not biased by the previously determined stoichiometry of four high affinity sites in calmodulin, the fitting program was forced to use three or five sites, respectively, when analyzing the titration data for intact calmodulin. When all variable parameters (including the protein concentration) were allowed to adjust their values, the error square sum of the optimal fit was 10-fold higher than for the case of four high affinity sites.

pH Titrations—pH titrations (from pH 6.5–11) were performed in 0 and 150 mM KCl in order to assess any salt effect on the pK_a values of ionizable groups which affect the Ca^{2+} affinity of calmodulin. Each fragment was first dissolved in 2 mM Tris/HCl buffer (at pH 7.5) containing 25 μM 5,5′-Br_2BAPTA and 0 or 0.15 M KCl. Small portions of Ca^{2+} were added and followed by measurements of the absorbance at 263 nm (A_{263}), until the system was approximately half-saturated. pH was then adjusted (with HCl) to 6.5, and A_{263} was recorded. The pH was adjusted in steps of ≈0.25 by adding small amounts (2–5 μl) of NH_3, and A_{263} was recorded at each step. The measured A_{263} was corrected for the dilutions caused by NH_3 additions and plotted *versus* pH. Since A_{263} reports the Ca^{2+} saturation level of the chelator, the Ca^{2+} affinity of which is constant in the pH interval 6.5–11 (Tsien, 1980), the plot gives a qualitative measure of pH effects on the Ca^{2+} affinity of the protein.

RESULTS AND DISCUSSION

The aim of the present work was to perform a detailed investigation of the Ca^{2+} affinity and cooperativity of calmodulin and its tryptic fragments. Titrations with Ca^{2+} in the presence of a chromophoric chelator have proven accurate enough to discriminate cooperative binding from independent binding (Linse *et al.*, 1987, 1988). Least squares fits to such titrations provide macroscopic Ca^{2+}-binding constants, which refer to the binding of the first, second, third, and so on Ca^{2+} ion to the protein, irrespective of what site is occupied in the protein. Three examples of experimental data with the chelator 5,5′-Br_2BAPTA (and intact calmodulin, TR_1C, or TR_2C, respectively) are shown in Fig. 1*A* together with the curves of best fit. Fig. 1*B* displays calculated curves for the chelator and a two-site protein with either two equally strong and noninteracting sites, positive cooperativity, or sequential binding. Evidently, positive cooperativity and sequential binding both result in S-shaped curves, but the curvature is of opposite sign. The trends in the experimental curves in Fig. 1*A* thus indicate cooperative binding. For intact calmod-

