Biochemistry

 Volume 22, Number 10 May 10, 1983

## Accelerated Publications

# Cadmium-113 Nuclear Magnetic Resonance Studies of Proteolytic Fragments of Calmodulin: Assignment of Strong and Weak Cation Binding Sites†

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ABSTRACT: Proteolytic fragments of bovine testis calmodulin were obtained by limited proteolysis with trypsin or thrombin. Cadmium-113 NMR studies showed that the tryptic fragment encompassing $Ca^{2+}$ binding domains III and IV ($TR_2C$) gave rise to a spectrum identical with that of the native protein. Two thrombic fragments containing either domains I, II, and III [$TM_1$-(1–106)] or the single domain IV [$TM_2$-(107–148)] both gave rise to one broad resonance only. These data indicate that domains III and IV comprise the two high-affinity $Ca^{2+}$ binding sites in intact calmodulin and that disturbance of the structural relationship between domain III and domain IV markedly reduces the affinity of these two sites for $Ca^{2+}$ ions. These observations are discussed with respect to other published accounts concerning the sequence in which the four calcium domains in calmodulin are filled.

The effects of the intracellular messenger $Ca^{2+}$ are often mediated through the heat- and acid-stable protein calmodulin ($M_r$ 16700). CAM[1] usually interacts with its receptor proteins only after binding of calcium ions [for reviews see Cheung (1980), Klee (1980), and Klee et al. (1980)]. Binding of up to four calcium ions causes large conformational changes in the protein. Amino acid sequencing studies have revealed the presence of four EF handlike calcium binding domains (Vanaman, 1980). Although many studies have addressed the question of the affinity of these four separate sites for $Ca^{2+}$ ions, unfortunately no unique answer has emerged yet [see, for example, Klee (1980)]. Moreover, no clarity exists at present as to how many $Ca^{2+}$ ions are bound in CAM–receptor protein complexes and, in addition, there is still considerable debate as to the order in which the sites are filled in purified apo-CAM (see Discussion).

Here we wish to describe our $^{113}Cd$ NMR studies comparing the binding of $Cd^{2+}$ to calmodulin and some of its proteolytic fragments that are obtained by limited proteolysis. Studies in our laboratory with at least six different calcium binding proteins have shown that the $Cd^{2+}$ ion is a convenient and reliable probe in the study of calcium binding sites [for review see Vogel et al. (1983)]. Our earlier studies with calmodulin (Forsén et al., 1980; Andersson et al., 1982) have shown that calmodulin contains two "high-affinity" sites that give rise to two resolved resonances in a $^{113}Cd$ NMR spectrum, whereas the two "low-affinity" sites are not observed due to exchange between free[2] and protein-bound $Cd^{2+}$ ions.[3] Only after addition of drugs that are known to bind to the hydrophobic surface of calcium or cadmium-saturated CAM is this exchange rate reduced and two additional resonances for the other two sites appear in the spectra (Forsén et al., 1980; Sudmeier et al., 1980; A. Andersson, unpublished results).

## Experimental Procedures

*Materials.* Calmodulin was prepared from bovine testis essentially as described by Jamieson & Vanaman (1979). Apo-CAM was prepared by passage of CAM over a Chelex-

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[1] Abbreviations: CAM, calmodulin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; UV, ultraviolet; CD, circular dichroism; TPCK, 1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol; DEAE, diethylaminoethyl.

[2] The term "free Cd" is only a relative nomenclature. $Cd^{2+}$ ions will interact weakly with (all) NH sites on the surface of any protein, resulting in a very broad resonance for these "free" ions that is usually less sharp than those for the tightly protein-bound $Cd^{2+}$ ions. $Cd^{2+}$ ions also interact with ions like Tris or $Cl^-$, which also cause changes in the Cd line width and chemical shift; hence, results are more easily interpretable when no buffer is used.

[3] Another possibility is a fast exchange between two conformations with different chemical shifts for these two metal ions.

