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KINETIC STUDIES OF CALCIUM BINDING TO CALMODULIN

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KINETIC STUDIES OF CALCIUM BINDING TO CALMODULIN

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ABSTRACT

Calmodulin is a 17,000 MW calcium-binding protein found in all eucaryotic cells. Calcium binding to the two high affinity sites essentially completes the 2.5-fold enhancement of the intrinsic tyrosine fluorescence of calmodulin. Equilibrium and kinetic constants were measured by equilibrium and stopped-flow fluorometry techniques, respectively. Calcium dissociation from low affinity sites was investigated using the fluorescent calcium chelator, QUIN-2. The data presented here are consistent with the following proposed minimal mechanism for calcium binding to four sites on calmodulin (CaM):

$$\mathrm{CaM} \underset{>100\ s^{-1}}{\overset{10^{7}\ M^{-1}s^{-1}}{\rightleftharpoons}} \mathrm{CaM\text{-}Ca_1} \underset{7\ s^{-1}}{\overset{\geq 10^{8}\ M^{-1}s^{-1}}{\rightleftharpoons}} \mathrm{CaM\text{-}Ca_2} \underset{>500\ s^{-1}}{\overset{\geq 10^{8}\ M^{-1}s^{-1}}{\rightleftharpoons}} \mathrm{CaM\text{-}Ca_3} \underset{>500\ s^{-1}}{\overset{\geq 10\ M^{-1}s^{-1}}{\rightleftharpoons}} \mathrm{CaM\text{-}Ca_4}$$

INTRODUCTION

Calmodulin has been found in all eucaryotic cells. One of the striking characteristics of calmodulin is its apparent lack of tissue or species species specificity. All calmodulins activate calcium-dependent phosphodiesterase which is used as a standard assay for calmodulin activity. The amino acid sequence of calmodulin from animal (invertebrate or vertebrate) and plant is highly conserved which presumably allows the non-species-dependent activation of phosphodiesterase. Calmodulins all contain large amounts of acidic residues and a relatively low amount of basic residues resulting in an isoelectric point near 4. The calmodulins have phe/tyr ratios near 8:2 and have no tryptophan residues. The mobilities, as shown by gel electrophoresis of various animal and plant species, display great similarity suggesting they may also have very similar physical and chemical properties (for comparison of species and tissue specificity see Cheung 1980, Wang and Waisman 1979, and Wolff and Brostrom 1979).

Calmodulin is an acidic, globular protein of molecular weight 16,680 (Watterson, Sharief and Vanaman 1980). It is a single polypeptide chain of 148 amino acids. One-third of the residues are aspartate or glutamate. The N-terminus is acetylated and there is one ε-N-trimethyl-lysine of unknown function. The intrinsic fluorescense spectra (see Figure 1) is predominately due to the two tyrosine

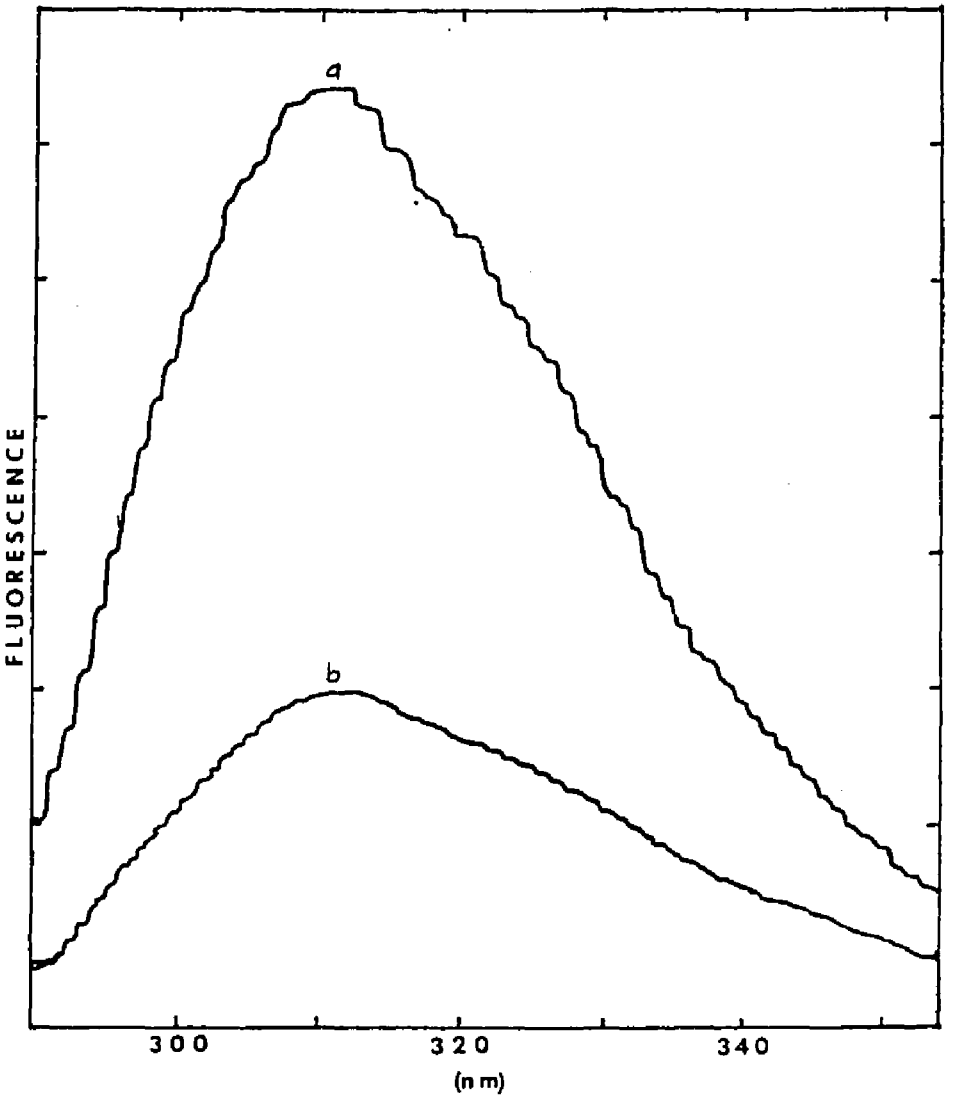

Figure 1. Quenching of calmodulin tyrosine fluorescence by calcium removal with EDTA. -- a) spectrum of calcium-saturated calmodulin, b) spectrum of calmodulin missed with EDTA to give calcium-free spectra. Conditions: calmodulin, 20 μM in 20 mM BTP, 0.1 M KCl, pH 8, 25°C, λex = 280 nm.

residues, Tyr 99 and Tyr 138. Calmodulin is very stable at low and neutral pH and at temperatures near boiling. Calcium-bound calmodulin shows a much greater stability to heat and denaturation and is more resistant to proteolytic anzymes (Wang et al. 1975, Lui and Cheung 1976, Ho et al. 1976).

Activation of calmodulin (CaM) regulated enzymes is thought to occur in two steps. The calmodulin molecule is activated by binding n = 1 - 4 calcium ions, this complex is then capable of binding to and activating the enzyme molecule (Equation 1).

$$CaM + nCa^{+2} \rightarrow CaM\text{-}Ca_n \text{ (active)} + E \rightarrow E\text{-}CaM\text{-}Ca_n \text{ (active)} \quad (1)$$

Phosphodiesterase has been shown to require more than two bound calcium ions per calmodulin molecule for activation (Crouch and Klee 1980). Whether all enzymes require the same calcium-calmodulin complex for activation or whether enzymes under calmodulin control require different degrees of calcium occupancy, possibly as a form of enzyme regulation, is unknown.

Calcium-binding sites have been proposed for calmodulin by comparison of the amino acid sequences of homologous calcium-binding proteins including parvalbumin, troponin-C and calmodulin (Goodman et al. 1979) and by analogy to the proposed structure of the homologous protein, troponin-C (Watterson et al. 1980). Troponin-C is the calcium modulator of the calcium-dependent contraction of skeletal muscle. The complete amino acid sequence of bovine brain calmodulin has been determined by Watterson et al. (1980). Calmodulin

was found to be homologous to troponin-C from bovine cardiac and skeletal muscle having identical residues in 50% of the positions. Another 30% are functionally and genetically conservative replacements. The amino acid sequence of troponin-C is composed of four homologous regions, each region contains a calcium-binding site. Calmodulin has an even greater level of internal homology. All four domains of calmodulin are similar in sequence, but the homology is highest between Domains I and III (residues 8-40 and 81-113, respectively) and between Domains II and IV (residues 44-76 and 117-148, respectively). Kretsinger and Barry (1975) predicted a 3-dimensional structure of troponin-C (rabbit skeletal) based on the crystal structure of parvalbumin and its sequence homology to troponin-C. In the region of troponin-C that best corresponds to parvalbumin, about 30% of the residues are identical (Collins et al. 1973). The regions of greatest similarity are the two 12 residue segments that correspond to the calcium-binding sites of parvalbumin. Eight regions in troponin-C can be predicted to be helical based on sequence data, which correspond well with known α-helical regions in parvalbumin. Kretsinger (1979) has described a basic evolutionary unit, the EF-hand, as the structure of the binding sites in these calcium-binding proteins. The hypothesized calcium-binding sites consist of a 12 residue loop containing potential liganding oxygen groups surrounded on each side by regions of α-helices. Watterson et al. (1980), based on Kretsinger's hypothesis have proposed that the four homologous domains in calmodulin constitute the calcium-binding sites. Each domain has

a 12 residue loop serving as the calcium-binding site, with both ends linked to a helical region of 8 residues. Six oxygen-containing residues provide the necessary ligands to calcium. The prediction of these four sites in troponin-C and calmodulin is supported by the experimental finding that both proteins bind four calcium ions per molecule (see Kretsinger 1980 for review).

Watterson et al. (1976) have concluded from equilibrium dialysis experiments that two classes of sites exist with dissociation constants of 1.1 μM and 8.6 μM. Most laboratories (see Lin 1982 for review) have found two classes of sites with dissociation constants between 0.2 and 20 μM. A few studies have reported a single class of binding sites (Kuo and Coffee 1976, Dedman et al. 1977, Haiech, Klee and Demaille 1981). Positive cooperativity between the first and second calcium ion bound has been observed by equilibrium dialysis, circular dichroism (Crouch and Klee 1980) and tyrosine fluorescense studies (Wang et al. 1982) finding Hill coefficients of 1.33, 1.22. and 2, respectively. Several investigators have suggested a possible sequence of calcium binding, but contradictions in the data exist that prevent any solid conclusions.

