Use of Engineered Proteins with Internal Tryptophan Reporter Groups and Pertubation Techniques To Probe the Mechanism of Ligand–Protein Interactions: Investigation of the Mechanism of Calcium Binding to Calmodulin†

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Received April 9, 1992; Revised Manuscript Received June 10, 1992

ABSTRACT: Stopped-flow kinetic and fluorescence spectroscopic analyses, including solvent and temperature perturbations, of five isofunctional structural mutants of calmodulin indicate that calcium binding to calmodulin follows the order site III, site IV, site I, site II, with dissociation occurring in the reverse order. Each of the isofunctional structural mutants contains a single tryptophan residue, introduced by site-specific mutagenesis, as an internal spectroscopic reporter group that was used as a probe of local conformational change. Calcium binding was studied by using flow dialysis or by using fluorescence spectroscopy and monitoring the change in the single tryptophan residue in each calcium-binding site. Calcium removal was examined by using EDTA and monitoring tryptophan fluorescence or by using Quin 2 and monitoring the change in the chromophoric chelator. Computational analysis of the data suggests a rate-limiting step for dissociation between calcium removal from sites I/II and sites III/IV. Unexpected results with the site IV isofunctional mutant (Q135W-CaM) indicated cross-talk between the amino and carboxyl terminal halves of CaM during the calcium-binding mechanism. Studies with ethylene glycol provided empirical data that suggest the functional importance of the electrostatic potential of CaM, or the molarity of water, in the calcium-binding process. Altogether, the data allowed a kinetic extension of the sequential, cooperative model for calcium binding to calmodulin and provided values for additional parameters in the model of calcium binding to CaM, a prototypical member of the family of proteins required for calcium signal transduction in eukaryotic cells. Further, the experimental approach of using isofunctional structural mutants in perturbation kinetic studies may prove to be a general strategy for the analysis of protein–ligand interactions.

Calcium is one of the intracellular messengers used by eukaryotic and prokaryotic cells to manage external information [see Carafoli (1987) for review]. This property of the calcium ion to be a specific and quantitative carrier of information inside a cell is due to the existence of two systems: one able to modulate the Ca^{2+} concentration in the cytoplasm in response to external stimuli and one able to decipher such modulations in the Ca^{2+} concentration. This last system is composed of a family of calcium-binding proteins able to reversibly bind Ca^{2+} in the physiological concentration range [see Van Eldik et al. (1982) for review]. Among these proteins, calmodulin (CaM)[1] is found in all eukaryotic cells tested so far and appears as a prototypical representative of this superfamily of proteins. A knowledge of the mechanism of calcium binding to calmodulin is, therefore, key to our understanding of how a calcium signal can initiate or modulate a cellular response to a stimulus.

In order to obtain insight into mechanism, at least two fundamental aspects of calmodulin function must be analyzed: the mechanism of calcium binding to calmodulin and the interaction of calmodulin with, and activation of, various target enzymes. Numerous studies over the past 10 years have been devoted to answering the first question, although few kinetic analyses have been reported [for a review, see Haiech et al. (1989)]. As a result, there are still several extant models to explain the mechanism of the four calcium ions binding to calmodulin. These models can be grouped into three types: (1) four independent and equivalent sites (Milos et al., 1986); (2) four independent sites, but two affinity classes, i.e., two pairs of independent sites (Forsen et al., 1986); and (3) cooperative binding among the sites (Haiech et al., 1981, 1989; Wang, 1985; Yazawa et al., 1987; Linse et al., 1991). The first model has the appeal of simplicity and is in agreement with the experimental data obtained by direct binding studies, such as those done by using dialysis methods. The second model is based mostly on early NMR and other spectroscopy studies in which spectral changes were monitored during titration experiments. The cooperative model assumes a coupling between the different sites of calmodulin, with either positive or negative cooperativity.

Two types of cooperative models have been proposed in order to take into account both direct binding data and spectrophotometric titration data. One cooperative model (Wang, 1985; Linse et al., 1991) assumes that (1) the two sites of a given lobe of calmodulin exhibit positive cooperativity, (2) the mean affinity of the C-terminal pair of sites is 6–10 times

† This work was supported by grants from the Centre National de la Recherche Scientifique (France), National Institute of Health Grant GM 30861 (USA), the National Science Foundation U.S.–France Cooperative Research Program, and the Association pour la Recherche contre le Cancer (France).

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CaM, calmodulin; Trp, tryptophan; EG, ethylene glycol; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; Quin 2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline.

0006-2960/92/0431-8098$03.00/0

higher than that of the N-terminal pair, and (3) there is no coupling between the N-terminal and the C-terminal lobes. The second type of cooperative model assumes coupling between contiguous sites in the CaM structure (i.e., cooperativity between sites that are adjacent in the sequence) and between noncontiguous sets of sites (i.e., between the sets of sites in the amino and carboxy termini). A variation of this second type of cooperativity model (Yasawa et al., 1987) postulates that coupling between the two halves of CaM occurs only in the presence of a calmodulin-binding structure, although a detailed description of this model is not available. Another variation of the second type of cooperativity model for calcium binding to calmodulin (Haiech et al., 1981) assumes that there is a possibility of cooperative interactions between individual sites or sets of sites either in the presence or in the absence of a calmodulin-binding structure and that there is an ordered and sequential binding of calcium. Thus, all of the cooperative models assume coupling between contiguous sites; the second type of model assumes coupling between all four sites, with the sequential model assuming an ordered site occupancy and coupling between all four sites.

From a mathematical point of view, it is interesting to note that the sequential model which has been described so far (Haiech et al., 1981), using four parameters, can simulate the model proposed by Wang (1985), which uses only three parameters. Similarly, the model proposed by Wang can simulate the two noncooperative models (the one with four independent and equivalent sites uses one parameter and the second one uses two parameters). However, the model of Wang cannot simulate the sequential model, and the two models without cooperativity are incompatible. A complete description of a protein with four sites for a ligand, will need 15 parameters corresponding to the actual occupancy of each site by calcium (four sites) and the coupling factors between sites (11 constants, with 6 constants describing the modification of the protein when one site is occupied, 4 constants when two sites are occupied, and 1 constant when three sites are occupied). Up to date, to take into account the whole set of experimental data gathered with all kinds of techniques, including the kinetic experiments (Martin et al., 1985) which cannot be explained by the model of Wang, a model with at least four parameters is needed.

