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Spin labeling studies of wheat germ calmodulin in solution

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Electron paramagnetic resonance was used to investigate the physical state of plant calmodulin in solution. Wheat germ calmodulin contains a single cysteine residue (Cys-27) on the first of four calcium binding loops. In this study the nitroxide spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) was covalently attached to Cys-27 to produce a Ca^{2+}-sensitive, biologically-active, labeled protein. The rotational correlation time of the spin label, a measure of its rotational mobility and reflective of the physical state of this region of the protein, was calculated under various conditions. Relative to control, changes in the physical state of the protein reflected by increased motion of the spin label were observed at high pH, low ionic strength and upon addition of Ca^{2+}. These results extend knowledge of the structure of the protein, previously known from solid state and biochemical studies, to calmodulin in solution.

Introduction

Proteins which bind Ca^{2+} specifically and tightly, and which are involved in the regulation and processing of Ca^{2+} flux across a cell membrane, fall into two groups, soluble proteins and membrane-intrinsic proteins. In general, Ca^{2+}-proteins buffer the amount of calcium ion in the cytosol and regulate the activity of other effector proteins and enzymes of the cell. The most prevalent and truly multifunctional (less specialized) of these Ca^{2+}-regulatory proteins is calmodulin (CaM). This protein is found in all eukaryotic cells, and since its discovery by Cheung [1] and Kakiuchi and Yamazaki [2] in 1970, CaM has been shown to regulate or bind more than 30 different proteins and enzymes in a calcium-dependent manner [3].

CaM is a member of the water soluble group of Ca^{2+}-regulatory proteins and thus is found, when unbound, in the cytosol. CaM contains only calcium-specific domains (no Ca^{2+}-Mg^{2+} sites) as shown by the high selectivity for Ca^{2+} over other physiological cations at physiological concentrations (K^+ 0.1 M, Mg^{2+} 1–3 mM) [4,5]. CaM has four of these domains which interact co-operatively allowing it to respond to rapid calcium transients which occur upon stimulation of many cell types [6].

When CaM binds three or more calcium ions a conformational change occurs which increases the α-helical content of the protein and exposes a hydrophobic region that allows the protein to interact with membrane-bound enzymes and transport proteins, peptides and pharmacological agents such as phenothiazines [5]. The three-dimensional crystal structure of CaM-$(Ca^{2+})_4$ complex (CaM isolated from rat testes) has been elucidated [7]. One unusual aspect of the CaM crystal structure is the long central helix which connects two globular lobes of the protein, each containing two Ca^{2+} binding domains. Babu et al. [7] suggest that this central helix may be buried in the absence of calcium, which is why this region is thought to be important in facilitating CaM's calcium-dependent interaction with drugs and proteins.

Recent studies of CaM in solution have suggested a difference between the crystal structure and solution structures of CaM. For example, Heidorn and Trewhella found a discrepancy between their X-ray solution scattering data and the crystal structure involving the central helix [8]. Their findings indicate that if the size and shape of the globular domains are the same in solution as in the crystal, the distances between the domains must be smaller by several ångstroms. More recently Bayley et al. suggest that the long continuous

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α-helix may be due in part to the conditions of crystallization [9]. Their study showed that the CaM-$(Ca^{2+})_4$ complex in solution contains significantly less α-helix than the X-ray crystal structure [9].

In the most direct proof of structural difference to date Persechini and Kretsinger [10] have shown that a genetically engineered CaM containing two cysteines at positions 3 and 146 (in domain I and IV, respectively) can be crosslinked to yield a fully active molecule even though the molecule is fully folded. Based on these studies, they have proposed that the central helix seen in the crystal structure acts as a 'flexible tether' in solution allowing CaM to assume a nearly globular shape.

Chemical modification studies with naturally occurring animal calmodulins, using either photoreactive synthetic peptides [11] or heterobifunctional arylazides [12] also indicate that the CaM-$(Ca^{2+})_4$ complex is globular in its active conformation (i.e., when complexed to a target protein or ligand).

CaM isolated from wheat germ has a single cysteine residue located at position 27 in the first binding domain [13]. The remainder of the amino acid composition is highly conserved relative to other calmodulins including those from mammalian sources [5]. The one sulfhydryl group makes the protein a favorable candidate for spin labeling with the sulfhydryl group specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) (see Fig. 1). In this paper, MAL-6 labeled CaM is used to investigate further the physical state of this protein in solution, and Ca^{2+}-induced changes in its conformation.