Most of the experimental data presented here is restricted to the binding of the first two calcium ions to calmodulin. The binding of these calcium ions will be considered independent from the binding of the third and fourth calcium ions to calmodulin. This simplification is allowed if the first two calcium sites bind with sufficiently higher affinity such that there is never a significant concentration

of calcium ions at the second two sites. This simplification is also true in the case where the last two sites filled do not exist until the first two sites are filled.

The simplest mechanism for calcium ions binding to two sites on calmodulin is the sequential two-step model of Equation 2.

$$CaM + Ca^{+2} \underset{k_{-1}}{\overset{k_1}{=}} CaM\text{-}Ca_1 \underset{k_{-2}}{\overset{k_2}{=}} CaM\text{-}Ca_2 \qquad (2)$$

The association constant for the binding of the first calcium ion, K_1, is equal to k_1/k_{-1} and for the second calcium ion, K_2 equals k_2/k_{-2}. An overall affinity constant for calcium binding to these sites can be written as equal to K_1K_2.

In addition to equilibrium data, transient kinetics provide an important tool for studying ligand binding mechanisms. Physiological conditions are generally not at equilibrium, therefore the determination of the rate constants at each step of the formation of the calcium-calmodulin complex may improve our understanding of how calmodulin activates a large variety of enzymes and calcium-dependent processes.

The focus of the experimental results presented here is to measure the kinetic properties of calcium binding to calmodulin by stopped-flow fluorometry. A discussion of the results of equilibrium fluorescence measurements with regard to theoretical models of fluorescence enhancement induced by ligand binding to a protein is also presented.

BACKGROUND

Effects of Calcium on the Physical Properties of Calmodulin

Calcium binding causes alterations in calmodulin that are evident from the observation of changes in the physical properties of the protein. The UV-absorbance spectrum shows multiple peaks at 253, 259, 265, 269 and 277 nm (Liu and Cheung 1976, Stevens et al. 1976, Watterson et al. 1976). Change in the absorbance spectrum has been shown to occur on binding calcium (Wang et al. 1975, Klee 1977). The occurrence of alterations in calmodulin upon binding calcium ions is also indicated by changes in the fluorescence emission (Wang et al. 1975, Dedman et al. 1977), circular dichroism (Dedman et al. 1977, Klee 1977, Wolff et al. 1977), nuclear magnetic resonance (Seamon 1980), optical rotatory dispersion (Liu and Cheung 1976), laser Raman spectroscopy (Seaton et al. 1983), and altered susceptibility to chemical modifications (Walsh and Stevens 1977, Richman and Klee 1978).

Other cations such as Mg^{+2} (Wolff et al. 1977, Haiech et al. 1981), Mn^{+2} (Wolff et al. 1977), K^{+} (Haiech et al. 1981) have been found to compete with calcium for binding sites on calmodulin, but either do not effectively cause activation of calmodulin-regulated enzymes or the binding affinities are much lower than for calcium. These cations induce alterations in the calmodulin molecule as

observed by fluorescence (Kilhoffer, Demaille and Gerard 1981) and laser Raman spectroscopy (Seaton et al. 1983), but the changes are not identical to those induced by calcium.

The fluorescence spectra of calmodulin are a frequently used monitor of the effects of calcium binding to calmodulin. The fluorescence of the calcium-free protein is dependent on ionic strength, but the calcium saturated protein is not (Kilhoffer et al. 1981). The change in the intrinsic fluorescence of calmodulin is complete with the binding of two calcium ions per calmodulin molecule (Kilhoffer, Demaille and Gerard 1980a, Wang et al. 1982). Octupus calmodulin, however, contains only one tyrosine residue which is analogous to Try 138 in mammalian calmodulin. Maximum fluorescence of octupus also occurs with two moles of calcium bound per mole of calmodulin (Kilhoffer, Demaille and Gerard 1980b). Fluorescence lifetime, fluorescence polarization measurements, and ionic quenching of tyrosine fluorescence have been used to compare ram testes and octupus calmodulin in order to differentiate between the effects of calcium binding on Tyr 99 and Tyr 138 (Kilhoffer et al. 1981). The conclusions, based on the assumption that the behavior of mammalian Tyr 138 is similar to the single tyrosine residue in octupus, indicate that the two tyr residues in mammalian calmodulin are in different microenvironments in the absence of calcium. Tyr 138 appears to be most exposed to solvent in the absence of calcium whereas Tyr 99 shows characteristics of a freely exposed residue in the presence or absence of calcium. Tyr 138 is almost completely quenched in the absence of calcium and becomes

dequenched on binding calcium. Much less initial quenching of Tyr 99 occurs undergoing a large dequenching in the presence of calcium characteristic of a large change in its microenvironment. The mammalian tyrosine residue, Tyr 99, appears to be responsible for nearly 70% of the tyrosine emission.

Investigations of the chemical reactivity of the tyrosine residues to acetylation and nitration support the existence of the Tyr 99 ring being freely exposed to solvent in the presence or absence of calcium (Richman 1978), but suggests that the largest calcium-induced change occurs in the microenvironment of the Tyr 138. The pKa of Tyr 99 is 10.1 and 10.4 in the presence and absence of calcium, respectively, which are similar to the pKa for a solvent-exposed tyrosine ring (Klee 1977). After nitration, the pKa of Tyr 99 is not perturbed by calcium binding and has an observed pKa of 7.3 (Richman and Klee 1978). The pKa of Tyr 138 in native calmodulin is 11.9 and appears calcium-independent. As a nitrotyrosine residue, the pKa of Tyr 138 is increased by calcium binding from 8.1 to 8.6 (Richman and Klee 1979). The environment of Tyr 138 appears to be significantly altered by bound calcium making it relatively inaccessible to solvent (Richman and Klee 1979). The conclusions reached by these investigators is that Tyr 138, but not Tyr 99, is in a microenvironment substantially altered by calcium binding to calmodulin.

In contrast to the fluorescence studies, but in agreement with the nitration studies, NMR data (Seamon 1980) suggest that calcium binding causes the largest change in the microenvironment at Tyr 138

whereas only a small change occurs at the Tyr 99 microenvironment. Although calcium binding to all four sites affects the resonances of the tyr and phe residues, binding of the first two calcium ions affects the trimethyllysine residue resonance. The first two calcium ions bound appear to affect a large portion of the calmodulin molecule whereas the last two calciums appear to cause only local perturbations.

Laser Raman spectroscopy also supports the existence of two overall levels or perturbation of the protein molecule by cations (Seaton et al. 1983). Calcium alone induces a perturbation of the peptide backbone with a small increase in α-helix and β-sheet structures. Both Ca^{+2} and Mg^{+2} induce a similar perturbation most likely the result of side chain reorientation.

Circular dichroism studies of calmodulin also indicate that calcium affects the environment of both tyr and phe residues with a nearly complete change occuring upon binding two moles of calcium per mole of calmodulin (Klee 1977, Crouch and Klee 1980, Kilhoffer et al. 1981). Ellipicity at 222 nm, which is a measure of the change in α-helix content, increases during calcium titration of calmodulin until the metal/protein ratio reaches 4:1. Therefore the binding of the third and fourth calcium ion also results in perturbations in the calmodulin molecule (Wang et al. 1982). CD studies indicate that calcium binding induces about a 10% increase in total α-helix content of calmodulin from ∿35% to ∿45% (Dedman et al. 1977, Klee 1977, Wolff et al. 1977).

The energy transfer properties of the lanthanide ion, terbium, have been exploited to investigate the order of metal binding to calmodulin. Competition experiments show that terbium and calcium ions reversibly compete for the same sites (Kilhoffer et al. 1980a, Wallace et al. 1982). Energy transfer can occur from both tyr residues (Wang et al. 1982), presumably in Domains III and IV. The enhancement of both the tyrosine fluorescence (Kilhoffer et al. 1980a, Wang et al. 1982) and the terbium luminescence (Kilhoffer et al. 1980a, Wallace et al. 1982, Wang et al. 1982) is complete during the addition of two terbium ions. One group of investigators (Wallace et al. 1982) have shown that the terbium emission enhancement occurs upon the binding of the second and third terbium ion to calmodulin, but other laboratories (Kilhoffer et al. 1980a, Wang et al. 1982) suggest that the enhancement occurs with the addition of the third and fourth terbium ion. Whether terbium and calcium bind to calmodulin in the same order is not known.

Kinetics of Calcium Interaction with Calmodulin

An observed calcium dissociation rate from calmodulin has been measured using stopped-flow fluorometry (Malencik et al. 1981). Rapid mixing of calcium-bound calmodulin with the calcium chelotor, EGTA, was found to give a first order dissociation rate constant of $\sim$10 s^{-1}. In the same study, a dansyl derivative of calmodulin produced a biphasic decrease in fluorescence upon addition of EDTA with rate constants of 10 s^{-1} and 0.31 s^{-1}. Monitoring the tyrosine fluorescence

of the dansyl derivative gave a first order rate constant of 8 s^{-1} suggesting that dansylation did not affect the two tyrosine residues and could be used to monitor changes occurring at the other calcium-binding sites. Walsh and Stevens (1977) have shown that oxidation of a single methionine can drastically reduce the calcium binding affinity of calmodulin. Since dansylation reacts with methionine residues, it is important to demonstrate that any covalent modification of calmodulin is not altering the calcium-binding properties of the protein.

MATERIALS AND METHODS

Calmodulin was prepared by a purification procedure developed in this laboratory based on the purification of parvalbumins. One volume of bovine testes was blended with two volumes of 10 mM TRIS, 1mM $MgCl_2$ and 1 mM EGTA, pH 7.5, and allowed to settle in the refrigerator at 4°C for 30 min. This mixture was then centrifuged for 10 min. at 5K rpm in a Beckman J-14 rotor to remove cellular debris. More buffer was added to the solid fraction and this step was repeated. The combined supernatants were fractionated by the slow addition of acetone at 4°C at 0-45, 45-55, and 55-75 (v/v)%. The precipitate of the 55-75 (v/v)% fraction, which is predominately calmodulin, was dialyzed in 1 mM $CaCl_2$, 20 mM TRIS and 0.5 M NaCl, pH 7.5, and centrifuged for 30 min. at 30K rpm (Beckman J-35) to remove any debris. The protein was diluted to 5 mg/ml and heated to 85°C, then rapidly cooled to 20°C and centrifuged for 15 min. at 10K rpm (Beckman J-14). Reagents were added to the supernatant to make the final concentration 5 mM $MgCl_2$, 5 mM EGTA and 40 g ammonium sulfate per 100 ml. After sitting for several minutes, any precipitate formed was removed by centrifugation for 10 min. at 10K rpm (Beckman J-14). Calmodulin was precipitated by reducing the pH to 4.0 with acetic acid. At this point the yield of calmodulin was 100 mg/Kg and was greater than 90 (w/w)% pure as judged by SDS gel

electrophoresis. Additional purification required for spectroscopic experiments was obtained by phenothiazine affinity chromatography (Jamieson and Vanaman 1979).