Because the CaM signal transducation pathway is a prototype for models of cellular and organismal responses mediated by calcium signals, it is necessary to dissect further these details of calcium binding to calmodulin in order to develop a more unified model of the calcium response mechanism in eukaryotic cells. The studies reported here are an attempt to provide deeper insight into the kinetic mechanism of calcium binding to calmodulin. The approach is one that combines modern methods of genetic engineering with classical stopped-flow kinetic/perturbation methods (Brossa et al., 1984; Barman et Travers, 1985). Specifically, we have combined the use of isofunctional structural mutations of CaM, in which single tryptophans have been introduced into each calcium binding loop in order to obtain an internal spectroscopic reporter group (Kilhoffer et al., 1988), with the use of temperature and solvent perturbations of calcium binding. During the course of attempts to perturb the binding by decreased temperature, we noted a discrete effect of the cryogenic solvent ethylene glycol and have utilized this effect as a perturbance during the kinetic analysis of calcium–protein interaction. This interdisciplinary approach allowed us to analyze calcium binding while monitoring the conformational changes in each calcium ligation site as reported spectroscopically by the different tryptophans. The results are presented in terms of a revised sequential model of calcium binding to calmodulin. While the results and model are specific to calcium binding to CaM, the approach may be a generally useful one for the analysis of ligand–protein interactions.

MATERIALS AND METHODS

Materials. Trichloracetic acid, RH Normapur, was obtained from Prolabo. Solutions were prepared fresh before use. Quin 2 was purchased from Calbiochem. All other chemicals were high-grade commercial products. Ultrapure water (Milli Q instrument from Millipore Corp.) was used throughout the experiments. Buffers were stored in acid-washed plasticware to minimized calcium contamination (calcium contamination was less than 5×10^{-7} M). Experiments were all performed in 50 mM Hepes, pH 7.5.

Methods. Production of Calmodulin Mutants. Five tryptophan-containing calmodulin mutants were produced. They are all derivatives of VU1-CaM or SYNCAM [Roberts et al. (1985); EMBL accession number M11334], a chimeric CaM that is indistinguishable from tissue-isolated vertebrate and plant CaMs, or CaM produced in *Escherichia coli* from a vertebrate cDNA, in its calcium-dependent activator activity with several vertebrate and plant enzymes (Roberts et al., 1984, 1985, 1986, 1987; Shoemaker et al., 1990). T26W-CaM, T62W-CaM, S81W-CaM, F99W-CaM, and Q135W-CaM differ from SYNCAM by the replacement of a single residue, Thr-26, Thr-62, Ser-81, Phe-99, and Asn-135, respectively, by tryptophan. Calmodulin mutagenesis and protein production were done in the VUCH-1 vector (Lukas et al., 1987) using cassette mutagenesis as previously described (Craig et al., 1987; Roberts et al., 1987; Kilhoffer et al., 1988). The correctness of constructs was verified by automated DNA sequence analysis as described in Zimmer et al. (1988), by amino acid compositional analysis (Schaeffer et al., 1987), by electrospray mass spectrometry (Van Doorselaer, unpublished results), and, in selected cases, by automated amino acid sequence analysis of the purified protein (Schaeffer et al., 1987). Fermentation and large-scale production of each protein was done at the CNRS Fermentation Center in Marseille, France (M. Scandellari, R. Toci, and M. Bauman).

Ca^{2+} Removal from Calmodulin Mutants. Ca^{2+} was removed from the different mutants using trichloracetic acid precipitation as described previously by Haiech et al. (1981).

Fluorescence Measurements. Steady-state fluorescence spectra were obtained with a Perkin-Elmer MPF 66 spectrofluorometer interfaced to a Perkin-Elmer 7500 computer. Excitation wavelength was set at 297 nm for selective excitation of tryptophan residues. Solution absorbance was always less than 0.11 at the excitation wavelength. Quantum yields (ϕ) of the tryptophan mutants were determined by taking tryptophan in water as a reference ($\phi = 0.14$ at 20 °C) and were corrected to account for the screening effect of scattered light (Hélène et al., 1971).

Linear Fluorescence Polarization Measurements. Fluorescence polarization is characterized by the degree of polarization:

$$p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are respectively the parallel and perpendicular components of the fluorescence. Measurements were carried out at 20 °C as described previously (Kilhoffer et al., 1989). Excitation wavelength was set at 297 nm and emission at 342 nm (interference filter with a band pass of 8 ± 0.5 nm).

Fluorometric Titrations of Calmodulin Mutants. Calcium titration of metal-free calmodulin mutants was performed by adding aliquots of $CaCl_2$ stock solution and following the change of a given fluorescence parameter (fluorescence intensity, shift in the fluorescence spectrum, or fluorescence polarization degree). Data were corrected for dilution.

Ca^{2+} Binding to Calmodulin Mutants. Flow dialysis experiments were performed as previously described (Haiech et al., 1981; Kilhoffer et al., 1988). Binding data were analyzed according to Haiech et al. (1991).

Protein Concentration. Protein concentrations were determined either by amino acid analysis or by UV absorption spectropy (Kilhoffer et al., 1988).

Kinetic Experiments. The kinetics of calcium dissociation from the different calmodulin mutants was measured by fluorescence stopped-flow spectroscopy using a home-built apparatus, the main part of which has been described elsewhere (Markley et al., 1981). Fluorescence was excited using a mercury lamp and a Jobin Yvon monochromator. Emission was measured at 90° to the incidence light.

For a typical experiment, the protein in buffer A (50 mM HEPES, pH 7.5, in the absence or presence of ethylene glycol) was loaded with calcium to a final stoichiometry of 10 Ca^{2+}/calmodulin. In the stopped-flow apparatus, calcium was then removed from calmodulin using either 10 mM EDTA (protein concentration $\sim 5 \times 10^{-5}$ M) or 100 μM Quin 2 (protein concentration $\sim 5 \times 10^{-6}$ M), depending on the experiment performed. EDTA or Quin 2 were dissolved in buffer A (the concentration of ethylene glycol being the same as the one used for the protein solution). When Ca^{2+} was removed with EDTA, the excitation wavelength was set at 297 nm and the emitted light was observed at $\lambda > 359$ nm using a cutoff filter (MTO A359C'). When Quin 2 was used, the excitation wavelength was set at 339 nm and emission was measured at $\lambda > 435$ nm. For each experiment, about three to five individual shots were averaged and analyzed according to one or two exponentials.

RESULTS AND DISCUSSION

Similarity in The Calcium-Binding Properties of the CaMs Containing Tryptophan and Those Lacking Tryptophan. The amino acid sequences of the various CaMs used in this study are summarized in Figure 1. Representative data are given in Figure 2 for T26W-CaM. The data show that this CaM is indistinguishable from the standard-of-comparison CaM (or SYNCAM) and from F99W-CaM, the CaM containing tryptophan at the corresponding calcium loop position in the third domain (Kilhoffer et al., 1988). Specifically, the data shown indicate a stoichiometry of 4 mol of calcium bound/mol of CaM, with a mean dissociation constant of 4×10^{-6} M. Similar results were obtained with the CaMs containing tryptophan in the second and fourth calcium-binding sites and the central helix (Table I). All of the tryptophan-containing CaMs used in this study are able to fully activate the vertebrate enzymes myosin light chain kinase and phosphodiesterase in the presence of a molar excess of calcium and the bacterial enzyme adenyl cyclase [Kilhoffer et al. (1988), Chabbert et al. (1991), and unpublished observations]. Altogether, these and previous data (Kilhoffer et al., 1988, 1989; Chabbert et al., 1989, 1991) demonstrate the validity of using CaMs containing tryptophans at the Y position of the calcium-binding loop as isofunctional structural mutants to probe calcium-binding mechanisms.