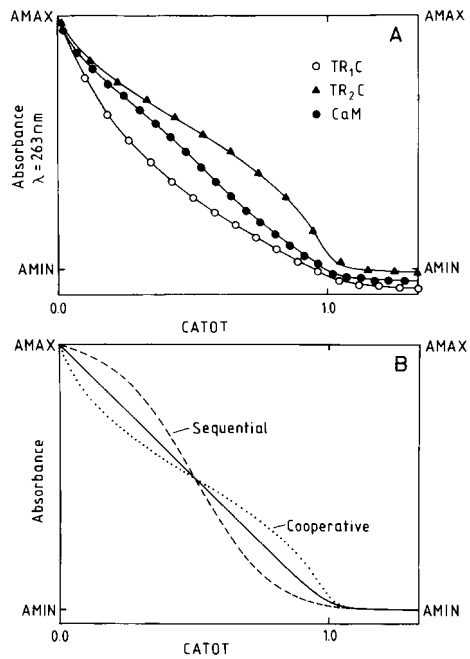

FIG. 1. *A*, examples of experimental data, absorbance at 263 nm *versus* total calcium concentration (CATOT) from titrations in the presence of 5,5′-Br_2BAPTA, for intact calmodulin (●), TR_1C (○), and TR_2C (▲). ——, optimal curves obtained by least squares fitting to the data points. The data have been normalized such that CATOT = 1.0 corresponds to total chelator concentration plus 2 times the protein concentration (fragments) or total chelator concentration plus 4 times the total protein concentration (calmodulin). For comparison, the three curves have been drawn with their respective AMAX and AMIN values at the same level. The actual values for the three titrations shown are: for intact calmodulin AMAX = 0.608 and AMIN = 0.220 (25.0 μM 5,5′-Br_2BAPTA); for TR_1C, 0.557 and 0.128 (27.5 μM); and for TR_2C, 0.575 and 0.185 (25.0 μM). *B*, calculated curves for a two-site protein (25 μM) in the presence of 5,5′-Br_2BAPTA (25 μM) with the following values of the two macroscopic Ca^{2+}-binding constants of the protein: ——, $K_1 = 2 \times 10^7$ M^{-1} and $K_2 = 5 \times 10^6$ M^{-1} as for equally strong and independent sites; – – –, $K_1 = 1 \times 10^8$ M^{-1} and $K_2 = 1 \times 10^6$ M^{-1} as for sequential binding; · · · · ·, $K_1 = 1 \times 10^6$ M^{-1} and $K_2 = 1 \times 10^8$ M^{-1} as for positive cooperativity.

ulin we can in fact see two consecutive, cooperative processes, each resembling the binding curve for one of the fragments. Fig. 2 shows an analysis of the precision in the different parameters obtained in the least squares fitting to the data for intact calmodulin as displayed in Fig. 1*A*. This analysis confirms positive cooperativity and that only a narrow range of values of the protein concentration ($F \cdot \mathrm{CPROT}_1$) give acceptable fits to the experimental data.

Chelator-Protein Interaction—One potential problem with the method used here is that the chelator might bind to the protein and that such an interaction could alter the Ca^{2+} affinity for the chelator and/or the protein. Chiancone *et al.* (1986) have used static difference absorbance experiments to show that the parent compound (BAPTA) interacts with calmodulin at low ionic strength. The concentration ranges used in that study were 220–260 μM for BAPTA and 18–75 μM for calmodulin. The product of these two concentrations was thus 10- to 30-fold higher than that in the present work. When applying the same method as that described by Chiancone *et al.* (1986) to 30 μM 5,5′-Br_2BAPTA and 30 μM calmodulin at low ionic strength, no interaction could be detected (detection limit $\Delta A \approx \pm 0.001$, data not shown). We find it reasonable to assume that interaction between chelator and protein is not an important factor in the present experiments.

Ca^{2+} Binding to the Tryptic Fragments—Each fragment binds two Ca^{2+} ions with affinities in the range accessible with the chelator used in this study ($\approx 10^6$–10^8 M^{-1} at low ionic strength). The two macroscopic binding constants, K_1 and K_2, obtained for TR_1C and TR_2C at different KCl concentrations are summarized in Table I. K_1 and K_2, expressed in molar concentration units, can be used to calculate the free energy of binding of two Ca^{2+} ions, $\Delta G_{tot} = -RT\ln(K_1K_2)$. For both fragments and at all salt concentrations, we find that $K_2 > K_1/4$ which implies positive cooperativity. (Note $K_2 > K_1/4$ always implies positive cooperativity, but if the two sites do not have identical affinities, positive cooperativity can exist even if $K_2 < K_1/4$.)

The cooperativity in a two-site system can be characterized by $\Delta\Delta G$, the effect of Ca^{2+} binding to one of the sites on the free energy of Ca^{2+} binding to the other site (Weber, 1975).