Calmodulin concentrations were determined by using an extinction coefficient of 3300 $M^{-1}cm^{-1}$ at 280 nm. The protein was stored at -20°C when not in use.

Calcium-free protein was obtained by the addition of EDTA "in site" or by removal of the bound calcium from salt-free lyophilized calmodulin by trichloroacetic acid precicipation (Haiech et al. 1981). Residual calcium was found to be 0.03-0.3 moles calcium per mole of calmodulin after acid precipitation by atomic absorption analysis. Calcium-free protein was also stored at -20°C, but used within three days because of possible reduced stability.

Special care was taken to avoid calcium contamination from external sources in these studies. All solutions were made from twice-distilled water which contains less than 0.5 μM calcium by atomic absorption analysis. Dialysis was performed with tubing boiled three times in EDTA-carbonate solution (pH 9-10) followed by boiling three times in twice-distilled water. Buffers and inorganic salts were reagent grade with the exception of specially purified potassium chloride (Suprapur grade EM Laboratories, < 0.1 ppm calcium) used in buffers for calcium-free protein. A solution of 0.2 M bis-tris-propane (BTP), 1 M KCl was run through a cyanogen bromide activated-sepharose column that was covalently crosslinked to carp parvalbumin to reduce its calcium content. All reagents used in

buffers were analyzed by atomic absorption for calcium content. Solutions of 20 mM BTP, borate, TRIS, HEPES, or PIPES were found to contain less than 1μM calcium, 20 mM MES contained 5 μM calcium. Reagents that were found to contribute a final concentration of less than 1 μM calcium to the protein buffer were used for buffers in the preparation of calcium-free protein. Plasticware were used almost exclusively to avoid calcium contamination from glass and washed prior to use in a EDTA-carbonate colution (pH > 9) to remove calcium.

Calcium buffers were made with the calcium chelators, EDTA, EGTA and NTA. Stock calcium solutions were prepared from either calcium chloride or calcium carbonate. The calcium chloride concentration was determined by titration of the chloride ion with silver nitrate. The calcium carbonate was oven-dried overnight before it was weighed and dissolved by the addition of HCl until the pH was 7. Calcium buffers were made at the required free calcium concentrations by varying the concentration ratio of calcium-chelator complex to free chelator at constant pH (± 0.02 pH units adjusted with HCl or NaOH). The different concentration ratios were achieved by combination of stock solutions of free chelator and calcium-bound chelator in varying volumetric proportions. The stock solutions of the calcium-chelator complexes (CaEDTA, CaEGTA and CaNTA) were made by titrating, at constant pH, a known concentration of free chelator with calcium. The titration was complete when a free calcium concentration was reached at which total chelator concentration equalled total

calcium concentration as calculated from the pK_{Ca}. Direct measurement of the free calcium ion concentration was made by a Radiometer calcium selectrode calibrated by calcium chloride standards.

The apparent pK_{Ca} at a given pH, PK'_{ca}, was calculated from Equation 3 using the pK_{Ca} of the deprotonated ligand, pK''_{Ca}, and the pK_a(s).

$$pK'_{Ca} = pK''_{Ca} + (\log [1 + 10(pK_{a1} - pH) + 10(pK_{a1}pK_{a2} - 2pH)]) \quad (3)$$

The pK'_{Ca} was calculated from the following literature values for conditions of 0.1 M KCl at 20°C: $NTA^{-3} + H^{+} = HNTA^{-2}$, 9.73 (Moddak and Dertel 1957); $NTA^{-3} + Ca^{+2} = CaNTA^{-1}$, 6.46 (Irving and Miles 1966); $EGTA^{-4} + H^{+} = HEGTA^{-3}$, 0.46 (Anderegg 1964); $HEGTA^{-3} + H^{+} = HEGTA^{-2}$, 8.85 (Anderegg 1964); $EGTA^{-4} + Ca^{+2} = CaEGTA^{-2}$, 11.0 (Irving and Miles 1966); $EDTA^{-4} + H^{+} = HEDTA^{-3}$, 10.23 (Grimes, Huggard and Wilford 1963); $HEDTA^{-3} + H^{+} = HEDTA^{-2}$, 6.16 (Grimes et al. 1963); $EDTA^{-4} + Ca^{+2} = CaEDTA^{-2}$, 10.59 (Grimes et al. 1963). The calculated free calcium concentrations of EDTA and EGTA were in agreement within a factor of two of the calcium selectrode determination. The agreement of the free-calcium concentrations for the NTA buffers was within 2%.

A fluorescent calcium chelator, QUIN-2, was purchased from Calbiochem-Behring. The concentration was determined with the extinction coefficient of 3,800 $M^{-1}cm^{-1}$ at 350 nm.

Fluorescence titrations and equilibrium measurements were performed on a Spex Fluorolog equipped with a thermobath (20°C or 25°C) and a magnetic stirrer. Calmodulin was excited at 280 ± 5 nm and fluorescence was monitored at 313 ± 5 nm.

Kinetic experiments were conducted on a laboratory-built stopped-flow fluorometer that has a deadtime of about 5 ms (described in White 1982). Protein and calcium buffers or free chelators were loaded into thermostated drive syringes, equilibrated at 20°C, then rapidly mixed in equal volumes. Solutions were degassed prior to use. The excitation was provided by a xenon or mercury arc lamp. The desired excitation and emission wavelengths were obtained by interference filters. The photomultiplier output was interfaced to a Data General Nova III minicomputer for calculations and data storage. The data from 4-7 identicals runs was fit to single exponentials with floating end points (Foss 1970). Calculated fits were compared to observed data by plotting both together or by direct comparison of printed data points. Alternatively, the photomultiplier output was recorded with an Explorer III oscilloscope (Nicolet Instruments Corporation). The traces were fit with an analog fitting procedure, the fit trace was superimposed on the observed trace and plotted for comparison.

RESULTS

The fluorescence spectra of calcium-saturated and calcium-free bovine calmodulin is shown by curves a and b in Figure 1, respectively. The fluorescence decrease with EDTA addition is 2.5-fold with no shift of the wavelength maximum in agreement with Dedman et al. (1977).

Titration of High Affinity Calcium-Binding Sites with EDTA and Calcium

A fluorescence titration of calcium-free calmodulin with $CaCl_2$, monitored at the wavelength of maximum fluorescence, 313 nm, is shown in Figure 2. Greater than 80% of the maximum fluorescence change occurs with the addition of two moles of calcium per mole of calmodulin. EDTA at pH $\geq$ 8 can stoichiometrically remove calcium from calmodulin since it has a binding affinity for calcium that is two orders of magnitude greater than that of calmodulin (2×10^8 M^{-1} compared to approximately 10^6 M^{-1} for calmodulin). The resulting fluorescence decrease observed upon addition of EDTA (Figure 2) correlated well with that of calcium addition demonstrating reversible calcium binding to calmodulin. Essentially identical results are found for calcium addition and removal over the pH range 7.5 to 9 with calmodulin concentrations from 4 to 90 M. Ideally, the fractional fluorescence change in Figure 2 would be expected to increase linearly reaching 100% fluorescence change at a ratio of 2 Ca/CaM if all calcium added to the

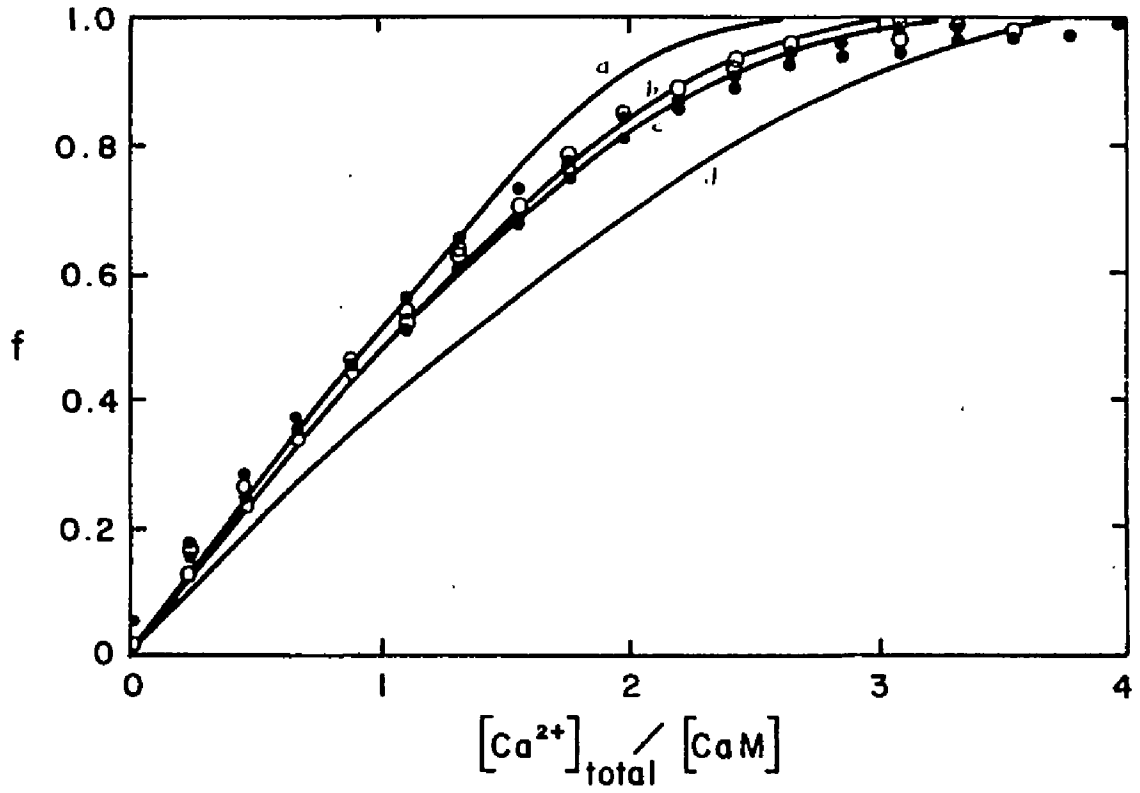

Figure 2. Stoichiometric fluorescence titration of calcium-free calmodulin with $CaCl_2$ (○) and subsequent removal of calcium with EDTA (●). -- Conditions: calmodulin, 18 μM in 20 mM borate, 0.1 M KCl, pH 9.0, 20°C, λex = 280 nm, λem = 313 nm. The theoretical curves assume the mechanism of Equation 8 using a) $K_1 = 10^5\ M^{-1}$, $K_2 = 10^7\ M^{-1}$, $K_3 = K_4 = 0$, [CaM] = 20 μM; b) $K_1 = 10^5\ M^{-1}$, $K_2 = 10^5\ M^{-1}$, $K_3 = K_4 = 2 \times 10^5\ M^{-1}$, [CaM] = 100 μM; c) $K_1 = 10^5\ M^{-1}$, $K_2 = 10^5\ M^{-1}$, $K_3 = K_4 = 2 \times 10^5\ M^{-1}$, [CaM] = 20 μM; d) $K_1 = K_2 = K_3 = K_4 = 10^6\ M^{-1}$.

solution was protein bound, i.e., stoichiometric binding. For less than stoichiometric binding, the ratio of calcium to calmodulin needed to achieve maximal fluorescence change would increase with decreasing protein concentrations. Since the observed fractional fluorescense change is independent of calmodulin concentration over the experimental concentration range (4-90 μM), the calcium binding must approach stoichiometric binding. The lack of a complete fluorescence increase between 0 to 2 Ca/CaM could be explained if some of the added calcium ions bound to lower affinity sites. (The theoretical curves in Figure 2 will be discussed later.) The observation in Figure 2 that the tyrosine fluorescence enhancement is associated with the binding of the first two calcium ions to the high affinity sites on calmodulin is in agreement with (Kilhoffer et al. 1980a, Wang et al. 1982).