Characteristic Fluorescence Spectrum and Quantum Yield of Each Tryptophan-Containing CaM Mutant. While the

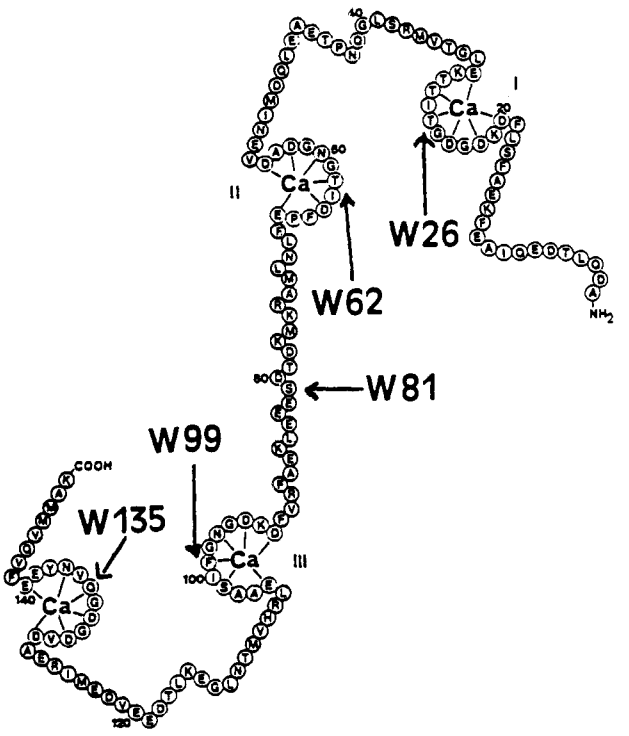

FIGURE 1: Schematic representation of SYNCAM, the reference mutant for calmodulin. The amino acid sequence corresponds to that published by Roberts et al. (1985). The figure shows the location of the tryptophanyl residues in the different calmodulin mutants: W26 in T26W-CaM, W62 in T62W-CaM, W81 in S81W-CaM, W99 in F99W-CaM, and W135 in Q135W-CaM.

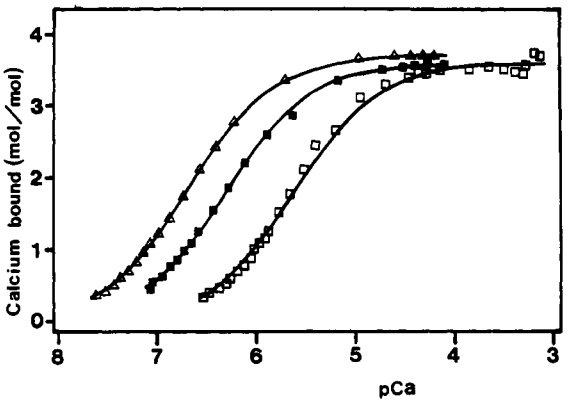

FIGURE 2: Calcium binding to T26W-CaM in 50 mM Hepes, pH 7.5, and 0% (□), 20% (■), and 40% EG (△). Results are expressed in moles of calcium bound per mole of protein as a function of pCa = $-\log [Ca]_F$, where $[Ca]_F$ represents the concentration of free calcium. Data were corrected for binding to the dialysis membrane. Experiments were performed in triplicate with concentrations ranging between 2.5×10^{-5} and 4×10^{-5} M.

fluorescence spectra of T26W-, T62W-, S81W-, and Q135W-CaM are characteristic of tryptophan emission and similar to that previously reported for F99W-CaM (Kilhoffer et al., 1989), with a maximum around 340–350 nm (Table II), they each show a slightly different emission maximum. These differences are probably due to their environments, in spite of each tryptophan being at the same relative location within its respective calcium-binding loop (position Y). In addition, calcium affects the fluorescence properties of each mutant in a different and specific manner, inducing a blue or a red shift in the fluorescence emission. Globally, the location of the emission maxima would go along with tryptophan residues located in a hydrophilic environment. The quantum yields of the various mutants (Table II) are generally around 0.14

Table I: Calcium-Binding Parameters of the Different Calmodulin Tryptophan Mutants in the Absence and the Presence of Ethylene Glycol (EG)[a]

mutants	EG (%)	K_d (μM)	n
T26W-CaM	0	3.85	4.4
	20	0.63	4
	40	0.27	3.5
T62W-CaM	0	2.5	3.7
	20		
	40	0.28	3.6
S81W-CaM	0	2.2	3.8
	20	0.68	3.7
	40	0.28	3.8
F99W-CaM	0	2.56	4.4
	20	1.1	3.8
	40	0.53	4
Q135W-CaM	0	2.9	3.9
	20	1.02	3.9
	40	0.48	4.2

[a] Experiments were performed in 50 mM Hepes, pH 7.5, in the absence or presence of ethylene glycol at the concentration indicated. K_d, the calcium dissociation constant, and n, the number of calcium binding sites, were obtained from flow dialysis experiments and determined using the Scatchard equation $\nu = nKx/1 + Kx$, where $K(=1/K_d)$ is the calcium association constant and ν and x are the concentration of bound and free calcium, respectively.

(which is the quantum yield of free tryptophan in water) and can reach values up to 0.31. The final values obtained during the calcium titration studies reported here are in agreement with those obtained for these calmodulins in the presence of a molar excess of calcium (Kilhoffer et al., 1988; Chabbert et al., 1991). The highest quantum yields are observed for T26WCaM and Q135W-CaM, i.e., for the tryptophan residues located in the Ca^{2+}-binding domains I and IV, not directly connected to the central helix. It can be added that residues 26 and 62 (respectively, residues 99 and 135) are in close proximity to each other in the three-dimensional crystal structure of the protein and should have very similar environments. Although interesting in terms of their spectral properties, the structural basis of the fluorescence properties of these tryptophans is beyond the scope of this study and was not pursued further.

Tryptophan Fluorescence Polarization as a Monitor of Local Conformational Changes in the Calcium-Binding Site and Fluorescence Intensity as a Monitor of Local and Remote Changes. For tryptophanyl residues in proteins, changes in the fluorescence polarization degree and modifications in the quantum yield (or intensity) may occur independently for several reasons. Namely, changes in the quantum yield of tryptophanyl residues may occur without changes in the fluorescence lifetime due to the presence of both a dynamic and a static component in the quenching mechanism of tryptophanyl fluorescence. On the other hand, changes in the fluorescence polarization degree may occur without changes in lifetime due to a modification of the mobility of the chromophore. Therefore, in proteins, measurements of both fluorescence polarization changes and fluorescence intensity changes may bring complementary information.