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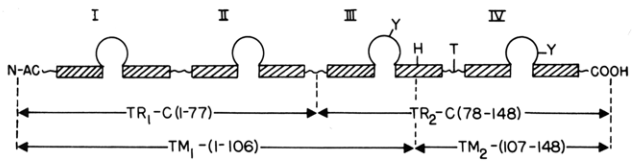


FIGURE 1: Schematic representation of the proposed structure of calmodulin outlining the four domains, each comprising a calcium binding loop and two flanking helix regions (hatched areas). Also indicated are the positions of the two tyrosine residues at positions 99 and 138 (Y) and the positions of the single histidine-106 (H) and trimethyllysine-115 (T) residues. Proteolytic fragmentation as induced by tryptic (TR) or thrombic (TM) cleavage is also indicated.

100 column; the residual amount of metal ion was determined by $^1$H NMR experiments and was always below 0.1. TPCK-treated trypsin, thrombin (grade I), and soybean trypsin inhibitor (type I-S) were obtained from Sigma Chemical Co. $^{113}$CdO (96.3% enriched in isotope $^{113}$Cd) was obtained from Oak Ridge National Laboratory. All other chemicals used were of analytical grade and were obtained from local suppliers.

*Methods.* Thrombic fragments of calmodulin were prepared following the method of Wall et al. (1981). Calmodulin (10 mg) was incubated for 2 h at 37 °C and pH 8.7 (50 mM Tris, 5 mM EDTA, and 1 mM DTT) with 100 units of thrombin. The peptides thus obtained were subsequently purified on a Sephadex G-50 column. Proteolytic fragmentation with trypsin followed essentially the procedures described by Drabikowski et al. (1977) and Walsh et al. (1978). Typically 1 mg of trypsin was added to 100 mg of CAM dissolved in 100 mL of freshly prepared 100 mM $NH_4HCO_3$–1 mM $CaCl_2$, pH 7.8. The reaction was stopped after 35 min at room temperature by the addition of 2 mg of soybean trypsin inhibitor. Fragments were purified on a DEAE-Sephadex A-25 column equilibrated in 50 mM $KH_2PO_4$, pH 7.2, which was eluted with a linear NaCl gradient up to a concentration of 0.5 M. Under these conditions CAM had completely disappeared, and peptide $TR_2C$ (see below) was the last to elute from the column. Peptides were checked for purity by NaDodSO$_4$ gel electrophoresis and agarose gel electrophoresis in the presence and absence of EDTA or $Ca^{2+}$. Moreover, their amino acid composition was checked by $^1$H NMR (361-MHz Nicolet WB) using the resonances for the two tyrosines and the single trimethyllysine and histidine residues as indicators (Seamon, 1980; Krebs & Carafoli, 1982). Figure 1 indicates the position of these moieties in intact calmodulin, and it can readily be seen that all four indicated fragments can be identified in this fashion.

$^{113}$Cd NMR spectra were obtained at room temperature on a home-built spectrometer equipped with an Oxford instrument 6-T magnet operating at a frequency of 56.55 MHz. A home-built probe with a horizontal sample orientation was used for these studies. Proteolytic fragments or whole CAM was dissolved in doubly distilled water treated with Chelex-100. The pH of all samples was kept between 7.0 and 7.5. Typical acquisition parameters have been described elsewhere (Forsén et al., 1979, 1980).

## Results

*Proteolytic Fragmentation.* Limited tryptic digestion of CAM in the presence of saturating amounts of $Ca^{2+}$ will result in cleavage only at lysine-77, thus giving rise to two peptides of equal size (see Figure 1) (Walsh et al., 1977; Drabikowski et al., 1977, 1982). Under our experimental conditions (see Methods) we found that peptide $TR_2C$ accumulated, whereas peptide $TR_1C$ was partially further degraded. Similar results

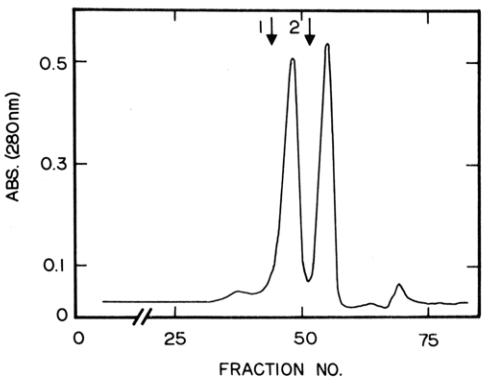


FIGURE 2: Column profile on Sephadex G-50 obtained after thrombic cleavage of CAM. Indicated by the arrows are the elution positions of CAM (1) and fragment $TR_2C$ (2). See text for further explanation.