Methods

Purification of calmodulin from wheat germ. CaM was isolated from wheat germ (Con Agra) as previously described [14] with slight modification. Briefly, the acetone powder produced by acetone extraction of fresh wheat germ was homogenized with 50 mM NaH_2PO_3/30 mM β-mercaptoethanol (β-ME)/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/5 mM EDTA (pH 5.9) buffer and centrifuged. CaM remains in the supernatant. Unwanted proteins in the supernatant are precipitated with 3% (w/v) trichloroacetic acid and centrifuged. CaM, still in the supernatant, is precipitated with 5% (w/v) trichloroacetic acid, centrifuged, resuspended in 2 M Tris buffer and adjusted to pH 7.5 with HCl. Unwanted proteins are precipitated with $(NH_4)_2SO_4$, added to a concentration of 55% of saturation, and stirred 30 min at 4°C. The resulting suspension is centrifuged at 23400 × *g* for 20 min at 4°C. The resulting supernatant was adjusted to 25 mM Hepes/0.5 mM $CaCl_2$/1 mM dithiothreitol (DTT); this solution was then stirred for 30 min at 4°C and centrifuged at 30000 rpm, for 1 h at 4°C. The supernatant was then loaded on a 2-trifluoromethyl-10-aminopropylphenothiazine (TAPP)-Sepharose column (rather than a phenyl-sepharose column), which had previously been stripped with 6 M guanidine-hydrochloride and equilibrated with column buffer (10 mM Hepes, 0.5 M $CaCl_2$ (pH 7.0)). The column was washed with 0.5 M HCl in column buffer until the absorbance at 279 nm was less than 0.005 AU (approx. 24 h). CaM was eluted from the column with buffer containing 1 mM EGTA/10 mM Tris-base/0.5 M KCl (pH 7.5). Protein fractions detected at 235 nm (approx. 50 ml) were combined and freeze-dried. The freeze-dried protein was reconstituted in a minimum volume of deionized water and desalted on a Sephadex G-50 (medium), 65 ml column using 10 mM NH_4HCO_3 buffer. The protein fractions were pooled, freeze-dried and reconstituted in a minimum volume of buffer (1 mM EGTA/10 mM Tris-base (pH 8.4)). Protein concentration was determined by amino acid analysis as previously described [15]. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis [16] and HPLC equipped with a C-3 reverse phase column [15].

Labeling of protein. The CaM was spin labeled under the following conditions, see Fig. 1. A 0.7 mM solution of MAL-6 in buffer (10 mM Tris-base/1 mM EGTA (pH 7.4)) was added to protein in solution to give a MAL-6 : CaM mole ratio of 5 : 1. The pH of the final solution was adjusted to between 6.5 and 7.0. The CaM and MAL-6 were allowed to incubate 1 h at room temperature, a time required to reach equilibrium (data not shown). The volume was then reduced to less than 4 ml with a N_2 pressurized, stirred cell embodying a filter

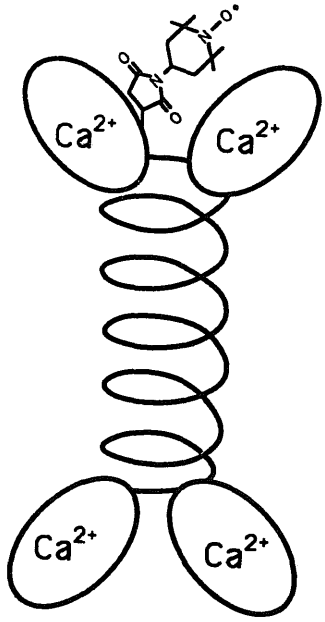

Fig. 1. Schematic of CaM with all four Ca^{2+}-binding domains occupied and the resulting exposed hydrophobic region of the protein. The maleimide spin label covalently attached to the single S-H group located in the first Ca^{2+}-binding domain is also shown.