For each CaM, there is a change in fluorescence polarization as a function of calcium binding (Figure 3). The results can be regrouped into two classes: one class for which the fluorescence change starts as soon as Ca^{2+} binds to the protein and shows a plateau at 2 mol of Ca^{2+}/mol of calmodulin and a second class where fluorescence polarization mainly changes when the third and the fourth calcium binds to the protein. The first group corresponds to reporter groups in the carboxy terminal part of the molecule (F99W-CaM and Q135W-CaM) whereas the second group includes the reporter tryptophans in the amino terminal half of CaM (i.e., T26W-CaM and T62W-CaM). Tryptophan at position 81 in the central helix (S81W-CaM) seems to be sensitive to calcium binding to both the carboxy and the amino terminal part of the molecule. Moreover, the signal change above 4 mol of Ca^{2+}/mol of protein may reflect the effect of calcium binding to weak nonspecific sites around Trp-81, for instance, the glutamic acid residues in positions 82–84. Overall, in the context of the generally accepted model of calcium binding to the carboxy terminal half of CaM first and then to the amino terminal half, the results are consistent with tryptophan fluorescence polarization being a monitor of local conformational changes in the calcium-binding site where the tryptophan has been introduced.

In contrast to fluorescence polarization changes with calcium titration, fluorescence intensities (Figure 4) do not simply monitor local conformational changes induced by calcium binding to the calmodulin lobe where tryptophan is located but also seem to be sensitive to calcium binding to a remote region of the molecule. For example, the fluorescence intensity of Q135W-CaM (tryptophan in site IV) is altered only 25% when the calcium/CaM molar ratio is $\sim$2, with most of the intensity change occurring under conditions where calcium should be binding to calcium-binding domains I and II. This comparatively large change in fluorescence intensity of tryptophan 135 coincident with binding to the first and second domains is consistent with an influence of the amino terminal part of the molecule on the structure of sites III and IV in the carboxy terminus, even though the carboxy terminal sites should already be filled with calcium. On the other hand, analysis of the fluorescence intensity changes that occur with T26W- and T62W-CaM (both reporter groups are in the amino terminal half of CaM) indicates that calcium binding to the carboxy terminal half can have a conformational effect on the amino terminal sites. Altogether, these results are a clear illustration of the cross-talk occurring between the two halves of the molecule.

Ethylene Glycol (EG) as a Pertubating Agent in the Study of CaM. Ethylene glycol and temperature changes have been used successfully previously as perturbating agents to study various systems (Brossa et al., 1984; Barman & Travers, 1985). EG allows us to work at subzero temperatures and thus to slow down the reactions. In addition, thermodynamic parameters associated with the reaction can be obtained. This was of interest for the study of the mechanism of Ca^{2+} binding to calmodulin. In order to use EG as a perturbating agent to study the kinetics of Ca^{2+} removal from calmodulin, we first undertook an analysis of the effect of this solvent on the calcium-binding mechanism of calmodulin at equilibrium using flow dialysis and fluorescence titrations.

(a) Ethylene glycol increases the apparent affinity of CaM for calcium but does not alter the number of calcium-binding sites. The effect of various EG concentrations on calcium binding to the CaM mutants is summarized in Table I. Representative data for one of the tryptophan-containing CaMs, T26W-CaM, is shown in Figure 2. EG does not modify the number of Ca^{2+}-binding sites but does appear to increase the affinity of Ca^{2+} for CaM. For the different CaM mutants, the mean dissociation constants (K_d) at a given EG concentration (Table I) are extremely similar. Because EG has at least two potential effects (a decrease in the dielectric constant of the solvent and a decrease in the molarity of water), it is not possible to attribute with confidence the effect of EG on the apparent calcium affinity to either an electrostatic phenomenom or a decrease in a "pseudocompetition" between water and calmodulin for calcium ions. Regardless, the ability

Table II: Fluorescence Parameters of the Different Tryptophan Calmodulin Mutants[a]

	$-Ca^{2+}$			$+Ca^{2+}$		
mutants	λ_{max} (nm)	ϕ	p	λ_{max} (nm)	ϕ	p
T26W-CaM	346	0.14	0.20 ± 0.0002	354	0.31	0.188 ± 0.0003
T62W-CaM	343	0.125	0.245 ± 0.0003	347	0.14	0.22 ± 0.0003
S81W-CaM	349	0.18	0.175 ± 0.0002	341	0.19	0.173 ± 0.0002
F99W-CaM	348	0.19	0.175 ± 0.0003	348	0.15	0.213 ± 0.0004
Q135W-CaM	350	0.23	0.182 ± 0.0002	355	0.28	0.137 ± 0.0003

[a] λ_{max} corresponds to the wavelength where fluorescence emission is maximum, ϕ is the fluorescence quantum yield, and p is the fluorescence polarization degree defined under Materials and Methods. Excitation wavelength was set at 297 nm. Buffer conditions: 50 mM Hepes, pH 7.5. $-Ca^{2+}$ corresponds to the protein obtained after Ca^{2+} removal. $+Ca^{2+}$ corresponds to the protein in the presence of 1 mM Ca^{2+}.

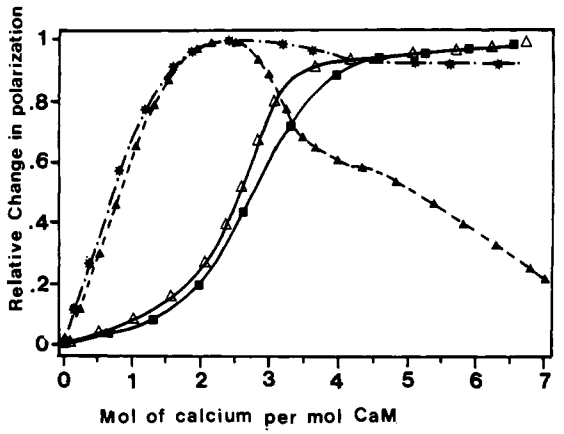

FIGURE 3: Changes in the fluorescence polarization degree (p) of the different tryptophan calmodulin mutants as a function of calcium added to the proteins. Calcium bound to the proteins was calculated by taking into account the calcium affinity constants obtained from calcium-binding studies. Under the conditions used, Ca^{2+} added corresponds to Ca^{2+} bound up to 4 mol of Ca^{2+} added per mole of protein. Experiments were performed in 50 mM Hepes buffer, pH 7.5, with an excitation wavelength set at 297 nm. On the ordinate, changes are expressed as the percentage of the maximum change, which depending on the mutant can correspond to an increase or to a decrease. Protein concentrations ranged between 3×10^{-5} and 5×10^{-5} M. Mutants: (▲) S81W-CaM (0.175–0.165); (*) F99W-CaM (0.178–0.213); (*) Q135W-CaM (0.184–0.134); (△) T26W-CaM (0.204–0.189); (■) T62W-CaM (0.246–0.219). For each mutant, polarization degrees in the absence of Ca^{2+} and at the maximum of the change, respectively, are indicated in parentheses.