$$\Delta\Delta G = \Delta G_{I,II} - \Delta G_I = \Delta G_{II,I} - \Delta G_{II}$$
$$= -RT\ln(K_{I,II}/K_I) = -RT\ln(K_{II,I}/K_{II})$$

where $\Delta G_{I,II}$ is the free energy of Ca^{2+} binding to site I when a Ca^{2+} ion is already bound to site II (*cf.* Fig. 3*A*) and $K_{I,II}$ etc. are the corresponding microscopic (site) binding constants. The titrations in the presence of 5,5′-Br_2BAPTA do not allow one to determine the distribution of Ca^{2+} between the sites of the protein and hence yield only the macroscopic binding constants. However, one can use the relations between macroscopic and microscopic binding constants ($K_1 = K_I + K_{II}$ and $K_1K_2 = K_IK_{II,I} = K_{II}K_{I,II}$) and write

$$-\Delta\Delta G = RT\ln(4K_2/K_1) + RT\ln((\eta + 1)^2/4\eta)$$

where $\eta = K_{II}/K_I$. The second term is zero for $\eta = 1$ (equally strong sites), and we may thus obtain a lower limit of $-\Delta\Delta G$ solely from the macroscopic binding constants as

$$-\Delta\Delta G_{\eta=1} = RT\ln(4K_2/K_1)$$

(Linse *et al.*, 1987, 1988). Thus, it is apparent from the results in Table I that each fragment binds two Ca^{2+} ions with positive cooperativity and relatively high affinity. At low ionic strength, the Ca^{2+} affinity for the C-terminal fragment, TR_2C, is on average 6-fold higher than for TR_1C if we compare $\sqrt{(K_1K_2)}$. There is a considerable decrease in the Ca^{2+} affinity on going from low ionic strength conditions to 150 mM KCl. For TR_2C, the product of K_1 and K_2 is reduced 5000-fold, corresponding to a 21 $kJ \cdot mol^{-1}$ increase in ΔG_{tot}, and for TR_1C the corresponding figures are 3200-fold and 20 $kJ \cdot mol^{-1}$.

K^+ Binding—On the assumption that the decrease in the Ca^{2+} affinity of calmodulin that occurs on adding KCl arises solely from competition between K^+ and Ca^{2+}, Haiech *et al.* (1981) have concluded that potassium binds competitively to the Ca^{2+} sites. Part of the KCl effect could, however, be simply due to nonspecific ionic strength effects: screening of electrostatic interactions and change in activity coefficients. To investigate this point, two-dimensional ^1H NMR COSY spectra were recorded for Ca^{2+}-depleted tryptic fragments at pH 7.5, 27 °C in the absence and presence of 150 mM KCl. The NH and $C^\alpha H$ chemical shifts of nearly all NH-$C^\alpha H$ cross-peaks were measured (a small number were missing owing to fast exchange). For TR_1C, the average shift difference between 0 and 150 mM KCl was 0.02 ppm for NH and 0.01 ppm for $C^\alpha H$, the largest difference being 0.07 ppm. For TR_2C, the average shift difference was 0.01 ppm for both NH and $C^\alpha H$, the largest difference being 0.04 ppm. These results are very similar to what was found in the case of calbindin D_{9k} and indicate that any binding of K^+ ions to calmodulin has only a minimal effect on the protein conformation. Thus, the effect of KCl on the Ca^{2+} affinities of the proteins is nonspecific. In the apo form of the protein, it is likely that the Ca^{2+} ligands repel each other and cannot fold in the proper conformation for Ca^{2+} coordination. It has been suggested that the charge on K^+ is too low to overcome the ligand repulsion and that K^+-binding would not result in the same arrangement of the ligands as in the Ca^{2+}-bound state (Snyder *et al.*, 1990).