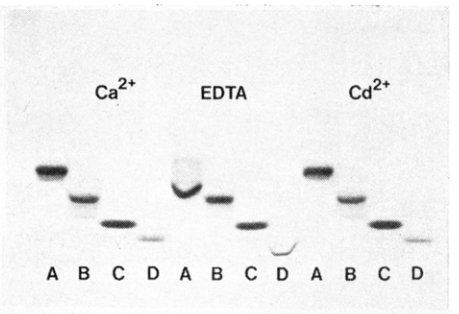


FIGURE 3: NaDodSO$_4$ gel electrophoresis of CAM and its proteolytic fragments (10–20% gradient gels, run at pH 8.7). Identification of the bands is as follows: (A) CAM; (B) $TM_1$; (C) $TM_2$; (D) $TR_2C$.

have been described by others (Klee, 1980).

Incubation of CAM with thrombin results in a highly selective cleavage at arginine-106, giving rise to two fragments of different size (see Figure 1) that can be readily fractionated on a Sephadex G-50 column (see Figure 2) (Wall et al., 1981). The small shoulder preceding the first peak probably comprises residual thrombin, whereas the first peak contains fragment $TM_1$ and the second fragment, $TM_2$. Also indicated in Figure 2 are the elution positions of CAM and fragment $TR_2C$ on the same column, indicating that the relative size of the fragments is as expected from Figure 1.

*Electrophoretic Studies.* In order to assess the affinities of these fragments for $Ca^{2+}$ and $Cd^{2+}$, we studied their behavior in the presence and absence of $Ca^{2+}$, $Cd^{2+}$, and/or EDTA on NaDodSO$_4$ gel electrophoresis. Similar experiments have been described in order to assess the binding of lanthanides to CAM (Wallace et al., 1982). It has been known for some time that abnormally high values for the molecular weight can be obtained for various highly acidic calcium binding proteins [see Klee (1980) and references cited therein] and that the mobility can be different in the presence of $Ca^{2+}$ or chelating agents. As can be seen in Figure 3, such behavior is found most notably for peptide $TM_2$, which runs on the NaDodSO$_4$ gel as if it has a molecular weight between $TM_1$ and $TR_2C$. From the results in Figure 3 it is clear that for CAM and $TR_2C$ different mobilities are observed when EDTA or cation is present. It is important to note that no difference between $Ca^{2+}$ and $Cd^{2+}$ is observed, showing that $Cd^{2+}$ is a good substitute for $Ca^{2+}$. Note further that the mobility of peptides $TM_1$ and $TM_2$ is not affected by the addition of cation or chelator.

*$^{113}$Cd NMR Studies of Fragment $TR_2C$.* Parts a and b of Figure 4 compare the $^{113}$Cd NMR spectra obtained after saturation of CAM and $TR_2C$ with $Cd^{2+}$. In both cases two sharp resonances are observed at chemical shift positions −87.5