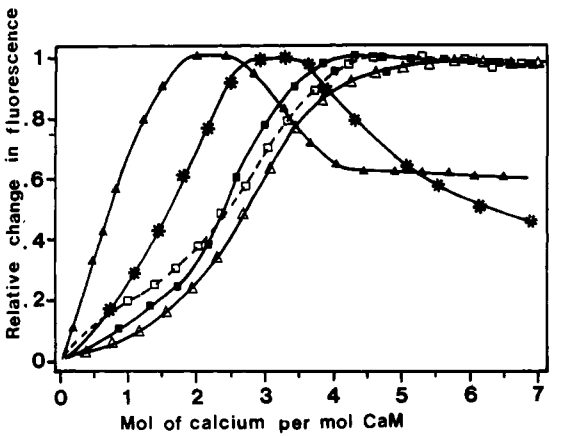

FIGURE 4: Changes in the fluorescence intensities of the different tryptophan calmodulin mutants as a function of calcium added to the proteins. Calcium bound to the proteins was calculated by taking into account the calcium affnity constants obtained from calcium-binding studies. Under the conditions used, Ca^{2+} added corresponds to Ca^{2+} bound up to 4 mol of Ca^{2+} added per mole of protein. Fluorescence intensities correspond to the areas under the fluorescence spectra, and changes (which can correspond to an increase or a decrease) are expressed as the percentage of the maximum change. Experiments were performed in 50 mM Hepes, pH 7.5. Protein concentrations ranged between 2.5×10^{-5} and 3.5×10^{-5} M. Mutants: (▲) S81W-CaM (5186–4786); (*) F99W-CaM (4908–5841); (□) Q135W-CaM (3894–5324); (△) T26W-CaM (4394–11 028); (■) T62W-CaM (2985–3435). For each mutant, the areas under the fluorescence spectra in the absence of Ca^{2+} and at the maximum of the change, respectively, are indicated in parentheses. Values are in arbitrary units.

to perturb calcium-binding affinity with maintenance of stoichiometry meant that EG alone was a sufficient perturbant for the subsequent kinetic studies.

(*b*) *In 40% EG, the sequential and ordered calcium binding to calmodulin is maintained.* In the presence of 40% EG (Figure 5), the change in fluorescence intensity as a function of calcium concentration reflects the order of the sequential binding. Fluorescence intensities of T26W-CaM and T62W-CaM are affected between 2 and 4 Ca^{2+} bound/calmodulin. In the first case, the change corresponds to an increase and in the second case to a decrease accompanied by a small red shift. The fluorescence intensities of F99W-CaM and Q135W-CaM are essentially affected between 0 and 2 Ca^{2+} bound/calmodulin, with a monophasic decrease for F99W-CaM and a red shift for Q135W-CaM. The tryptophan residue located in the central helix of S81W-CaM is only slightly affected, and its spectrum shows a small increase with a slight blue shift (which explains the decrease in fluorescence intensity at 343 nm reported in Figure 5) between 0 and 2 Ca^{2+}/calmodulin. These results are consistent with the maintenance of a sequential and ordered calcium binding in the presence of 40% EG. Moreover, the titration curves of the different mutants give some insight as far as the calcium-binding mechanism of calmodulin in the presence of 40% EG is concerned. Tryptophan 99 fluorescence intensity varies between 0 and ~1 calcium/calmodulin, whereas signals from tryptophans 135 and 81 are monitoring the binding of the two first calcium ions. This is a signature for a sequential binding mechanism. On the other hand, tryptophan 26 exhibits a change in fluorescence intensity between 2 and 3 mol of calcium/mol of CaM whereas for tryptophan 62 the change takes place between 2 and 4 mol of calcium/mole of CaM. These observations suggest the following overall sequence of calcium binding: III–IV–I–II with tryptophan 99 monitoring the occupancy of site III, tryptophans 135 and 81 that of sites III and IV, tryptophan 26 that of site I, and tryptophan 62 that of sites I and II.

Biphasic Kinetics of Calcium Removal from Calmodulin Mutants. Taken together, the different results obtained so far tend to indicate that (a) the five different mutants are similar to the original SYNCAM as far as calcium binding is concerned (number of sites, mean affinity constant, and overall shape of the binding isotherm); (b) EG does not qualitatively modify the calcium-binding mechanism of calmodulin (maintenance of a sequential and ordered calcium binding); and in 40% EG, tryptophans 99, 135, and 81 are probes sensitive to the binding of the first two calcium ions, whereas tryptophans 26 and 62 are sensitive to the binding of the two last calcium ions.

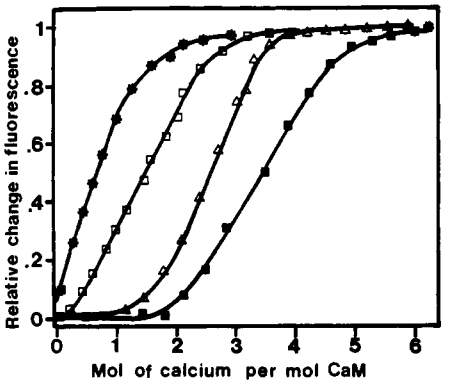

FIGURE 5: Changes in the fluorescence intensities of the different tryptophan calmodulin mutants in ethylene glycol as a function of calcium added to calmodulin. Calcium bound to the proteins was calculated by taking into account the calcium affinity constants obtained from calcium-binding studies. Under the conditions used, Ca^{2+} added corresponds to Ca^{2+} bound up to 4 mol of Ca^{2+} added per mole of protein. Changes were expressed as percentage of the maximum change. Protein concentrations ranged between 2.5×10^{-5} and 3.5×10^{-5} M. Buffer conditions: 50 mM Hepes, 40% EG, pH 7.5. Mutants: (*) F99W-CaM (I_{344} 57–33); (□) Q135W-CaM (I_{334} 76–59); (□) S81W-CaM (I_{343} 52–47); (△) T26W-CaM (I_{353} 104–202); (■) T62W-CaM (I_{339} 87–65). For each mutant, the intensities measured at a given wavelength in the absence of Ca^{2+} and at the maximum of the change, respectively, are indicated in parentheses. Values are in arbitrary units.

The different mutants have therefore been used to analyze the kinetics of calcium removal from calmodulin.

(a) Ca^{2+} Removal with EDTA. At first, EG was used to work at subzero temperature in order to slow down the calcium dissociation rate. However, when the relaxation rates of the different mutants are compared (Table III) to those obtained for SYNCAM in the absence of EG (Haiech et al., 1989), it appears that EG by itself slows down the dissociation rate.

Fluorescence relaxation of tryptophan 26 and tryptophan 62 is associated with a fast process, whereas the relaxation of tryptophans 81, 99, and 135 is associated with a slow process. On the other hand, Arrhenius plots of these data show a break for tryptophans 26 and 62 and are linear for tryptophans 81, 99, and 135 (Figure 6). Values of $\Delta H^{\ddagger}$ an $\Delta S^{\ddagger}$ deduced from these Arrhenius plots are similar for tryptophans 26 and 62 on the one hand and for tryptophans 81, 99, and 135 on the other hand (Table IV).