(Omegacell) with a nominal molecular weight limit of 5000. The concentrated solution was then loaded on the Sephadex G-50 desalting column, described above, using 10 mM NH_4HCO_3 buffer; protein fractions were pooled and freeze-dried. The lyophilized, labeled protein was reconstituted in a minimum volume of buffer (10 mM Tris-base/1 mM EGTA (pH 8.4)), and a tabletop centrifuge was used to remove any particulate matter. Protein concentration of the supernatant was determined by bicinchonic acid (BCA) assay [17] using unlabeled CaM as a standard. The purity of the modified CaM was checked in the same manner as the native CaM. CaM activator activity was determined with a phosphodiesterase (PDE) activity assay [18] with one minor change. Rather than using AGX-1 resin absorption of uncleaved cAMP and subsequent detection of tritiated adenosine, the inorganic phosphate released from 5′-AMP after the 10 min incubation with 5′-nucleotidase was detected using Lanzetta reagent [19].

Specificity of spin label binding. Specificity of label binding was determined by competition with *p*-(hydroxymercuri)benzoic acid, sodium salt (PMB), a reagent which reacts specifically with free sulfhydryl groups of proteins to form mercaptides [20]. Two aliquots of protein (one treated with PMB and one control) were then subjected to normal labeling conditions as described above.

Spectral data. All samples contained 0.25 mg spin-labeled CaM in 300 μl (except where indicated) of 10 mM Tris-base/1 mM EGTA buffer containing various amounts of $CaCl_2$ and KCl as needed. EPR spectra were obtained at room temperature on a Varian E-109 X-band spectrometer interfaced to a computerized data acquisition system. Constant temperature was maintained for each sample by allowing N_2 to flow through the cavity. Spectrometer conditions were: field sweep: 50 gauss; modulation amplitude: 0.32 gauss; modulation frequency: 100 kHz; microwave power: 16 mW.

Ionic strength, [Ca^{2+}], and pH studies. Ionic strength studies were performed with 0.25 mg spin-labeled CaM in 250 μl buffer. Samples containing Ca^{2+} were adjusted to 1.3 mM $CaCl_2$ giving a final ratio of free calcium ion (calculated from equilibrium data [21]) to CaM of 5 : 1. Ionic strength was adjusted with KCl and pH was adjusted to 7.4 with minimal amounts of 1.0 M HCl or 0.5 M KOH. Each of these three reagents was essentially Ca^{2+}-free containing a maximum of 0.0005% Ca^{2+}.

The effect of Ca^{2+} on spin-labeled CaM was observed at constant pH and ionic strength (150 mM, adjusted with KCl). The pH was adjusted to 7.4 as above.

The effect of pH on spin-labeled CaM was observed in ranges of ionic strength and calcium ion concentration in which no marked change in spin-label motion was seen. The ionic strength was adjusted between 148 mM and 156 mM with HCl. Samples containing Ca^{2+} were adjusted to a free calcium ion concentration of between 310 and 350 μM.

The PDE-activator activity study was performed as described above, but the pH of the concentrated buffer was adjusted with NaOH or HCL prior to its use in the assay.

Results

Specificity of spin label bound to CaM

MAL-6 labeled CaM was prepared as described in Methods. EPR spectroscopy was performed on both the PMB-treated CaM and control CaM. The EPR spectrum of PMB-treated CaM incubated with MAL-6 is shown in Fig. 2a. No evidence of the MAL-6 spin label was observed in contrast to control (Fig. 2b). The latter spectrum corresponds to a 1 : 1.06 mole ratio of MAL-6 : CaM, based on double integration of the EPR spectrum and comparison to standards. Since PMB is specific for the cysteine group of CaM these results indicate that there is no non-specific binding of the spin label. One MAL-6 molecule is incorporated per protein molecule through reaction at the single SH group (Cys-27).