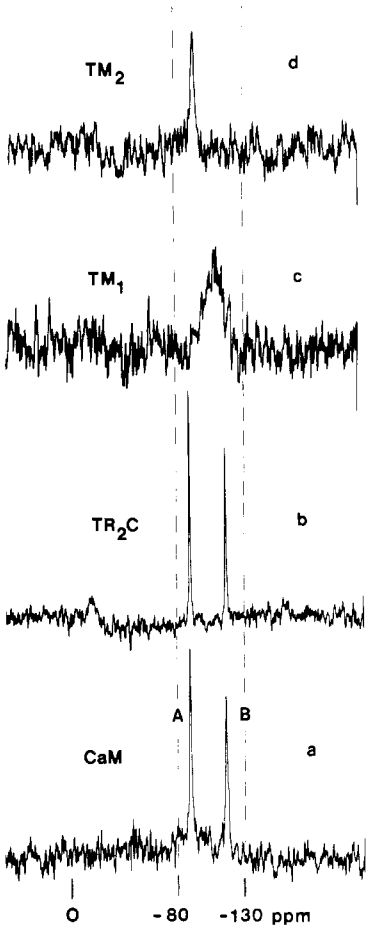


FIGURE 4: $^{113}Cd$ NMR spectra of calmodulin (a) and its fragments (b–d). Conditions used were as follows: (a) 1.2 mM CAM, 4.8 mM $Cd^{2+}$, $2.5 \times 10^4$ acquisitions; (b) 0.3 mM $TR_2C$, 0.6 mM $Cd^{2+}$, $3 \times 10^5$ acquisitions; (c) 0.92 mM $TM_1$, 2.7 mM $Cd^{2+}$, $5 \times 10^4$ acquisitions; (d) 1.30 mM $TM_2$, 2.5 mM $Cd^{2+}$, $5 \times 10^4$ acquisitions.

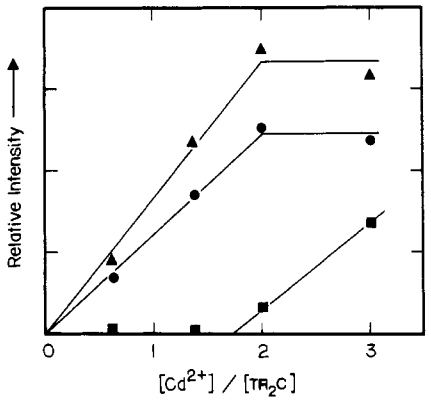


FIGURE 5: Increase in signals A (▲), B (●), and free $Cd^{2+}$ (■) upon titration of apo-$TR_2C$ (0.3 mM) with $Cd^{2+}$ (other conditions as in Figure 4b).

and −114.2 ppm.[4] Thus we conclude that resonances A and B in the intact protein represent $Cd^{2+}$ bound to domains III and IV. Figure 5 shows the peak intensities observed for both signals upon $^{113}Cd^{2+}$ titration of apo-$TR_2C$. Clearly, as with CAM (Forsén et al., 1980), both signals increase in parallel rather than sequentially, indicating either that domains III and IV have identical binding constants or that binding occurs in a positive cooperative fashion (Klee, 1977; Crouch & Klee, 1980). Since we have observed the same parallel increase for skeletal and cardiac troponin C, we feel that positive cooperativity is the more likely explanation.

[4] The difference in intensity between signal A and signal B arises from a slight difference in $T_1$ for these two sites.

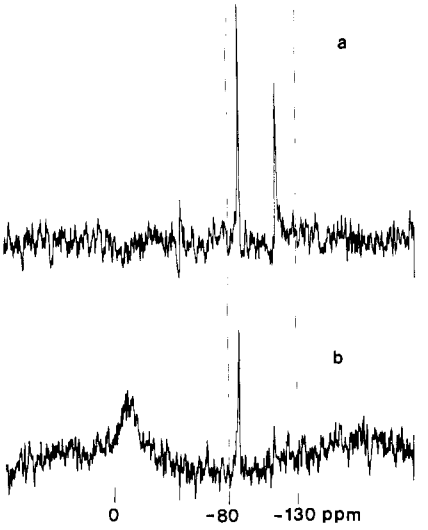


FIGURE 6: $^{113}Cd$ NMR spectra of $Cd^{2+}$-saturated $TR_2C$ (0.3 mM) before (a) and after (b) addition of 1.3 equiv of $Ca^{2+}$ ($1.3 \times 10^5$ acquisitions).