At least, two different processes in the calcium-binding mechanism of calmodulin are evidenced from these results: a fast process with a low $\Delta H^{\ddagger}$ that is reported through tryptophans 26 and 62 and a slow phase with a high $\Delta H^{\ddagger}$ reported by tryptophans 81, 99, and 135.

(b) Ca^{2+} Removal with Quin 2. Quin 2 has already been used to directly follow removal of calcium from calmodulin (Martin et al., 1985, 1986; Suko et al., 1986). In order to determine the relationship between calcium dissociation and the conformational changes monitored by the tryptophan residues, we studied calcium removal from the different calmodulin mutants using Quin 2.

Experiments were performed between –5 and 30 °C on the different mutants with special emphasis on T26W-CaM (Figure 7). Similar results were obtained in all cases (data not shown) and in all cases two phases were observed, a fast one and a slow one, which differ by ~2 orders of magnitude on the time scale. A quantification of the fluorescence change of Quin 2 (performed separately) indicates that each phase accounts for the removal of two calcium ions. At temperatures below –5 °C, the rapid phase seems to split into two phases, each accounting for the removal of one calcium ion.

Table III: Kinetic Parameters for Ca^{2+} Dissociation from the Different Calmodulin Tryptophan Mutants at Different Temperatures[a]

t (°C)	k (s^{-1})	t (°C)	k (s^{-1})	t (°C)	k (s^{-1})
		T26W-CaM/EDTA			
31.5	221	10	101	–8.5	31
25.5	166	5.5	87	–13.5	19
21	152	0	81	–17.5	6
15.5	144	–4.5	43		
		T62W-CaM/EDTA			
21	228 (13)	5.5	121	–9	70
15.5	214 (21)	0	102 (15)	–13	51
10.5	143 (3)	–4	84 (26)	–17	19
		S81W-CaM/EDTA			
32	4.5	10.5	0.46	–8.5	0.064
25	1.7	5.5	0.22	–13.5	0.048 (0.0085)
21.5	1.3	0.5	0.17	–17	0.020
15	0.6	–4	0.098		
		F99W-CaM/EDTA			
21	2.8 (0.52)	6	0.6	–9	0.1
15.5	1.7	0.5	0.38 (0.04)	–17	0.055
10.5	1.1	–4	0.18		
		Q135W-CaM/EDTA			
21	4.1	5.5	1.5 (0.65)	–8.5	0.3
15	3.7 (0.1)	0	1	–17	0.077
		T26W-CaM/Quin 2 (Slow Phase)			
32	0.91	16	0.26	5.5	0.06
25	0.58	10.5	0.14	0.5	0.034
21	0.45				
		T26W-CaM/Quin 2 (Rapid Phase)			
32	228	16	113	0.5	47
25	195	10.5	102	–4	37
21	101	5.5	73		

[a] Calcium was removed from the calmodulin mutants with a stopped-flow apparatus using EDTA or Quin 2, when indicated. Changes in the fluorescence intensity of tryptophan or Quin 2 were monitored, respectively. The fluorescence change of Quin 2 induced by Ca^{2+} removal from T26W-CaM was clearly biphasic, leading to two sets of kinetic constants (related to a slow and to a rapid phase). Values given in parentheses are calculated SD. Buffer conditions: 50 mM Hepes, 40% EG, pH 7.5.

Table IV: Thermodynamic Parameters Relative to the Dissociation of Ca^{2+} from the Different Calmodulin Tryptophan Mutants[a]

mutants	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H_1^{\ddagger}$ (kJ/mol)	$\Delta S_1^{\ddagger}$ (J mol^{-1} K^{-1})
T26W-CaM/EDTA	20 ± 5	–135 ± 17	81 ± 11	90 ± 41
T62W-CaM/EDTA	25 ± 2.5	–113 ± 9	91 ± 13	139 ± 40
S81W-CaM/EDTA	62 ± 2.4	–31 ± 9		
F99W-CaM/EDTA	65 ± 2.3	–14 ± 8		
Q135W-CaM/EDTA	65 ± 6	–9 ± 12		
T26W-CaM/Quin 2 (rapid phase)	30 ± 3.5	–99 ± 12		
T26W-CaM/Quin 2 (slow phase)	68 ± 5.5	–22 ± 19		

[a] Thermodynamic functions were deduced from the Arrhenius plot of the kinetic data presented in Table III. Calcium was removed using either EDTA or Quin 2. Ca^{2+} removal from T26W-CaM using Quin 2 led to two phases: a slow one and a rapid one. Thermodynamic functions associated to each phase are given under T26W-CaM (slow phase) and T26W-CaM (rapid phase). For T26W-CaM and T62W-CaM, the Arrhenius plot showed a break (Figure 7). Two sets of thermodynamic functions could therefore be obtained ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta H_1^{\ddagger}$, $\Delta S_1^{\ddagger}$). Buffer conditions: 50 mM Hepes, 40% EG, pH 7.5.

Although interesting, this last point will not be studied further in this paper. The Arrhenius plot of the data leads to a high $\Delta H^{\ddagger}$ (68 kJ/mol) for the slow phase and to a low $\Delta H^{\ddagger}$ (30 kJ/mol) for the rapid phase. These results are in good agreement with the work of Martin et al. (1985). Between

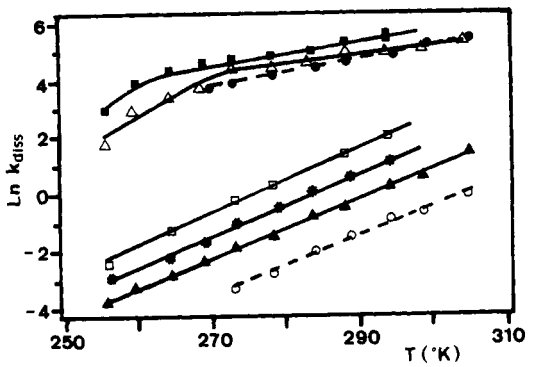

FIGURE 6: Arrhenius plots of the kinetic data relative to calcium removal with EDTA from the T26W-CaM (△), T62W-CaM (■), S81W-CaM (▲), F99W-CaM (*), and Q135W-CaM (□) and with Quin 2 from T26W-CaM (- - -). (●) corresponds to the rapid phase and (○) to the slow phase. k corresponds to the kinetic constant of calcium dissociation. Values of k are given in Table III.