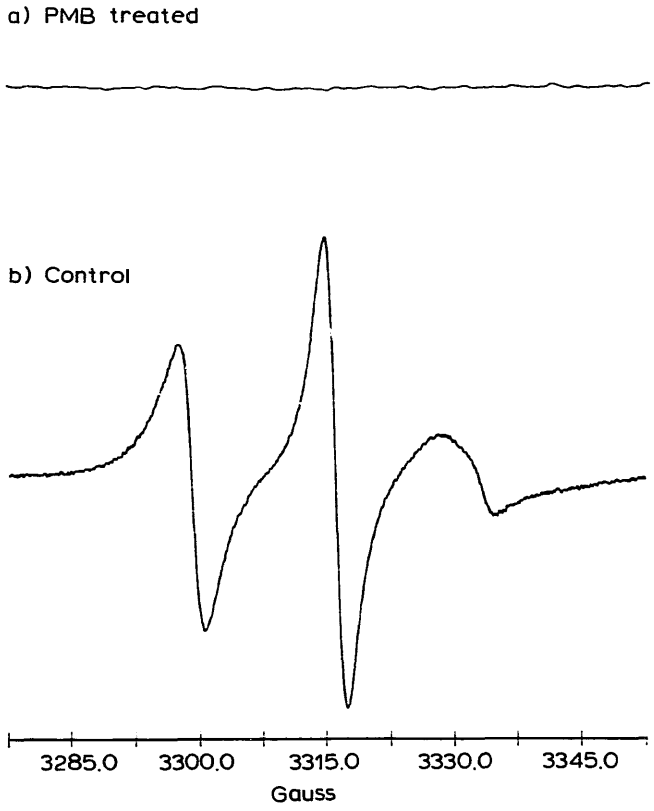

Fig. 2. Comparison of the EPR spectra of PMB-blocked CaM and control CaM after incubation with MAL-6. The PMB-treated CaM (a) shows no evidence of spin label. The control CaM (b) reflects a MAL-6 : CaM ratio of 1 : 1.06 determined by comparison to standards.

Characterization of MAL-6-labeled CaM

No difference in the PDE activator activity of spin-labeled CaM relative to native protein was found, indicating that the presence of the spin label did not affect the activity of the protein. The motion of the spin label on Cys-27 was estimated by an apparent rotational correlation time (τ) employing a well-established, composite equation based on Kivelson theory [22] and the g and T-tensor values of tempone [23] shown in Eqn. 1. τ can be envisaged as the time

$$\tau_c = 6.5 \cdot 10^{-10} \Delta H_{pp}(0) \left[\left(\frac{A(0)}{A(+1)} \right)^{1/2} - 1 \right] \quad (1)$$

necessary for the spin label to rotate through an angle of one radian. Decreased (increased) values of τ suggest faster (slower) rotational motion of the spin label.

Since CaM is a calcium-binding protein, the effect of Ca^{2+} on the motion of MAL-6 on Cys-27 was investigated. In Fig. 3 a half-maximal effect is seen at a free Ca^{2+} concentration of approx. 125 μM. This corresponds to a ratio of 2.5 mol Ca^{2+} : 1 mol CaM. Increasing the Ca^{2+} concentration further appears to decrease tau to a plateau value at concentrations greater than 300 μM. It was assumed therefore that at 300 μM Ca^{2+} all four calcium binding loops are occupied. In the remainder of the studies a 350 μM concentration of free Ca^{2+} was used to ensure that Ca^{2+} occupied all binding loops.

Fig. 4 shows the effect of ionic strength on spin-labeled CaM. In the presence of Ca^{2+}, spin label motion was essentially constant over the entire range of ionic strength studied. However, in the absence of Ca^{2+}, tau is rapidly increased as a function of ionic strength, but then remains relatively constant for ionic strengths greater than 135 mM. Therefore, an ionic strength of 150 mM was maintained for all experiments. The effect of increasing pH on apocalmodulin and calmodulin with all four binding loops occupied with Ca^{2+} is shown in Fig. 5. The isoelectric point of CaM is 4.0. Thus, the higher values of τ at low pH are consistent with decreased motion of a precipitated protein. At very high pH the protein denatures. Hence, the relatively fast motion of MAL-6 is expected. Fig. 5 also addresses the change in the PDE-activator activity of CaM as a function of pH. The maximum activator activity occurs