Ca^{2+} Binding to Calmodulin—The four macroscopic binding

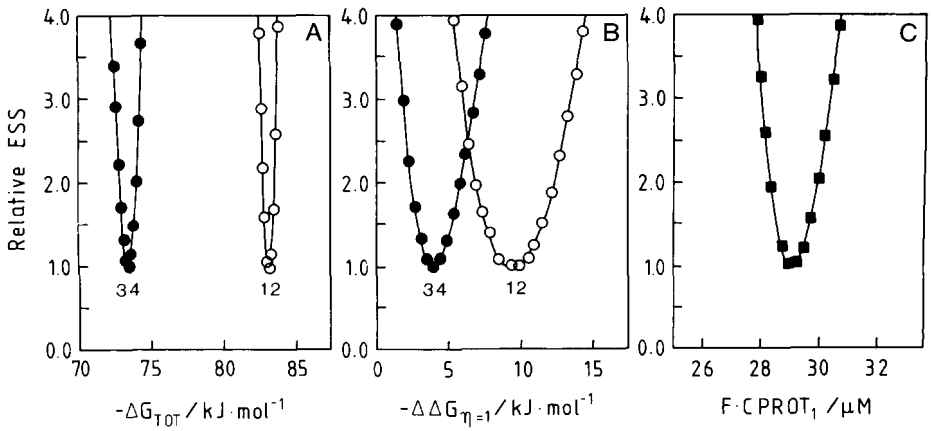

FIG. 2. **Minimum error square sum (ESS) *versus* (*A*) $-\Delta G^{12}_{tot}$ (○), $-\Delta G^{34}_{tot}$ (●), (*B*) $-\Delta\Delta G^{12}_{\eta=1}$ (○), $-\Delta\Delta G^{34}_{\eta=1}$ (●), and (*C*) $F \cdot \mathrm{CPROT}_1$ (■).** The experimental data for intact calmodulin as displayed in Fig. 1 has been analyzed as regards the dependence of ESS on the values of the different parameters. Each point in the curve for ΔG^{12}_{tot} is obtained by keeping the product of K_1 and K_2 (and hence ΔG^{12}_{tot}) constant while letting AMAX, AMIN, F, K_3, K_4, and the individual values of K_1 and K_2 adjust their values to obtain the lowest possible ESS for the specific value of ΔG^{12}_{tot}. Points for the other parameters are obtained in a similar fashion.

TABLE I

Macroscopic binding constants of TR_1C and TR_2C

$\Delta G_{tot} = -RT\ln(K_1K_2)$ and $-\Delta\Delta G_{\eta=1} = RT\ln(4K_2/K_1)$. lgK stands for $^{10}\log K$. The uncertainties are ±0.2 in $\lg K_1$ and $\lg K_2$, and ±0.5 in ΔG_{tot}, unless otherwise stated. Note that $\lg(K_1K_2)$ is more well determined than the individual $\lg K_1$ and $\lg K_2$. The uncertainties for $\lg K_1$ and $\lg K_2$ are linked such that if the value for one is underestimated by 0.1 then the value for the other is overestimated by ≈0.1.

[KCl]	TR_2C				TR_1C			
	$\lg K_1$	$\lg K_2$	ΔG_{tot}	$-\Delta\Delta G_{\eta=1}$	$\lg K_1$	$\lg K_2$	ΔG_{tot}	$-\Delta\Delta G_{\eta=1}$
mM			*kJ·mol⁻¹*	*kJ·mol⁻¹*			*kJ·mol⁻¹*	*kJ·mol⁻¹*
Low	6.9	7.7	−83.4	8.5 ± 1.5	6.56 ± 0.05	6.54 ± 0.05	−74.7 ± 1.0	3.3 ± 0.5
50	5.1	6.8	−68.9	≥10	4.8 ± 0.2	5.4 ± 0.2	−58.5 ± 1.5	6.8 ± 1.5
100	5.0	6.4	−65.5	≥10	4.0 ± 0.3	5.8 ± 0.3	−56.0 ± 1.5	≥10
150	4.8	6.1	−62.4 ± 1.0	≥10	4.0 ± 0.3	5.6 ± 0.3	−54.9 ± 2.0	≥10