Determination of the Equilibrium Constants of Calcium Binding to the High Affinity Sites

The equilibrium and kinetic studies presented here use intrinsic tyrosine fluorescence as an indicator of the binding of calcium to calmodulin. Since the tyrosine fluorescence change is associated with the binding of calcium to the two high affinity sites, only the equilibrium and rate constants for calcium binding to these two sites can be measured by changes in tyrosine fluorescence.

The equilibrium dependence of calcium binding on free calcium concentration was studied with the aid of the calcium chelators EDTA, EGTA and NTA, and their corresponding calcium-chelator complexes

CaEDTA, CaEGTA and CaNTA. The calcium chelators at concentrations greater than that of calmodulin served as calcium buffers to maintain a constant free calcium concentration during each fluorescent measurement. The pH at which each experiment was performed was selected so that the calcium-binding affinity of the chelator employed was similar to that of calmodulin. Fluorescence measurements were made of protein solutions containing a fixed concentration of the free chelator EDTA, EGTA or NTA to which the fully calcium-bound chelator CaEDTA, CaEGTA, or CaNTA was added causing a change in the free calcium concentration. Alternatively, protein solutions were prepared with a fixed concentration of calcium-bound chelator and the free calcium concentration changed by the addition of free chelator. The free calcium concentrations were obtained by measuring the free calcium concentration at identical ratios of calcium-bound chelator to free chelator with a calcium selectrode. The data were analyzed according to the method of Hill (Hill 1925) in which f is the fractional fluorescence enhancement. Figure 3 shows the data obtained in the CaNTA buffered experiments at pH 9. The dependence of $\log(f/1-f)$ on $\log([Ca^{+2}])$ is linear over three logarithmic scales and has a slope (Hill coefficient) of 2.0 ± 0.2. The free calcium concentration at which the ratio f/1-f is equal to one in Figure 3 is $1.1 \pm 0.2 \times 10^{-6}$ M. The significance of this calcium concentration will be discussed below. Similar data obtained by use of the calcium chelators EDTA at pH 6 and EGTA at pH 6.8 were reduced by linear regression and are summarized in Table 1. The observed Hill coefficient of 2.0 ± 0.6 indicates cooperative lignad

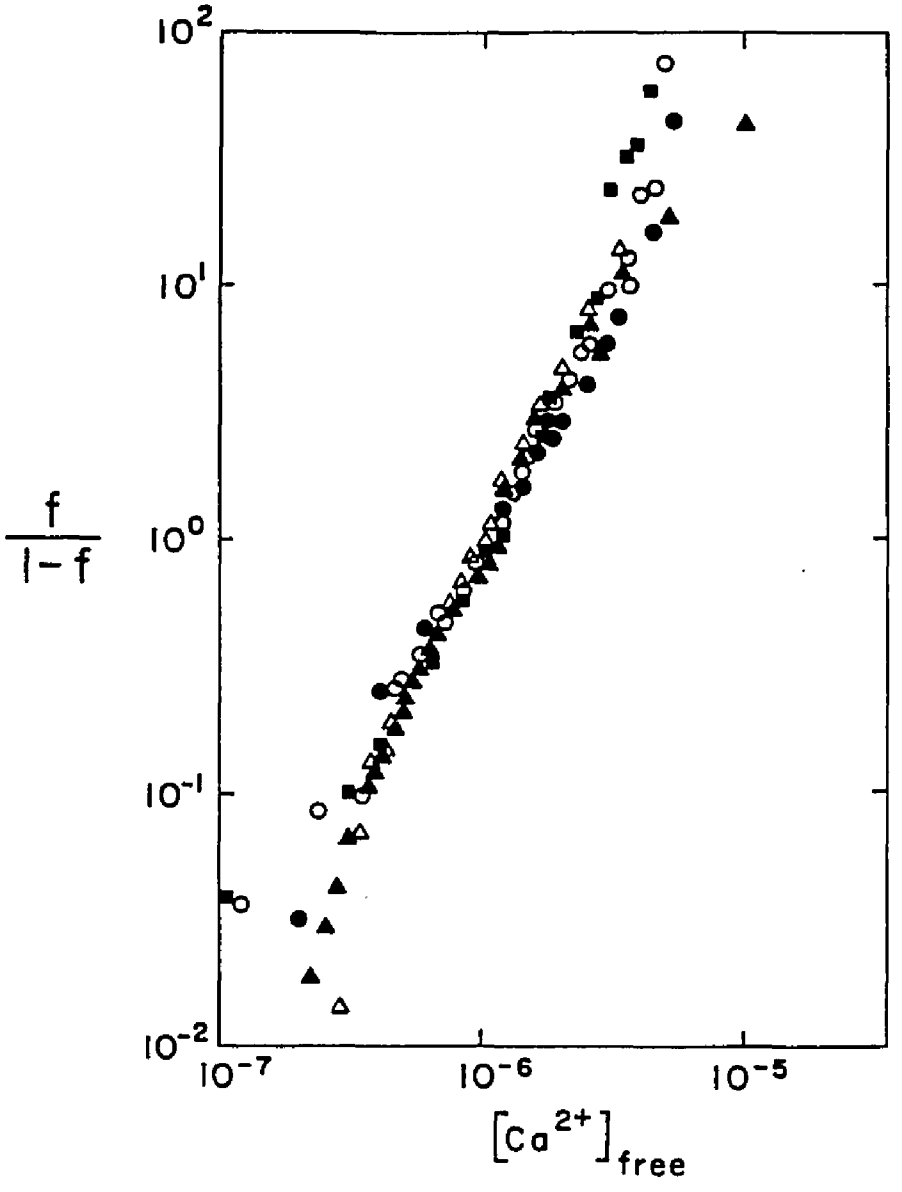

Figure 3. Hill plot of the equilibrium fluorescence dependence of calmodulin on free calcium concentration. -- The symbols (○, ●, △, ▲,■) represent individual but identical experiments.--f = fractional fluorescence change. Conditions: calmodulin, 15 μM in 20 mM BTP, 0.1 M KCl, pH 9, 25°C, λex = 280 nm, λem = 313 nm. The free calcium concentration was maintained by NTA.

Table 1. Summary of the equilibrium dependence of the fractional fluorescence change on free calcium concentration

Chelator	pH	$[Ca^{2+}]_{free}$ at f/1-f = 1 (μM)	Hill Coefficient
EDTA	6.0	2.7 ± 0.3	1.9 ± 0.2
EGTA	6.8	1.3 ± 0.3	2.0 ± 0.5
NTA	9.0	1.1 ± 0.2	2.0 ± 0.2

binding. The fluorescence equilibrium data therefore support the reported positive cooperative binding between the calcium ions bound to the two high affinity sites on calmodulin (Crouch and Klee 1980, Wang et al. 1982). The free calcium concentration at which f/1-f = 1 ranges from 1.1 - 2.7 μM for pH 9-6. The decrease in the affinity of calmodulin for calcium with decreasing pH may be due to protonation of one or more of the carboxylic acid side chains in the binding site. Direct competition between H^{+} and Ca^{+2} for available ligands or electrostatic repulsion between calcium ions and calmodulin due to the protein being more positively charged at lower pH may be causing the lowered calcium affinity.

The curves in Figure 4 represent the theoretical dependence of ratio f/1-f on free calcium concentration. The fractional change in fluorescence can be written as the ratio of the sum of the concentrations of the molecular species of calmodulin contributing to the fluorescence enhancement to the sum of all calmodulin species. The total fluorescence change seen when two calcium ions bind to calmodulin could require only the binding of the first calcium ion, require the binding of both calcium ions, or partial change could occur upon the binding of each calcium ion. In order to vary the extent of cooperativity between the equilibrium constants from Equation 2, K_1 and K_2 can be represented as αK and K/α, respectively, such that