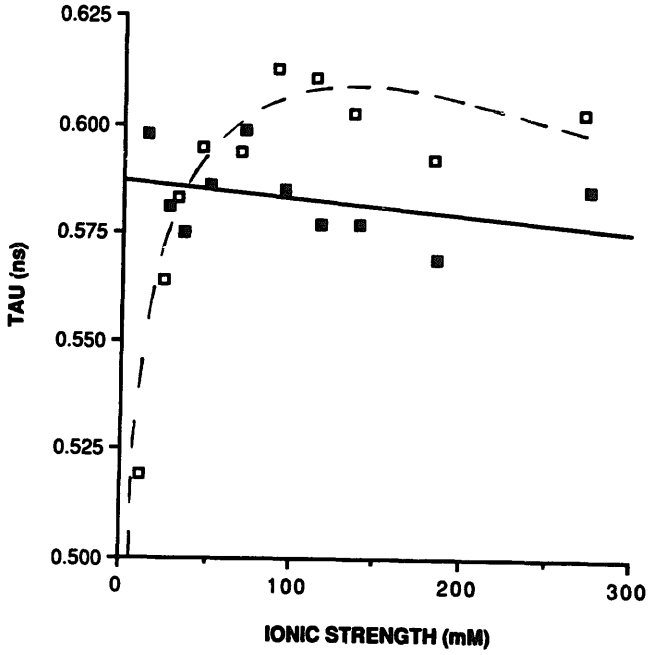

Fig. 4. Effect of ionic strength on MAL-6-labeled CaM. Samples contained 60 μM CaM in buffer (10 mM Tris-base/1 mM EGTA (pH 7.4)). Ionic strength was varied by addition of KCl. ApoCaM is represented by open squares. Solid squares designate samples containing 350 μM free Ca^{2+}.

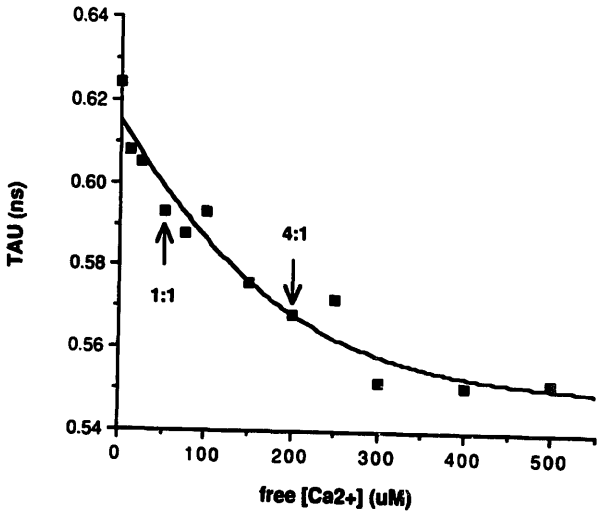

Fig. 3. Effect of free calcium ion concentration on MAL-6-labeled CaM. Samples contained 50 μM CaM in buffer (10 mM Tris-base/1 mM EGTA (pH 7.4)) and ionic strength was adjusted to 150 mM with KCl. Abscissa reflects [Ca^{2+}] available for domain occupation.

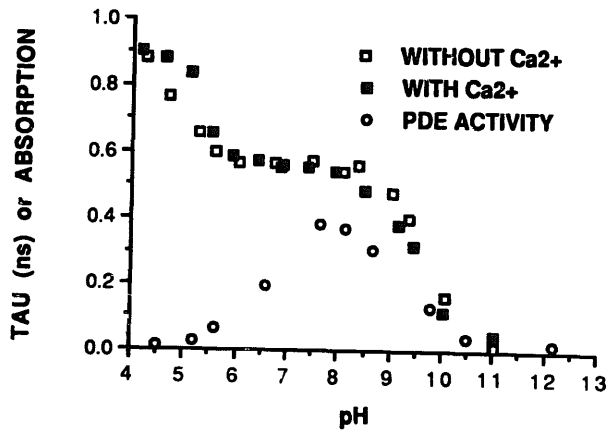

Fig. 5. Effect of pH on MAL-6-labeled CaM or PDE-activator activity of CaM. Spin-labeled samples contained 50 μM CaM and were adjusted to an ionic strength of 150 mM with KCl. Those samples with a Ca^{2+} : CaM ratio of 5 : 1 are indicated by a solid square. The ability of CaM to activate PDE is represented by the absorbance of inorganic phosphate detected following enzymatic activation (denoted by circles).

at physiologic pH and decreases to complete inactivity at extremes of pH. The physiological pH is located in the center of the plateau where τ was approximately constant and at the peak of PDE activator activity. Therefore, pH 7.4 was deemed the appropriate pH for all studies.

Discussion

Since the spin label, MAL-6, is bound only to Cys-27 of CaM as shown in Fig. 2, then the motion of the spin label reflects the local environment of this amino acid. Environmental changes which are known to cause conformational changes in CaM are reflected in the spin-label motion. We are therefore assured that, under the conditions set forth in this paper, conformational changes in CaM can be monitored with this highly sensitive magnetic resonance technique.