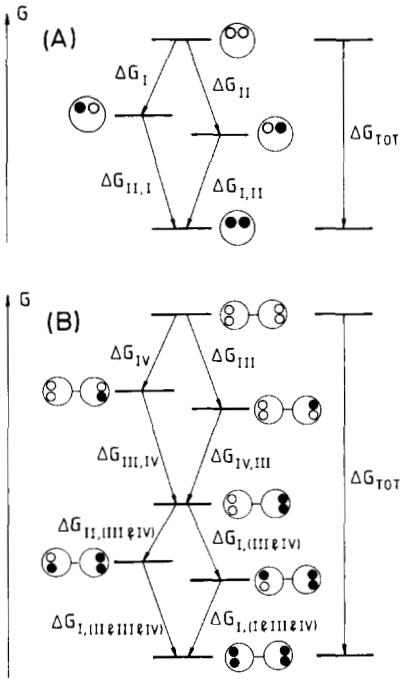

FIG. 3. *A*, free energy diagram for Ca^{2+} binding to a two-site protein. *B*, free energy diagram for Ca^{2+} binding to a four-site protein in which K_{III} and $K_{IV} \gg K_I$ and K_{II}. The free Ca^{2+} ions are not included in these diagrams, but, of course, contribute to the free energies.

constants, K_1, K_2, K_3, and K_4, of calmodulin at different KCl concentrations are summarized in Table II. In this case, we have calculated the free energy of binding four Ca^{2+} ions, $\Delta G_{tot} = -RT\ln(K_1K_2K_3K_4)$. We note that at all KCl concentrations the values of ΔG_{tot} for calmodulin equals the sum of the ΔG_{tot} values for the tryptic fragments.

Ca^{2+} binding to a protein with four Ca^{2+} sites could be exceedingly complex due to possible interactions between any pair among the four sites. The analysis is simplified if two of the sites have considerably higher Ca^{2+} affinity than the other two. For example, if K_{III} and K_{IV} are much greater than K_I and K_{II}, then the sites bind in a semisequential manner as shown in Fig. 3*B* and

$$K_1 \approx K_{III} + K_{IV}$$

$$K_1K_2 \approx K_{III}K_{IV,III} = K_{IV}K_{III,IV}$$

$$K_3 \approx K_{I,(III\text{ and }IV)} + K_{II,(III\text{ and }IV)}$$

$$K_3K_4 \approx K_{I,(III\text{ and }IV)}K_{II,(I\text{ and }III\text{ and }IV)} = K_{II,(III\text{ and }IV)}K_{I,(II\text{ and }III\text{ and }IV)}$$

where $K_{I,(III\text{ and }IV)}$ is the site binding constant for site I when Ca^{2+} is already bound to sites III and IV, and so on (*cf.* Fig. 3*B*). We can calculate the free energy of binding the first two ($\Delta G^{12}_{tot} = -RT\ln[K_1K_2]$) and last two ($\Delta G^{34}_{tot} = -RT\ln[K_3K_4]$) Ca^{2+} ions and lower limits of the cooperativity within these pairs ($-\Delta\Delta G^{12}_{\eta=1} = RT\ln(4\,K_2/K_1)$ and $-\Delta\Delta G^{34}_{\eta=1} = RT\ln(4\,K_4/K_3)$, respectively). The values of the four macroscopic binding constants show that the sites in calmodulin are grouped into two pairs of different strength, each pair displaying positive cooperativity. If we calculate ΔG^{12}_{tot}, ΔG^{34}_{tot}, $-\Delta\Delta G^{12}_{\eta=1}$, and $-\Delta\Delta G^{34}_{\eta=1}$ (Table III) and compare with the results for the fragments (Table I), we can conclude that the two sites of the C-terminal domain in calmodulin bind Ca^{2+} most strongly in a cooperative manner. The N-terminal domain binds Ca^{2+} 6 times more weakly but also cooperatively. The condition K_{III} and $K_{IV} \gg K_I$ and K_{II} does not hold strictly for calmodulin. The above treatment nevertheless gives *lower* limits to the cooperativity, and no false indications of positive cooperativity could arise. As can be seen in Fig. 2, the precision in ΔG^{12}_{tot} and ΔG^{34}_{tot} (or the products K_1K_2 and K_3K_4) is high, whereas the precision in $\Delta\Delta G^{12}_{\eta=1}$ and $\Delta\Delta G^{34}_{\eta=1}$ (or the ratios K_2/K_1 and K_4/K_3) is considerably lower.