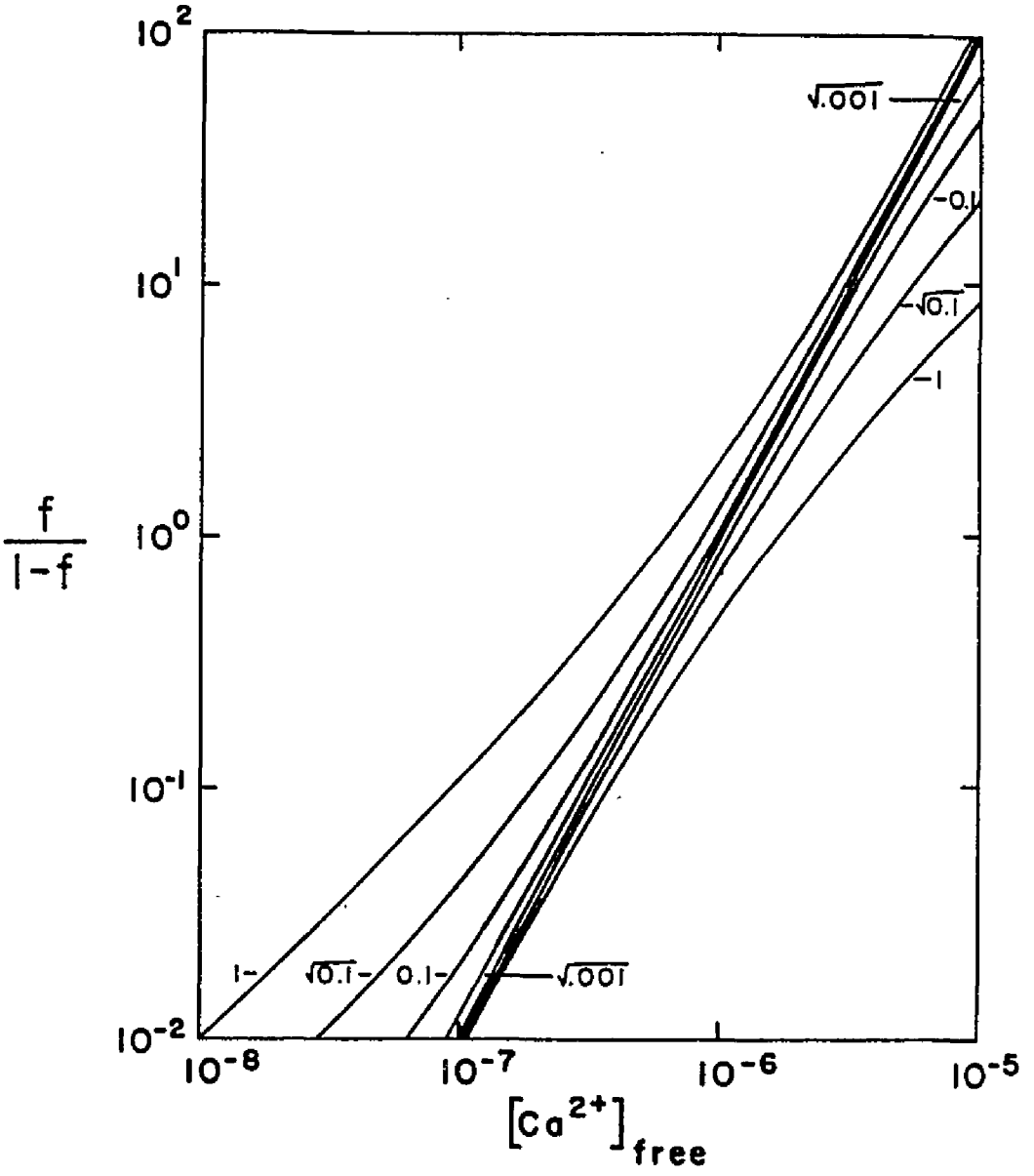

Figure 4. Theoretical curves for the equilibrium fluorescence dependence of free calcium concentration. -- The fractional fluorescence change, f, was calculated from Equation 6 using $K = 10^6\ M^{-1}$, $B = 0$ or 1, and $\alpha = 1$, $\sqrt{0.1}$, 0.1, and $\sqrt{0.001}$. For comparison the data from Figure 3 is represented by the shaded area.

$K_1K_2 = K^2$. Using the equilibrium expressions for each calcium ion bound to calmodulin in (Equation 4 and 5),

$$K_1 = \alpha K = [CaM\text{-}Ca_1] \;/\; [CaM]\,[Ca^{+2}] \tag{4}$$

$$K_2 = K/\alpha = [CaM\text{-}Ca_2] \;/\; [CaM\text{-}Ca_1]\,[Ca^{+2}] \tag{5}$$

f can be written as a function of $[Ca^{+2}]$, and K as shown in Equation 6.

$$f = \frac{\alpha B\, K\, [Ca^{+2}] + K^2\, [Ca^{+2}]^2}{1 + \alpha K\, [Ca^{+2}] + K^2\, [Ca^{+2}]^2} \tag{6}$$

The variable B is equal to one in the case where the fluorescence change requires only the first calcium ion bound. If the fluorescence change requires the binding of both calcium ions, B equals zero. For the cases where fluorescence change occurs during the binding of each calcium ion, $0 < B < 1$.

Assuming $K = 10^6\ M^{-1}$, theoretical curves were plotted in Figure 4 using Equation 6 for B = 0 and 1. Values of $\alpha = 1$, $\sqrt{0.1}$, 0.1, $\sqrt{0.001}$ make $K_2 > K_1$ by a factor of 1, 10, 100 and 1000, respectively. The curves that could be constructed with $0 < B < 1$ lie within the boundaries of the curves shown in Figure 4. At values of $\alpha = 0.1$, the theoretical curves for all values of B are close together and nearly parallel near f/1-f = 1. As α becomes less than 0.1, the theoretical curves begin to merge.

The shaded area in Figure 4 represents the data from Figure 3. The close agreement of the data at pH 9 with the theoretical curves at $\alpha \geq 0.1$ suggests that the binding affinity of calmodulin for the second calcium ion is tighter than the affinity for the first calcium ion by a factor $\geq 10^2$. Therefore the equilibrium data indicates cooperative binding of calcium to the high affinity sites with $K_2 > 100K_1$.

For the case where the fluorescence change upon binding of the first calcium ion by calmodulin is equal to the fluorescence change upon binding the second calcium ion (B = 0.5), the free calcium concentration is equal to 1/K when f/1-f = 1. For cases of unequal fluorescence change associated with the binding of each calcium ion ($B \neq 0.5$), 1/K approaches the free calcium concentration at f/1-f = 1 as α decreases. In Figure 4, the free calcium concentration tends toward 1 μM at f/1-f as α decreases. At $\alpha = \sqrt{0.001}$, the free calcium concentration is within 2% of the value of 1/K (recall K equals 10^6 M^{-1} for the theoretical curves in Figure 4). Therefore, as the binding of calcium at the high affinity sites on calmodulin becomes more positively cooperative, the free calcium concentration at f/1-f = 1 approaches 1/K. This is consistent with the disappearance of the single liganded calmodulin molecule at highly cooperative binding such that f/1-f is equal to the ratio of the concentration of calmodulin bound to two calcium ions to the concentration of calcium-free calmodulin (Equation 7).

$$\frac{f}{1 - f} = \frac{[CaM - Ca2]}{[CaM]} \qquad (7)$$

From the combination of Equations 4, 5 and 7 it can be seen that at f/1-f = 1, the free calcium concentration, $[Ca^{+2}]$, is equal to $(K_1K_2)^{-1/2}$ under conditions of positive cooperativity. Thus a comparison of the equilibrium data with the theoretical curves in Figure 4 indicates that $(K_1K_2)^{-1/2}$ is equal to 1 μM and $K_2 \geq 100K_1$ for the two step mechanism in Equation 2. Thus, $K_1 \leq 10^5\ M^{-1}$ and $K_2 \geq 10^7\ M^{-1}$.

The theoretical curves plotted in Figure 2 describe the simplest model for four calcium ions binding to calmodulin. The sequential, four step mechanism is shown in Equation 8.

$$CaM \overset{K_1}{=} CaM\text{-}Ca_1 \overset{K_2}{=} CaM\text{-}Ca_2 \overset{K_3}{=} CaM\text{-}Ca_3 \overset{K_4}{=} CaM\text{-}Ca_4 \qquad (8)$$

The affinity constants for each step are represented by K_1, K_2, K_3 and K_4. Using the results of the equilibrium experiments (Figure 3 and 4), the high affinity constants are set at $K_1 = 10^5\ M^{-1}$ and $K_2 = 10^7\ M^{-1}$. Curve (a) shows the fluorescence change when only two calcium ions bind to calmodulin ($K_3 = K_4 = 0$). The fluorescence change is greater than 90% complete at a ratio of 2 Ca/CaM. Reported literature values for the binding constants of the low affinity sites, K_3 and K_4, are near $10^5\ M^{-1}$ (Watterson et al. 1980). If the binding affinities K_3 and K_4 are set equal to $2 \times 10^5\ M^{-1}$, the curves (b) and (c) result for [CaM] = 100 μM and 20 μM, respectively. The effect of calcium binding to the lower affinity sites is to decrease the fluorescence enhancement to 80-84% completion at a ratio of 2 Ca/CaM. Changing the calmodulin concentration by a factor of five caused

only a slight change in the fluorescence enhancement. With the binding affinities set equal such that all sites have high affinity for calcium and are non-cooperative, $K_1 = K_2 = K_3 = K_4 = 10^6\ M^{-1}$. This case is shown in curve (d). Here, the fluorescence enhancement is only about 70% complete at a ratio of 2 Ca/CaM. The good agreement between the data and curve (c) in Figure 2 provides additional evidence for cooperative binding between the two high affinity sites and indicates that the fluorescence increase on binding two calcium ions to the high affinity sites is probably affected by calcium binding to the lower affinity sites.

Kinetics of Calcium Dissociation from Calmodulin

In Figure 5, the kinetics of calcium dissociation from calmodulin were measured by rapid mixing of calcium-saturated calmodulin with excess EDTA at pH > 7.5. The resulting fluorescence decay is fit very accurately by a single exponential with an observed rate constant of $8.4 \pm 0.3\ s^{-1}$. The observed dissociation rate constant over the pH range of 6.5 to 9 was found to be $8.5 \pm 1.7\ s^{-1}$ by observing the tyrosine fluorescence decay of calmodulin using the calcium chelators EDTA and EGTA at several concentration ratios of chelator to protein (Table 2). No relationship between chelator or chelator concentration and the rate constant was observed as long as there was a sufficient amount of chelator to remove all calcium from the protein. This observation is important because it demonstrates that the observed fluorescence change is not due to the chelator