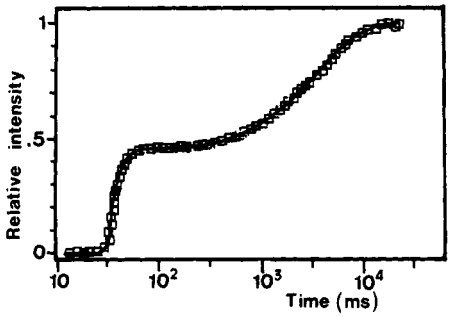

FIGURE 7: Kinetic of calcium removal from Ca^{2+}-loaded T26W-CaM using Quin 2. T26W-CaM at a concentration of 5×10^{-6} M in the presence of 5×10^{-5} M Ca^{2+} was mixed with a solution of Quin 2 (100 μM) in a stopped-flow apparatus. The fluorescence change of Quin 2 was monitored as a function of time using buffer A (50 mM Hepes, 40% EG, pH 7.5); temperature, 15.7 °C.

0 and 30 °C, all these processes exhibit an Arrhenius plot without break (Figure 6).

Conformational Change as a Limiting Step in the Slow Phase of the Kinetics of Ca^{2+} Removal from Calmodulin. The sequential model presented earlier (Haiech et al., 1981) has been improved to take into account the various results obtained on calmodulin, including both steady-state and kinetic results. The so-called kinetic extension of the sequential model is presented in Figure 8. This model associates to each calcium-binding step (1, 3, 5, and 7) a conformational step (0, 2, 4, 6, and 8). As it can be agreed (from our results and those of others) that the two first calcium ions bind to the C-terminal part of the protein, and the two last calcium ions to the N-terminal part, the binding steps 1 and 3 on the one hand and 5 and 7 on the other hand can be associated to calcium binding to the C-terminal and N-terminal part of calmodulin, respectively. The conformational steps 0 and 2, respectively, 6 and 8, may reflect the coupling between the two sites in the C-terminal half and N-terminal half of the molecule. Finally, step 4 has to be associated with a coupling between the two lobes of calmodulin. These conformational steps are probably associated with the exposure of hydrophobic patches on the calmodulin surface and with the interaction with compounds such as ANS or peptides. A quantification of certain steps of the model can probably be made using results obtained in recent T-jump experiments (Tsuruta & Sano, 1990). Indeed, T-jump experiments have shown that equilibrium constants between two conformations are around 2 and 3 and that the kinetic rates of the conformational changes are extremely rapid, occurring in the micro-to-millisecond range. As will

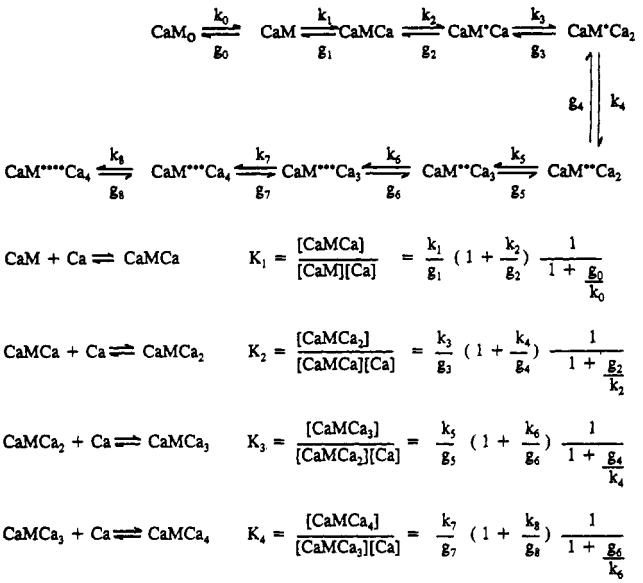

FIGURE 8: Kinetic model of calcium binding to calmodulin. In the model, steps 0, 2, 4, 6, and 8 are related to conformational changes whereas steps 1, 3, 5, and 7 are related to the calcium-binding steps. K_1, K_2, K_3, and K_4 correspond to the macroscopic association constants.

be suggested in the following section, step 4 of the kinetic scheme is probably a limiting step. Therefore, results obtained by Tsuruta and Sano (1990) argue for kinetic rates below the millisecond for steps 0, 2, 6, and 8 and for conformation equilibrium constants between 2 and 3 for these same steps. Also, time-resolved spectra of the five calmodulin mutants in the presence of 1 mM calcium suggest that the tryptophans exist in at least two conformations which interconvert on a time scale above the nanosecond (Chabbert et al., 1991). The ratio between the two conformations (conformation equilibrium constant) deduced from the preexponential factors was reported to lie between 1 and 5.

More information on different parameters of the model and of certain g_i (Figure 8) can be obtained from the analysis of the kinetics of calcium removal performed in 40% ethylene glycol. At this concentration of ethylene glycol, the fluorescence properties of each tryptophan seem to be sensitive to calcium binding to the sites in the vicinity of the probe and thus the tryptophans constitute good reporter groups to follow the conformational changes in a given region of the protein. On the other hand, ethylene glycol does not seem to qualitatively modify the mechanism of calcium binding to calmodulin. With and without EG, a sequential model of binding is implied by the data.

Ca^{2+} removal from calmodulin has been studied by using either EDTA and monitoring tryptophan fluorescence changes or Quin 2 and monitoring the fluorescence change of that chromophore. Information on the conformational changes associated with calcium removal is awaited in the first case, whereas in the second case, one can follow the departure of each calcium from calmodulin. Kinetic analysis clearly shows two different types of processes. One process with a ΔH^* of ~60 kJ/mol is monitored by Trp-81, Trp-99, Trp-135, and the slow phase of Quin 2, and a second process with a ΔH^* around 30 kJ/mol is monitored by Trp-26, Trp-62 and the rapid phase of Quin 2. As will be discussed below, this rapid phase may be strictly related to the calcium-binding steps. Indeed, in the rapid phase recorded by Quin 2, two calcium ions are removed from calmodulin with a relaxation rate of ~200 s^{-1} at 25 °C. From Figure 8 this relaxation rate is related to either g_5, g_6, g_7, or g_8. g_6 and g_8 are related to

conformational changes which, according to the discussion at the beginning of the paragraph, are probably extremely rapid processes (kinetic rates in the range of 10 ns to 1 ms). In the present study, information on the conformational steps 6 and 8 can be obtained by analyzing the Arrhenius plots of T26W- and T62W-CaM in 40% EG. For these mutants, the Arrhenius plot is biphasic and points to a rapid step with a rate constant k_1 of 2003 and 10 856 s^{-1}. These values are of the same order of magnitude as the kinetic rates of CaM conformational changes previously deduced from the results of Tsuruta et al. (1990) and Chabbert et al. (1989). We may therefore assume that they are a measure of the conformational steps 6 and 8. As the calculations we make are approximations, we do not attribute a precise value to the kinetic rates of steps 6 and 8. And, for the sake of simplicity, we will consider that the two rate constants are identical and with a value around 10 000 s^{-1}, at 25 °C, in 40% EG. Also by reference to the conformation equilibrium constants discussed earlier, k_8 and g_8 (respectively k_6 and g_6) appear to be of the same order of magnitude. Therefore, K_4, the macroscopic binding constant associated to the binding of the fourth calcium (Figure 8), is almost equal to k_7/g_7 and g_7 is $\sim 200\ s^{-1}$. At 25 °C, K_4 equals $5 \times 10^5\ M^{-1}$ (a value obtained from direct calcium-binding studies). k_7 therefore is $\sim 10^8\ M^{-1}\ s^{-1}$ at 25 °C and in 40% ethylene glycol. Taking into account the viscosity of the medium which is ~ 10 times that of water, the value found for k_7 is compatible with a diffusion-limited process in a medium containing 40% ethylene glycol.