Since the two globular domains of calmodulin bind in a sequential manner, it is not straightforward to assess whether Ca^{2+} binding to one globular domain affects the Ca^{2+} affinities

TABLE II

Macroscopic binding constants of calmodulin

$\lg K$ stands for $^{10}\log K$. The uncertainties are ±0.2 in $\lg K_1$ and $\lg K_2$ unless otherwise stated. Note that $\lg(K_1K_2)$ and $\lg(K_3K_4)$ are more well determined than the individual $\lg K$ values. The uncertainties for $\lg K_1$ and $\lg K_2$ (or $\lg K_3$ and $\lg K_4$) are linked such that if the value for 1 is underestimated by 0.1 then the value for the other is overestimated by ≈0.1.

[KCl]	$\lg K_1$	$\lg K_2$	$\lg K_3$	$\lg K_4$	ΔG_{tot}
mM					*kJ·mol⁻¹*
Low	7.0	7.6	6.5	6.4	−156.4 ± 1.5
10	6.2	7.4	6.0	6.0	−145.0 ± 1.5
25	5.8	7.0	5.4	5.7	−136.0 ± 1.5
50	5.4	6.7	4.6	5.5	−126.8 ± 2.0
100	4.9	6.6	4.4 ± 0.3	5.6 ± 0.3	−122.6 ± 2.0
150	4.7	6.2	4.0 ± 0.3	5.3 ± 0.3	−115.7 ± 3.5

TABLE III

Free energy of binding the first two ($\Delta G^{12}_{tot} = -RT\ln(K_1K_2)$) and last two ($\Delta G^{34}_{tot} = -RT\ln(K_3K_4)$) Ca^{2+} ions to calmodulin and a lower limit of the cooperativity within each pair ($-\Delta\Delta G^{12}_{\eta=1} = RT\ln(4\,K_2/K_1)$ and $-\Delta\Delta G^{34}_{\eta=1} = RT\ln(4\,K_4/K_3)$, respectively)

[KCl]	ΔG^{12}_{tot}	$-\Delta\Delta G^{12}_{\eta=1}$	ΔG^{34}_{tot}	$-\Delta\Delta G^{34}_{\eta=1}$
mM	*kJ·mol⁻¹*	*kJ·mol⁻¹*	*kJ·mol⁻¹*	*kJ·mol⁻¹*
Low	−83.5 ± 0.5	8.0 ± 1.5	−73.6 ± 1.0	3.7 ± 0.5
50	−69.0 ± 0.5	≥10	−57.6 ± 1.5	9.0 ± 1.5
100	−65.7 ± 0.5	≥10	−56.9 ± 1.5	≥10
150	−62.4 ± 1.0	≥10	−53.3 ± 2.5	≥10

of the sites in the other domain. However, the free energy values calculated for intact calmodulin (Table III) are, within the error limits, identical with those of the isolated fragments (Table I). Thus, the globular domains in calmodulin appear to bind Ca^{2+} independently of each other. In contrast, cooperativity between the globular domains has been observed when calmodulin is complexed with peptides or fragments of target enzymes (Yazawa *et al.*, 1987; Ikura *et al.*, 1989).