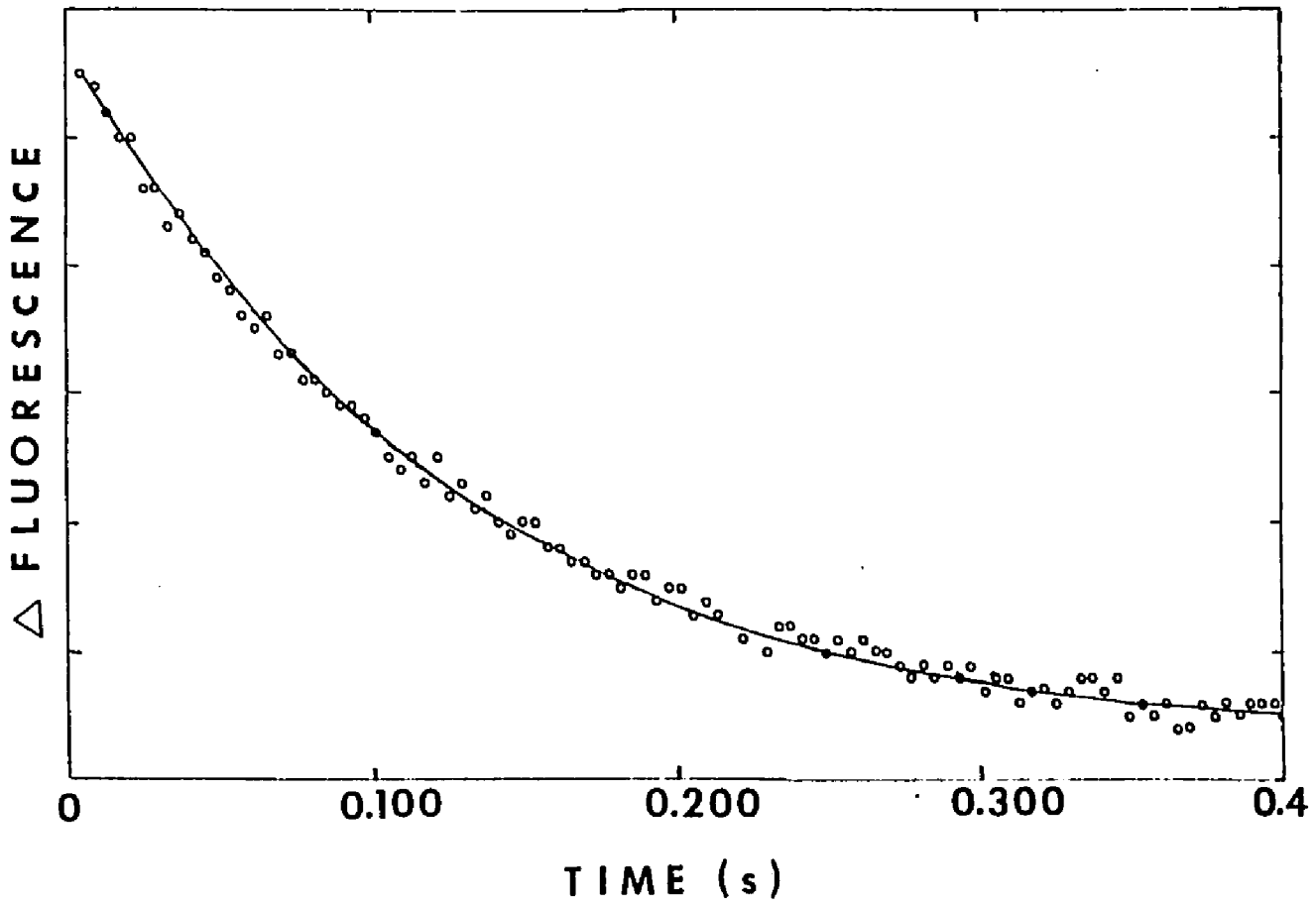

Figure 5. Calcium dissociation from calmodulin as observed by tyrosine fluorescence change upon addition of 1 mM EDTA. -- Conditions: calcium-saturated calmodulin, 21 μM in 20 mM BTP, 0.1 M, pH 7.8, 20°C, em = 280 nm, em = 313 nm. The solid line is a single exponential with a rate constant of 8.4 s^{-1}.

Table 2. Summary of the observed association and dissociation rate constants for calcium binding to the high affinity sites on calmodulin

		Association		Dissociation	
Chelator	pH	k ($M^{-1}s^{-1}$)	Range of [Ca^{2+}] Free	l (s^{-1})	$\frac{[Chelator]}{[Protein]}$
EDTA	5.5	$4.5 \pm 2.0 \times 10^6$	1 - 25 μM		
	5.7				
	7.8			8.4 ± 0.3	50
	7.9			8.6 ± 0.4	8
	9.0			6.8 ± 0.8	30
EGTA	6.5	$6.3 \pm 1.0 \times 10^6$	0.5 - 8 μM		
	6.5			10.3 ± 0.4	130
	6.5			9.7 ± 0.3	130
	6.5			10.4 ± 0.6	33
	7.8			8.3 ± 0.6	50
NTA	9.3	$1.2 \pm 0.1 \times 10^7$	0.5 - 6 μM		
QUIN-2	9.0			6.8 ± 0.2	27
	9.0			6.7 ± 0.2	13
	9.0			6.9 ± 0.2	7

binding to calmodulin. The observed dissociation rate constant appears to decrease from about 10 s^{-1} to 7 s^{-1} with increasing pH from 6.5 to 9.

Measurement of the kinetics of calcium dissociation was also made by rapidly mixing the fluorescent, calcium-chelator QUIN-2 (Tsien 1980) with calcium-saturated calmodulin and monitoring fluorescence enhancement of QUIN-2 (λex = 340 nm) with a 440 nm cut-off filter. Traces a and b in Figure 6 show the observed first order fluorescent enhancement of QUIN-2 over an observation of time of 1 s and 50 ms, respectively. The observed rate constant was found to be 6.8 ± 0.3 s^{-1} at pH 9.0. The agreement between these two rate constants indicates that the QUIN-2 fluorescence enhancement is due to calcium coming off the high affinity sites of calmodulin. This conclusion is further supported by the observation that the QUIN-2 fluorescence change corresponds to the addition of two moles of calcium per mole of calmodulin. Trace b demonstrates that no additional faster reactions were measurable with half times less than ~ 1 ms. The baseline fluorescence of QUIN-2 (chelator mixed with buffer) is shown in Trace c. The rapid initial fluorescence enhancement between Trace c and Trace a indicates that the rate constants for calcium dissociation from the low affinity sites are greater than 500 s^{-1} which is the fastest measurable rate under the conditions of the experiment. The amplitude of this fast jump corresponds to the binding of less than two moles of calcium per mole of calmodulin which is probably due to a lack of calcium saturation of the calmodulin.

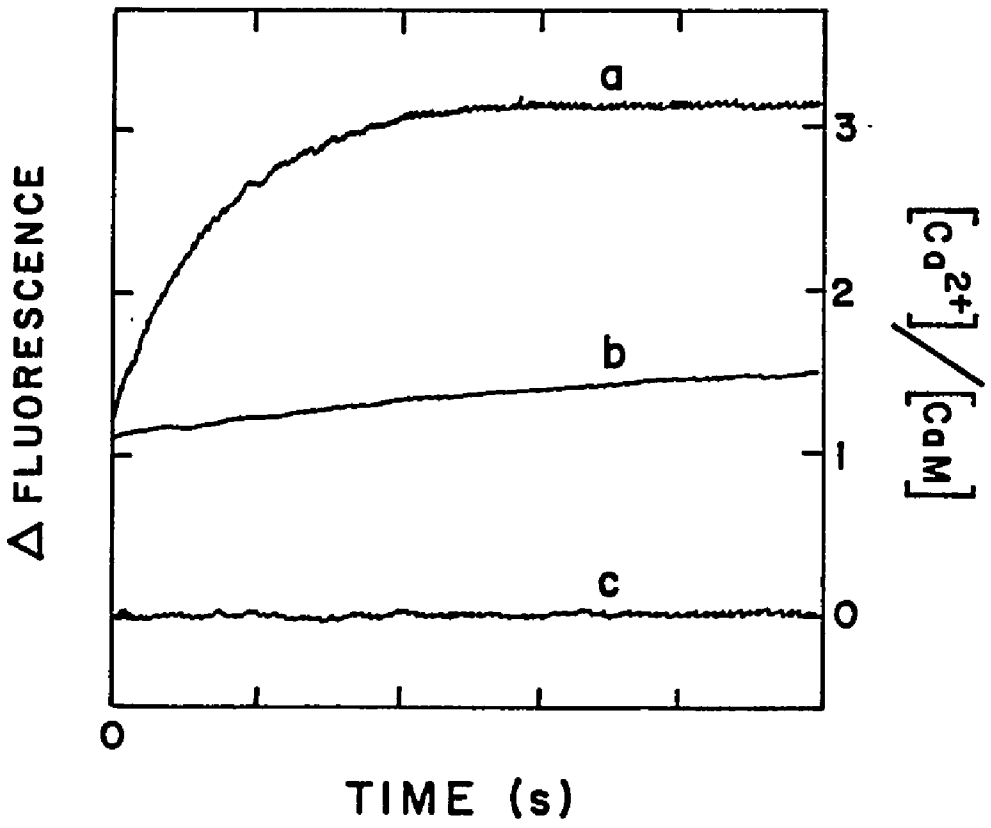

Figure 6. Calcium dissociation from calmodulin as observed by fluorescence enhancement of QUIN-2. -- a) 1.0 s and b) 50 ms observation time; c) baseline: QUIN-2 mixed with buffer. The data was fit to a single exponential with a rate constant of 6.8 s^{-1}. Conditions: calmodulin, 15 μM in 20 mM borate, 0.1 M KCl, pH 9.0, 20°C, λex = 340 nm, λem $\geq$ 440 nm.

Kinetics of Calcium Binding to Calmodulin

The kinetics of calcium binding to calmodulin were measured by rapid mixing of calcium-free calmodulin with EDTA, EGTA and NTA calcium buffers to give the desired free calcium concentration. The tyrosine fluorescence enhancement induced upon calcium binding to calmodulin is seen in Figure 7. In initial experiments, 100 moles of EDTA per mole of protein was mixed with calmodulin to remove calcium from the protein (prior to rapid mixing with a calcium buffer). Alternatively, calcium-free protein was prepared by precipiation of the protein with trichloroacetic acid. The results were the same for both methods. The fluorescence enhancement upon calcium binding are fit very accurately by a single exponential over the range of 0.5-6 μM free calcium at pH 9 as illustrated by Figure 7 at 5.7 μM free calcium.

The dependence of the observed rate constant on free calcium concentration is shown in Figures 8-10 for each of the calcium chelators employed. Each observed rate constant represents the average of 4-7 identical runs. The free-calcium concentration was determined by theoretical calculation as described in the Methods section and by direct measurement with a calcium electrode.