*$^{113}Cd$ NMR Studies with Thrombic Fragments.* Parts c and d of Figure 4 show the spectra obtained with fragments $TM_1$ and $TM_2$ after the addition of 3 and 2 equiv of $Cd^{2+}$ ions, respectively. The broad signal for $TM_1$ appears at −109 ppm and probably represents binding to domain III; the signal for $TM_2$ (domain IV) appears at −95 ppm. Unfortunately, the proteolytic cleavage of the protein between domain III and domain IV has caused changes in the calcium binding loops III and IV such that the chemical shift positions for the thrombic fragments no longer correspond to those observed for CAM. However, since $Cd^{2+}$ bound to $TM_1$ resonates closest to B and that of $TM_2$ resonates closest to A, it is tempting to speculate that resonances A and B represent $Cd^{2+}$ bound to domains IV and III, respectively, in CAM. Spectra obtained at lower and higher ratios of $Cd^{2+}$ with respect to thrombic peptide gave essentially the same chemical shifts. The resonances for $TM_1$ are somewhat sharper when 2 or 4 equiv are present, whereas the resonance for $TM_2$ becomes broadened somewhat when more $Cd^{2+}$ is added (data not shown).

*Metal Ion Competition.* Figure 6 shows the results obtained after the addition of 1.3 equiv of $CaCl_2$ to a sample of $Cd^{2+}$-saturated $TR_2C$. Addition of only 1 equiv abolishes resonance B whereas after addition of 2.7 equiv about 40% (and after addition of 4.0 equiv about 25%) of resonance A is still present (data not shown). Identical results have been reported for intact bovine testis CAM (Forsén et al., 1980).

## Discussion

Recent studies with proteolytic or synthetic fragments of calcium binding proteins have provided some new insights in the functioning of calcium binding proteins. In general, it has been observed that fragments containing only one binding domain (like $TM_2$) have lower affinity for $Ca^{2+}$ than fragments containing a pair of related domains (like $TR_2C$). The latter behave often very similar to the intact protein [for review and discussion see Seamon & Kretsinger (1983) and Vogel et al. (1983)]. For example, in the case of skeletal troponin C, a protein which is very similar with CAM, studies with fragments demonstrated unequivocally that domains III and IV contain the two high-affinity $Ca^{2+}$ binding sites [Leavis et al.,