Salt Effects on pK_a Values of Ionizable Groups—The pH titrations of the fragments in the presence of the chelator and half-saturationg concentrations of Ca^{2+} show different behavior at 0 and 150 mM KCl. For both fragments, it is found that at low ionic strength there is a considerable increase in Ca^{2+} affinity when pH is raised from 6.5 to 8.5, whereas at 150 mM KCl the Ca^{2+} affinity is constant in the same pH interval. It thus seems that KCl in some way affects the ionizable groups in the pH range 6.5–8.5. It is not possible to judge from the present experiments whether these effects are predominantly nonspecific or if K^+ ions compete with protons for specific side chains. The linewidth of the ^{39}K NMR signal is considerably increased in the presence of calmodulin[2] which indicates that K^+ ions are weakly associated to calmodulin (*cf.* a similar study on calbindin D_{9k}: Linse *et al.*, 1991). In any case, theoretical treatment of the Ca^{2+} affinity of calmodulin at pH 7.5 is likely to overestimate the salt effects, if the protein surface charge is kept the same at all salt concentrations. In addition, the pH titrations show for both fragments a considerable increase in Ca^{2+} affinity in the pH range 9–11, both at low and high salt concentration. The latter effect is most likely a result of deprotonation of lysine side chains. If H^+ binding to some ionizable groups affects pK_{Ca}, Ca^{2+} binding will affect pK_a of the same ionizable groups. In fact, Ca^{2+} binding to calmodulin and its tryptic fragments has been reported to result in proton uptake or release (Milos *et al.*, 1986; Sellers *et al.*, 1991).

Comparison with Previous Studies—Many of the previous studies of Ca^{2+} binding to calmodulin have failed to distinguish between cooperative and independent binding, either because the method used is not sensitive enough or the data analysis has extracted information (*e.g.* affinities for individual sites) that is not present in the data itself. Nevertheless, if we disregard all studies that assume identical and independent sites, the values for the products of the first two and last two macroscopic binding constants (K_1K_2 and K_3K_4) should be closely similar in all studies that are free from large systematic errors. The values reported here for $\lg(K_1K_2)$ and $\lg(K_3K_4)$ of calmodulin in 150 mM KCl and 2 mM Tris/HCl (10.9 and 9.3, respectively), fall in the range of those of previous studies at comparable conditions (10.4–11.1 and 8.0–9.4, respectively). To our knowledge no measurements have previously been performed at an ionic strength as low as in the present work (0 M KCl, 2 mM Tris/HCl). We may, however, note that the results we report for 10 mM KCl and 2 mM Tris/HCl ($\lg(K_1K_2) = 13.6$ and $\lg(K_3K_4) = 12.0$) are, as expected, only slightly lower than those published by Haiech *et al.* (1981). For 0 M KCl and 10 mM Tris/HCl, pH 7.55, they report $\lg(K_1K_2) = 13.7$ and $\lg(K_3K_4) = 12.1$.

Conclusion—A detailed reinvestigation of the calcium-binding constants of calmodulin, using the chromophoric chelator 5,5′-Br_2BAPTA, has provided strong evidence for cooperative Ca^{2+} binding within each globular domain, both at low and high ionic strength. There is no indication of cooperativity between the two domains. Rather, comparisons with the tryptic fragments strongly suggest that the two domains bind Ca^{2+} independently of each other. The Ca^{2+} affinities of the sites in the C-terminal domain are on average 6-fold higher than those of the sites in the N-terminal domain.

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[2] T. Drakenberg, personal communication.