The difference observed between the rotational correlation time of apoCaM and the CaM-$(Ca^{2+})_4$ complex is real (Fig. 3) but small when compared to the much larger changes imposed by changes in pH (Fig. 5) (note the different ordinate scales used). Fig. 5 shows the relationship among τ, PDE-activator activity, and pH. Maximal activator activity occurs at physiological pH where τ appears relatively constant. However, at high pH, where the protein is denatured and activator activity is minimal, the spin label would be more exposed to solvent and behave as if it were free in solution. This is consistent with the low value of τ at high pH (Fig. 5). In contrast, when the pH approaches the isoelectric point (approx. pH 4) the spin-label motion slows considerably (high τ value) and there is complete loss of PDE-activator activity. The crystal structure of CaM was obtained from a crystal formed at pH 5.6. Fig. 5 shows that spin-label motion already starts to decrease (increased τ) both in presence and absence of Ca^{2+} at a pH of 5.6, and the ability of CaM to activate PDE is minimal at this pH. Recently, it has been reported that at physiological ionic strength, the α-helical content is increased at pH 5.5 compared with that at pH 7.4 in experiments performed in absence or presence of Ca^{2+} [24]. Therefore, these considerations, together with reported differences in other physical properties of CaM in solution compared to the solid state (α-helical content [9], X-ray solution scattering [8]) may suggest that the structure of CaM inferred from the crystal is not fully transferable to that in solution.

CaM becomes active only when it binds Ca^{2+}. The protein has four Ca^{2+} binding domains, and is fully active upon filling three to four of these [3]. Over the range of Ca^{2+} concentration studied, spin-label motion increases (Fig. 3), consistent with the interpretation that as Ca^{2+} exposure to CaM increases, Cys-27 in the first Ca^{2+}-binding domain becomes less hindered. As mentioned above, this change is slight relative to differences induced at pH extremes. This result is reasonable since variations in pH have been shown by CD studies to alter the α-helical content of CaM [24]. In contrast, at a pH of 7.4 at physiological ionic strength, the α-helical content remained constant upon addition of Ca^{2+} [24]. Likewise, at pH 5.5, the α-helical content, though larger than that at pH 7.4, also remained constant upon addition of Ca^{2+} [24]. Therefore, our results (Figs. 3 and 5) suggest that EPR spin-labeling methods can, depending on the circumstances, reflect large secondary structural changes in CaM also observed in CD [24] and smaller, more subtle conformational changes not detectable by CD.

The ionic strength study (Fig. 4) shows that at very low ionic strength the difference in motion between apoCaM (white squares) and CaM-Ca^{2+} complex (black squares) is more pronounced than the difference at high ionic strength. This result is consistent with the findings of others who show that at low ionic strength and physiological pH, Ca^{2+} binding to apoCaM induces an increase in α-helical content [24]. These workers also showed, as noted above, that at high ionic strength and physiological pH, the α-helical content remained relatively constant.

In the presence of Ca^{2+}, the conformation of CaM seems to be stable with respect to increasing ionic strength (Fig. 4). This substantiates the work of Richman and Klee [25] who found that ionic strength did not affect the UV or CD spectra of CaM in the presence of Ca^{2+}. In contrast, at low ionic strength these workers further showed the same changes in the CD spectra of CaM resulted when either the ionic strength was increased in the absence of Ca^{2+} or when Ca^{2+} was added to apoCaM at low ionic strength [25], a finding recently confirmed be Kowluru et al. [24]. This result is also consistent with our findings in Fig. 4 which show a higher value of τ resulted from either an increase in ionic strength in the absence of Ca^{2+} or by addition of Ca^{2+} to apoCaM at low ionic strength.

Ten years ago, Hewgley and Puett [26] reported the use of an iodoacetamide spin label to study bovine brain CaM. Their label was bound to multiple methionine residues and gave evidence of Heisenberg spin exchange in the presence of Ca^{2+}. In contrast, our method uses a maleimide spin label bound to a single cysteine residue located in the first Ca^{2+}-binding domain of wheat germ CaM. Upon addition of Ca^{2+} we observed an increase in motion in this domain with no evidence of Heisenberg spin exchange.

In this paper we showed that at pH 7.4 and in the presence of 350 μM free Ca^{2+} at physiological ionic strength, spin labeled CaM yields a highly reproducible EPR spectrum reflective of a stable conformation of this protein. Therefore, such a system can now be used to monitor conformational changes in the physical state of CaM in response to a number of different environ-

ments and stimuli. Such studies are currently in progress.

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