Data obtained with NTA calcium buffers show an initial lag in the free calcium concentration dependence of the observed association rate constant (Figure 8). The data are consistent with the sequential two step mechanism shown in Equation 2 fit with the rate constants listed in the legend of Figure 8. The slope of the linear portion of the fit corresponds to a second order rate constant

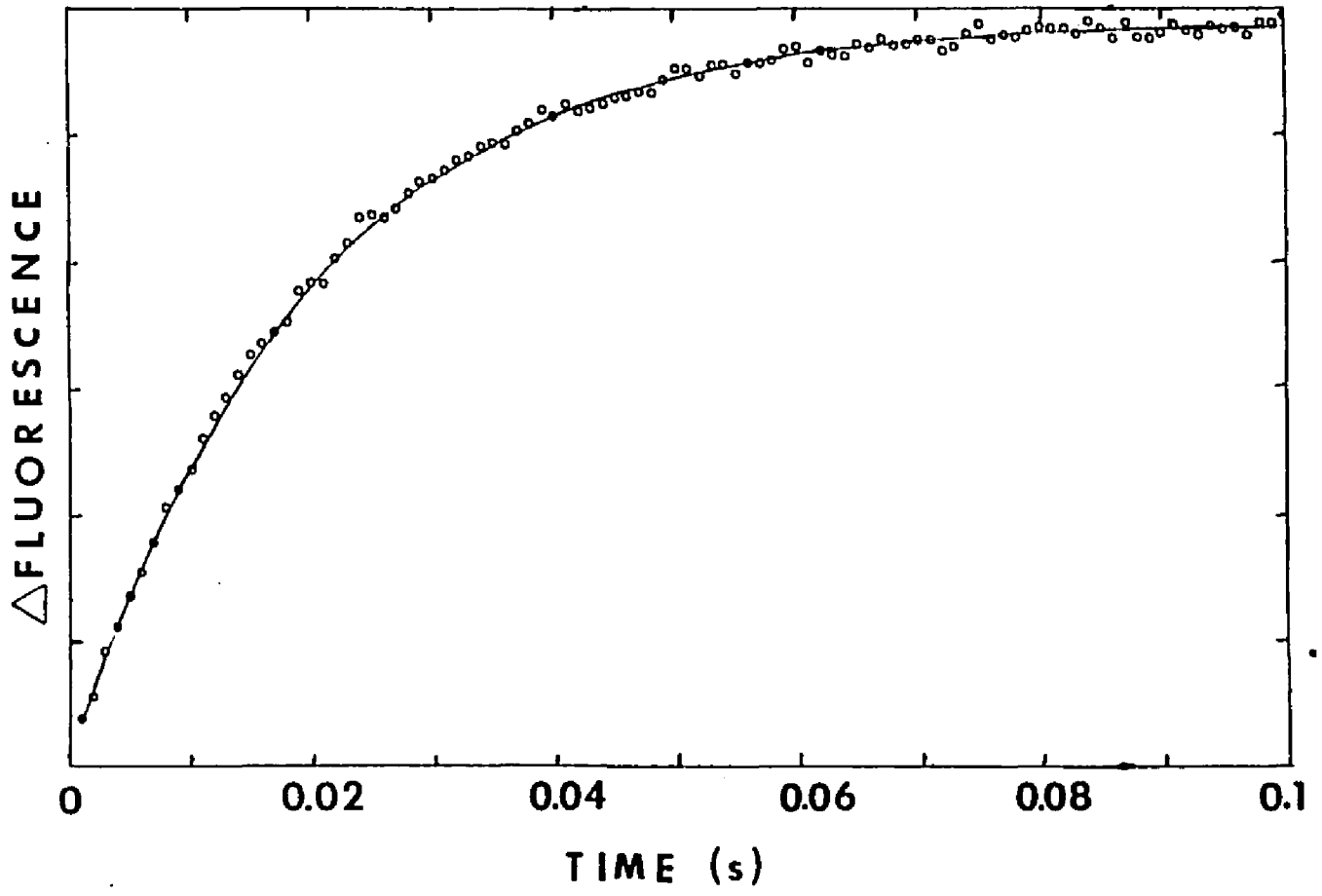

Figure 7. Calcium binding to calmodulin observed by tyrosine fluorescence change upon addition of calcium. -- The free calcium concentration was maintained by 10 mM NTA calcium buffer. The solid line is a single exponential with a rate constant of 60 s^{-1}. Conditions: calmodulin, 15 μM in 20 mM borate, 0.1 M KCl, pH 9.3, 20°C, λex = 280 nm, λem = 313 nm.

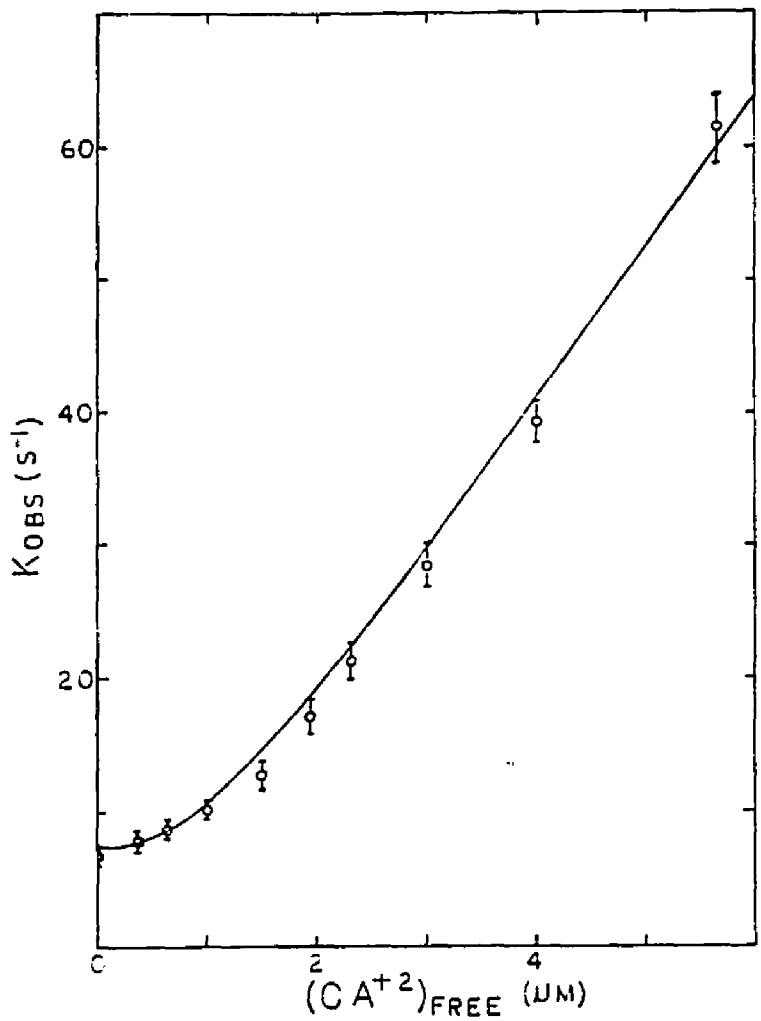

Figure 8. Dependence of the observed calcium association rate on free calcium concentration at pH 9. -- The data was fit with the mechanism of Equation 2 (solutions of the required differential equations were found by Arne Strand, unpublished) using $k_1 = 1.2 \times 10^7$ $M^{-1}s^{-1}$, $k_2 = 10^8$ $M^{-1}s^{-1}$, $k_{-1} = 80$ s^{-1} and $k_{-2} = 9$ s^{-1}. Conditions are the same as in Figure 7.

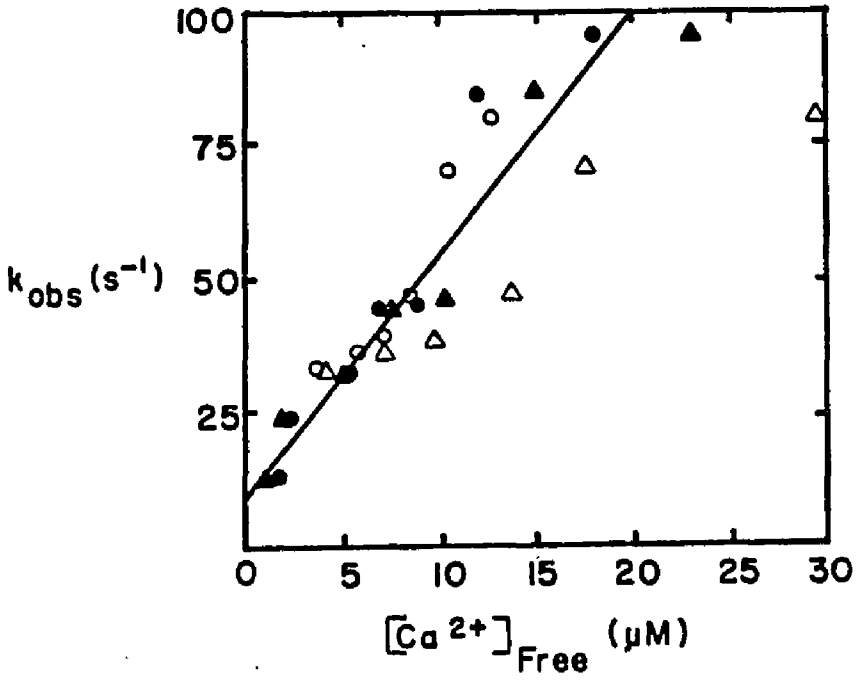

Figure 9. Dependence of the observed calcium association rate on free calcium concentration at pH 5.5 and 5.7. -- The free calcium concentrations were measured by a calcium selectrode (△,▲) or calculated as described in Materials and Methods (○,●). The open and closed symbols represent data at pH 5.5 and 5.7, respectively. The line through the data gives a slope of $4.5 \pm 2.0 \times 10^6$ $M^{-1}s^{-1}$. The y-intercept was set at 10 s^{-1} (see text). Conditions: calmodulin, 15 μM in 20 mM MES, 0.1 KCl, pH 5.5 or 5.7, 20°C, λex = 280 nm, λem = 313 nm. 10 mM EDTA were used to maintain a free calcium concentration.

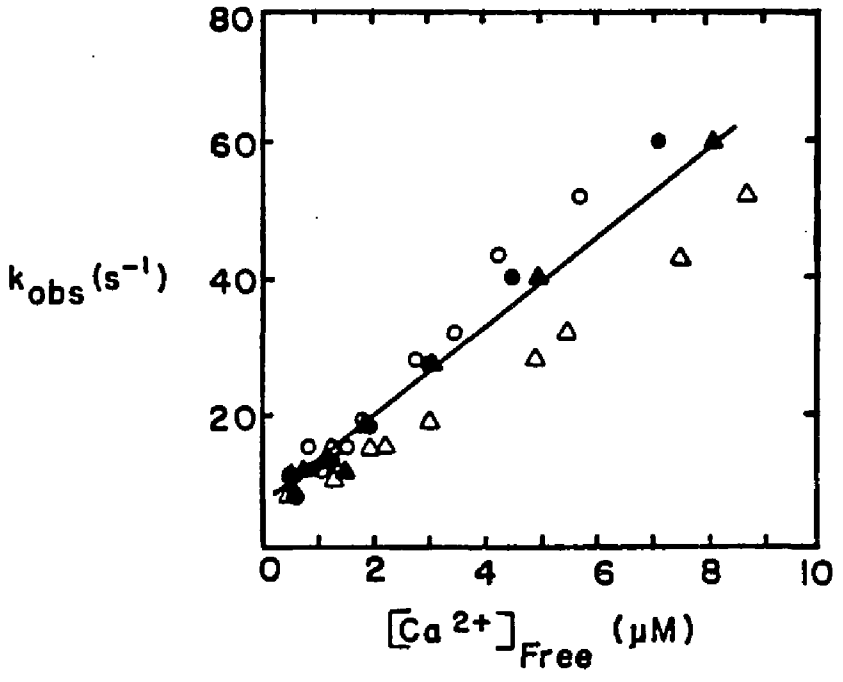

Figure 10. Dependence of the observed calcium association rate on free calcium concentration at pH 6.5. -- Symbols and conditions are the same as in Figure 9 with the exception of the pH. The slope of a line through the data gives $6.3 \pm 1.0 \times 10^6$ $M^{-1}s^{-1}$. The y-intercept is 7 s^{-1}.

of 1.2×10^7 $M^{-1}s^{-1}$ for calcium binding to the first site. The dissociation rate constant of 9 s^{-1} corresponds to the y-intercept and is only a slightly higher value than 6.8 ± 0.8 s^{-1} (Table 2), obtained by direct measurement of the dissociation rate constant at pH 9. The good agreement of the data with the theoretical curve for this mechanism provides additional evidence for cooperative binding between the first two calcium ions bound to calmodulin.