On the other hand, we may assumed that the discussion concerning calcium binding onto the C-terminal half of calmodulin can be transposed to the events occurring in the N-terminal half. In other terms k_0, g_0 (respectively, k_2 and g_2) would have the same properties as k_6, g_6 (respectively, k_8 and g_8) and thus the macroscopic calcium-binding constant relative to the binding of the first calcium ion would be around k_1/g_1. Kinetic studies using Quin 2 have shown that two calcium ions are removed from calmodulin at a slow rate ($\approx 1\ s^{-1}$). With reference to the model in Figure 8, this relaxation rate is linked to either g_1, g_2, g_3, or g_4. If we assume that no conformational step is limiting, g_1 will be $\sim 1\ s^{-1}$, and taking the value of 8 10 $10^6\ M^{-1}$ measured for K_1, k_1 will be $\sim 8 \times 10^6\ M^{-1}\ s^{-1}$, a value well below a diffusion-limited process which normally governs a calcium-binding step. This conclusion therefore argues for a conformational limiting step, which would appear after the removal of the two calcium ions, i.e., at step 4, and would correspond to g_4 in the kinetic scheme.

More precision on the kinetics of the calcium removal process can be obtained from the relaxation rates of the different mutants at 25 °C (Table V). Indeed, the relaxation rates related the rapid phase are in the order

$$t_{w62} > t_{quin2r} > t_{w26}$$

where t_{w62}, t_{w26}, and t_{quin2r} correspond, respectively to the relaxation rate of Trp-62 and Trp-26 and to the rapid phase monitored by Quin 2. Such a sequence would let us hypothesize that Trp-62 is sensitive to the removal of the first calcium ion and Trp-26 to the removal of at least the second calcium. This confirms our results at equilibrium and our proposed sequence of calcium binding. Concerning the slow phase, the relaxation rates of Trp-135 (t_{w135}), Trp-99 (t_{w99}), and Quin 2 (t_{quin2s}) are as follows: $t_{w135} \approx 2t_{w99} \approx 4t_{quin2s}$. This indicates that either the limiting step in calcium removal can be divided in at least three steps, one monitored by Trp-135, one by Trp-99, and one limited by the removal of the last two calcium ions, or that the slow phase is extremely variable and sensitive to the different point mutations. Indeed, if our first hypothesis was true, that would mean that Trp-99 has monitored the binding of two calcium ions, which is in contradiction with the results at equilibrium. Therefore, we compared the kinetics of calcium removal from one of the mutants (S81W-CaM) by following the change in fluorescence of tryptophan 81 (removing Ca^{2+} with EDTA) and of Quin 2 (removing Ca^{2+} with Quin 2). The results are practically the same, arguing therefore, for a sensitivity of the limiting step (step 4) to point mutation in the calmodulin mutant.

Table V: Relaxation Rates of Ca^{2+} Removal from the Different Calmodulin Mutants at 25 °C[a]

mutant	k (s^{-1})	k_1 (s^{-1})
T26W-CaM	175	2003
T62W-CaM	278	10856
S81W-CaM	1.76	
F99W-CaM	4	
Q135W-CaM	8.8	
T26W-CaM/Quin 2 (slow phase)	0.52	
T26W-CaM/Quin 2 (fast phase)	167	

[a] Relaxation rates k and k_1 were obtained from parameters in Table IV by use of the following equation: $\ln k_{diss} = \ln(k_B T/n) + \ln K = \ln(k_B T/n) - \Delta H^*/RT + \Delta S^*/R$. $\ln k_{diss}$ corresponds to the natural logarithm of the kinetic constant of calcium dissociations (k or k_1), k_B to the Boltzmann's constant, n to the Planck's constant, and R the gas constant. K^* is an equilibrium constant related to the activation reaction, taking into account the concentration of molecules in the activated and initial state. ΔH^* and ΔS^* are the enthalpy and entropy of the activation step (Johnson et al., 1963).

CONCLUSIONS

The present study brings up two main points: one concerns the importance of the electrostatic potential and/or the water molarity in the process of calcium binding to calmodulin; The second is related to the mechanism of calcium binding to calmodulin.

Concerning the second point, direct binding studies of calcium to the five calmodulin mutants lead to a good approximation of experimental data by a Scatchard equation, apparently pointing to a model with four independent and equivalent sites. On the other hand, calcium titrations followed by fluorescence show that calcium binds first to the carboxy terminal pair of sites and then to the amino terminal pair of sites. This apparent discrepancy between these two sets of data illustrates the nonequivalence between the Scatchard equation and the Satchard model. To reconcile the results, it is necessary to consider a model with coupling between the different calcium-binding sites. Such a model, although with cooperative interaction between sites, leads to a Scatchard equation to describe the direct binding of Ca^{2+} to CaM. As titration of Q135W-CaM strongly argues for a coupling between the two lobes of calmodulin, the simplest model which so far explains the whole set of data appears to be sequential binding model. A sequence of calcium binding can be deduced from experiments performed in 40% EG, and we may assume that the same sequence stands also in the absence of EG.

The kinetic scheme presented in this paper allows one to take into account the whole set of experimental data on the calmodulin calcium-binding mechanism gathered to date. Results on Ca^{2+} binding or Ca^{2+} removal from calmodulin halves, corresponding to the N-terminal part (residues 1–77) or to the C-terminal part (residues 78–148), (Minowa & Yagi, 1984; Martin et al., 1985), can also be simulated using the scheme described in Figure 8 and taking steps 5–8 for calcium binding to the N-terminal moiety and steps 0–3 for binding

to the C-terminal moiety (J.H., unpublished observations). The number of steps proposed in our model corresponds to the minimum required to explain the different data, and maybe more will be required when experiments are done to link each kinetic step to a specific molecular mechanism. Such a sequential kinetic mechanism may be taken as a first working model to analyze the specificity and the mechanism of interaction of calmodulin with target peptide. In addition, if one makes the logical assumption that the sequential Ca^{2+}-binding mechanism also applies to the CaM:CaM-binding protein complex, an insight into the mechanism of action of calmodulin could be obtained, as the kinetic sequential model would allow calmodulin to differentially activate the various target enzymes as a function of time.

ACKNOWLEDGMENT

We are indebted to Drs. D. M. Watterson and T. J. Lukas for helpful discussion throughout this work. The expert editorial assistance of Mrs. M. Wernert is greatly acknowledged.

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