REFERENCES

Andersson, A., Forsén, S., Thulin, E. & Vogel, H. J. (1983) *Biochemistry* **22,** 2309–2313
Babu, Y. S., Sack, J. S., Greenhough, T. G., Bugg, C. E., Means, A. R. & Cook, W. J. (1985) *Nature* **315,** 37–40
Babu, Y. S., Bugg, C. E. & Cook, W. J. (1988) *J. Mol. Biol.* **204,** 191–204
Burger, D., Cox, J. A., Comte, M. & Stein, E. A. (1984) *Biochemistry* **23,** 1966–1971
Chiancone, E., Thulin, E., Boffi, A., Forsén, S. & Brunori, M. (1986) *J. Biol. Chem.* **261,** 16306–16308
Cox, J. A., Malnoë, A. & Stein, E. A. (1981) *J. Biol. Chem.* **256,** 3218–3222
Cox, J. A., Comte, M., Mamar-Bachi, A., Milos, M. & Schaer, J.-J. (1988) in *Calcium and Calcium Binding Proteins* (Gerday, C., Bolis, L. & Gilles, R., eds) pp. 141–162, Springer Verlag, Berlin
Crouch, T. H. & Klee, C. B. (1980) *Biochemistry* **19,** 3692–3698
Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252,** 8415–8422
Forsén, S., Vogel, H. J. & Drakenberg, T. (1986) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. 6, pp. 113–157, Academic Press, New York
Haiech, J., Klee, C. B. & Demaile, J. G. (1981) *Biochemistry* **20,** 3890–3897
Heidorn, D. B. & Trewhella, J. (1988) *Biochemistry* **27,** 909–915
Iida, S. & Potter, J. D. (1986) *J. Biochem.* (*Tokyo*) **99,** 1765–1772
Ikura, M., Hasegawa, N., Aimoto, S., Yazawa, M., Yagi, K. & Hikichi, K. (1989) *Biochem. Biophys. Res. Commun.* **161,** 1233–1238
Ikura, M., Kay, L. E. & Bax, A. (1990) *Biochemistry* **29,** 4659–4667
Jarrett, H. W. & Kyte, J. (1979) *J. Biol. Chem.* **254,** 8237–8244
Keller, C. H., Olwin, B. B., LaPorte, D. C. & Storm, D. R. (1982) *Biochemistry* **21,** 156–162
Kretsinger, R. H. & Nockolds, C. E. (1973) *J. Biol. Chem.* **248,** 3313–3326
Kretsinger, R. H. & Weissman, L. J. (1986) *J. Inorg. Biochem.* **28,** 289–302
Linse, S., Brodin, P., Drakenberg, T., Thulin, E., Sellers, P., Elmdén, K., Grundström, T. & Forsén, S. (1987) *Biochemistry* **26,** 6723–6735
Linse, S., Brodin, P., Johansson, C., Thulin, E., Grundström, T. & Forsén, S. (1988) *Nature* **335,** 651–652
Linse, S., Johansson, C., Brodin, P., Grundström, T., Drakenberg, T. & Forsén, S. (1991) *Biochemistry* **30,** 154–162
Martin, S. R., Andersson Teleman, A., Bayley, P. M., Drakenberg, T. & Forsén, S. (1985) *Eur. J. Biochem.* **151,** 543–550
Milos, M., Schaer, J.-J., Comte, M. & Cox, J. A. (1986) *Biochemistry* **25,** 6279–6287
Ogawa, Y. & Tanokura, M. (1984) *J. Biochem.* (*Tokyo*) **95,** 19–28
Sellers, P., Laynez, J., Thulin, E. & Forsén, S. (1991) *Biophys. Chem.* **39,** 199–204
Snyder, E. E., Buoscio, B. W. & Falke, J. J. (1990) *Biochemistry* **29,** 3937–3943
Thulin, E., Andersson, A., Drakenberg, T., Forsén, S. & Vogel, H. J. (1984) *Biochemistry* **23,** 1862–1870
Tsien, R. (1980) *Biochemistry* **19,** 2396–2404
Vogel, H. J., Lindahl, L. & Thulin, E. (1983) *FEBS Lett.* **157,** 241–246
Wang, C.-L. A. (1985) *Biochem. Biophys. Res. Commun.* **130,** 426–430
Wang, C.-L. A., Leavis, P. C. & Gergely, J. (1984) *Biochemistry* **23,** 6410–6415
Yazawa, M., Ikura, M., Hikichi, K., Ying, L. & Yagi, K. (1987) *J. Biol. Chem.* **262,** 10951–10954
Yoshida, M., Minowa, O. & Yagi, K. (1983) *J. Biochem.* (*Tokyo*) **94,** 1925–1933