A straight line was drawn through the set of data obtained by the EDTA (Figure 9) and the EGTA (Figure 10) calcium buffers. The intercept of the EDTA data was set at 10 s^{-1} because of the direct measurement of calcium dissociation rate constant is near 10 s^{-1} (see Table 2). The data obtained with EDTA calcium buffers (pH 5.5 and 5.7) in Figure 9 give a slope corresponding to a second order rate constant of $4.5 \pm 2.0 \times 10^6$ $M^{-1}s^{-1}$. The intercept at pH 6.5 falls at 8 ± 2 s^{-1} which is in the range of the dissociation rate constants listed in Table 2 for pH 6.5. The apparent second order rate constants found for calcium binding to calmodulin are listed in Table 2. The differences found in the second order association rate constant may reflect a decrease in the binding affinity of calmodulin for calcium with a decrease in pH from 9 (CaNTA buffered) to 5.5 (CaEDTA buffered). A parallel decrease in the association constant was observed.

DISCUSSION

Assignment of the rate constraints for the binding of calcium to calmodulin were made by consideration of both the equilibrium and the kinetic data presented here. The conclusions of these studies are shown in the following mechanism for calcium binding to four sites on calmodulin (Equation 9).

$$
\begin{array}{lll}
k_1 = 1.2 \times 10^7 M^{-1}s^{-1} & k_2 \geq 6 \times 10^7 M^{-1}s^{-1} & k_3 \geq 10^8 M^{-1}s^{-1} \\
CaM = CaM - Ca_1 = & CaM - Ca_2 = & CaM - Ca_3 \\
k_{-1} \geq 130s^{-1} & k_{-2} = 7s^{-1} & k_{-3} > 500s^{-1} \\
k_2 \geq 10^8 M^{-1}s^{-1} & & \\
= CaM - Ca_4 & & \\
k_{-4} > 500s^{-1} & &
\end{array}
\qquad (9)
$$

The values of k_1 and k_{-2} were measured directly (Table 2). The equilibrium data from Figure 3 indicate cooperative binding between the two high affinity sites with $K_2 \geq 100K_1$. With this equality, the affinity constants K_1 and K_2 were determined from Table 1 since $(K_1K_2)^{-\frac{1}{2}} = 10^{-6}M$ from Figure 3. Lower limits on the rate constants k_{-1} and k_2 were calculated using $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. Experiments employing the calcium chelator, QUIN-2 (Figure 6) demonstrate that the dissociation rate constants for the low affinity sites, k_{-3} and k_{-4}

are > $500s^{-1}$. Given that the binding constants for the low affinity sites, K_3 and K_4, are near $10^5 M^{-1}$, the second order rate constants, k_3 and k_4, must be at least $10^8 M^{-1}s^{-1}$ or greater.

Equation 9 is the most detailed mechanism that can be written based on the data presented. The second order rate constants for the binding of all but the first calsium ion are near the diffusion-limited rate (about $10^8 M^{-1}s^{-1}$) expected for calcium binding to a macromolecule. The second order rate constant for the binding of the first calcium ion is much slower than expected for a diffusion-limited rate constant and suggests a two step mechanism. Speculative mechanisms for the binding of the first calcium can be made. Calcium colliding and binding weakly to calmodulin followed by a major structural rearrangement of the protein is one possible mechanism for the binding of the first calcium ion (Equation 10).

$$CaM = CaM - Ca_1 = CaM - Ca_1^* \qquad (10)$$

The asterisk designates the structurally rearranged species. Another possibility is the establishment of a rapid equilibrium prior to a diffusion rate-limited binding of the calcium ion (Equation 11).

$$CaM = CaM^* = CaM - Ca_1^* \qquad (11)$$

In either case, formation of an altered single-liganded calmodulin complex is followed by fast binding of calcium to the three remaining sites limited only by the rate of diffusion of calcium ion the protein.

Binding four ligands to a protein can be, kinetically, very complicated. In principle, there can exist 16 possible populations:

1 ligeand-free, 4 single liganded, 6 diliganded, 4 trilinganded, and 1 fully liganded protein population (see Figure 11). Again, for simplicity, consider the binding of calcium to the high affinity sites on calmodulin (Equation 12).

$$\begin{array}{ccccc} & & \overset{K_1}{=} & \overset{K_2}{=} & \\ & & \text{Ca - CaM-} & & \\ \text{CaM} & & & \text{Ca - CaM - Ca} & \\ & & \text{-CaM - Ca} & & \\ & & \underset{K_1'}{=} & \underset{K_2'}{=} & \end{array} \qquad (12)$$

The association constants K_1, K_2, K_1' and K_2' represent the equilibrium conditions among the four molecular species. Note that two possible populations of single liganded calmodulin can exist. The type of interaction between the two calcium-binding sites is reflected in the relative size of the association constants. The calcium sites can be independent such that $K_1 = K_2'$ and $K_2 = K_1'$. Identical sites binding cooperatively would result in $K_1 = K_1'$, with $K_1 << K_2$ and $K_1 << K_2$. A balance of the equilibrium equations for the binding of calcium to two sites requires that $K_1K_2 = K_1'K_2'$. Thus, cooperative binding to two non-identical sites could result in four nonequal association constants although K_1K_2 must equal $K_1'K_2'$. Negative cooperativity could occur in one branch such that $K_1' >> K_2'$. Although the kinetic and equilibrium data obtained here do not rule out branched mechanisms of the type shown in Equation 12 and Figure 11, the data are most simply explained by the sequential, cooperative binding of calcium to the high affinity sites, followed by calcium binding to the low affinity sites.

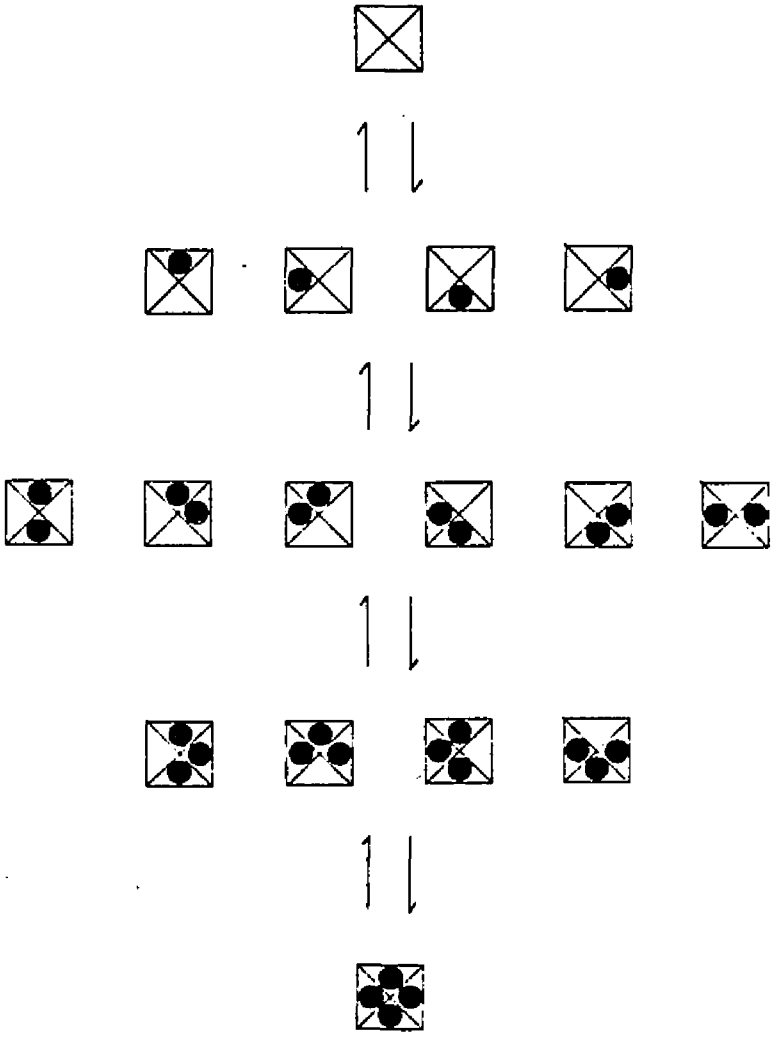

Figure 11. General mechanism for ligand binding to four sites on a macromolecule. -- The squares represent the protein and the filled circles represent calcium ions.

Calmoduling is one of a family of homologous calcium-binding proteins. Parvalbumin, a 11,000 MW calcium-binding protein found in muscle, has two calcium-binding sites that show positive cooperativity (Cave et al. 1979, White 1983). Both parvalbumin and the high affinity sites of calmodulin bind two calcium ions following the general mechanism of Equation 13, P represents either protein.

$$P + Ca^{+2} \overset{\text{non-diffusion-limited}}{=} P - Ca_1 \overset{\text{diffusion-limited}}{=} P - Ca_2 \qquad (13)$$

For Whiting parvalbumin (White 1983), the non-diffusion-limited second order rate constant is $1.4 \times 10^6 M^{-1}s^{-1}$ and $K_2 \geq 100K_1$. For both proteins, binding of the first calcium ion alters the protein molecule allowing fast binding of calcium to the remaining sites limited only by the rate of diffusion to the protein. The similarities between the calcium-binding properties of parvalbumin and calmoduling suggest that the mechanism of Equation 13 may be a general model for calcium binding to this group of homologous calcium-binding proteins. To date no kinetic data is available from the other homologous calcium-binding proteins of this family to further support this model.

APPENDIX A

ABBREVIATIONS USED IN THE TEXT

CaM, calmodulin

CaM - Ca_n, calmodulin bound to n calcium ions

CD, circular dichroism

E, enzyme

E - CaM - Ca_n, calmodulin-activated enzyme

EDTA, ethylenediaminetetracetic acid

EGTA, ethylene glycol bis(β - aminoethyl ether) - N, N, N′, N′-tetraacetic acid

HEPES N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid

k, rate constant

K, affinity or equilibrium constant

MES, 2(N-morpholino) ethane sulfonic acid

NMR, nuclear magnetic resonance

NTA, nitrilotriacetate

PIPES, 1,4-piperazinediethane sulfonic acid

TRIS, tris(hydroxymethyl)aminomethane

UV, ultra-violet

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