1978; for discussion see McCubbin & Kay (1980)]. Spectroscopic studies provided evidence that the structural characteristics are maintained in some of these fragments and that $Ca^{2+}$ binding often gives rise to similar changes in the spectroscopic parameters as for the intact protein (Leavis et al., 1978; Evans et al., 1980). Although CAM and skeletal troponin C are homologous (Vanaman, 1980), the situation is less clear as to the location of the two high-affinity sites in CAM. Estimates of the dissociation constants have indicated that all four sites have dissociation constants within one order of magnitude ($K_D \approx 10^{-5}$–$10^{-6}$ M) and that the four sites can be divided into two subclasses, two with lower and two with higher affinity (Klee, 1980). Earlier $^{113}Cd$ and $^{43}Ca$ NMR studies have also provided evidence for the existence of two high-affinity and two low-affinity sites. The former are characterized by a much slower metal ion off-rate than the latter[5] (Forsén et al., 1980; Andersson et al., 1982). The experiments reported here show unequivocally that the two sites with slower metal off-rates are domains III and IV, since the $^{113}Cd$ NMR spectra obtained with the tryptic fragment $TR_2C$ are identical with those obtained with CAM (compare parts a and b of Figure 4). Since $^{113}Cd$ NMR chemical shifts are known to be extremely sensitive to small changes in protein structure, as exemplified by studies on a series of homologous parvalbumins (Cavé et al., 1982), the structure of the binding sites in CAM and $TR_2C$ must be identical. Note that *no* two sharp resonances are observed for peptide $TM_1$ (containing domains I, II, and III) (see Figure 4c), suggesting that domains I and II contain the protein bound $Cd^{2+}$ ions that are not observable (due to exchange broadening) for CAM (Figure 4a; Forsén et al., 1980). Rather, we observed only one broad resonance, probably representing the $Cd^{2+}$ bound to domain III. Also, for $TM_2$ (domain IV) we only observed one broadened resonance, although the broadening here is not as marked as observed for $TM_1$. This broadening effect for these two resonances is of interest. In these particular experiments the line width can be viewed as approximately inversely related to the affinity:[5] thus the order of affinity CAM and $TR_2C > TM_2 > TM_1$. The notion that $TM_2$ has a higher affinity for metal ions than $TM_1$ is of interest since studies with synthetic peptides have shown that the presence of an $\alpha$-helix on either side of the calcium binding loop increases the affinity for $Ca^{2+}$ ions (Reid et al., 1981). $TM_1$ has only three amino acids on its carboxy-terminal end of the calcium binding loop, which are capable of forming an $\alpha$-helix, whereas the calcium binding loop of $TM_2$ is surrounded by its native $\alpha$-helices, and the latter is thus expected to have a higher affinity for the metal ion. In addition, it should be noted that both $TM_1$ and $TM_2$ have a lower affinity for $Cd^{2+}$ than peptide $TR_2C$. From the data presented in Figure 3 it is also clear that both $Ca^{2+}$ and $Cd^{2+}$ have a lower affinity for the thrombic fragments, since their electrophoretic mobility is not altered upon addition of metal ion or chelator. This is consistent with the idea that a pair of related calcium binding domains has a higher affinity than one isolated domain (Seamon & Kretsinger, 1983; Vogel et al., 1983). In this respect it is of interest that the only two crystal structures reported to date for calcium binding proteins (Kretsinger & Nockolds, 1973; Szebenyi et al., 1981) show not only that the structure of the calcium binding loops and the surrounding helices are conserved but also the approximate 2-fold symmetry within a pair of domains. This structural relationship is also likely to be important for the cooperativity between the two high-affinity sites (Klee, 1977; Crouch & Klee, 1980; Forsén et al., 1980). Upon titration of apo-CAM (Forsén et al., 1980) or apo-$TR_2C$ (Figure 5) with $Cd^{2+}$, signals A and B increase in parallel in both cases, suggesting positively cooperative interactions between domain III and domain IV that are identical in the peptide and in intact CAM.

It could be argued that affinity for $Ca^{2+}$ and $Cd^{2+}$ ions is not similar. Although both ions have the same charge and a very similar size (0.097 and 0.099 nm, respectively), some calcium binding proteins have a higher (Drakenberg et al., 1978) and some a lower (Murakami et al., 1982) affinity for $Cd^{2+}$ than for $Ca^{2+}$. Several lines of evidence suggest however that $Cd^{2+}$ is a good probe in the studies with proteolytic fragments outlined above. First, our studies with $NaDodSO_4$ gel electrophoresis show that $Ca^{2+}$ and $Cd^{2+}$ induce the same change in mobility. Second, the metal ion competition between $Ca^{2+}$ and $Cd^{2+}$ observed with $TR_2C$ (Figure 6) is identical with that observed for the intact protein (Forsén et al., 1980). Third, two sites with high and low affinity are observed for CAM both in "perturbing" $^{113}Cd$ NMR and in "nonperturbing" $^{43}Ca$ NMR studies, indicating the high similarity between the outcome of experiments with the two different probes. Studies with $^{43}Ca$ NMR of proteolytic fragments are presently in progress in our laboratory. Finally, $^1H$ NMR titration studies with both $Ca^{2+}$ and $Cd^{2+}$ show identical changes in resolved resonances for intact calmodulin as well as for the proteolytic fragments (our unpublished results).

Our results discussed above indicate that III and IV are the first domains to be filled in intact apoprotein. Various other studies have been reported addressing this same problem. UV and near-UV–CD experiments, fluorescence experiments, and $^1H$ NMR measurements of CAM indicate that tyrosines-99 and -138 that are located in calcium binding loops III and IV (see Figure 1) are most effected by binding of the first two calcium ions and are thus consistent with the notion that domains III and IV comprise the two high-affinity sites (Klee, 1977; Crouch & Klee, 1980; McCubbin et al., 1979; Dedman et al., 1977; Seamon, 1980; Krebs & Carafoli, 1982). In addition, UV, CD, and fluorescence studies (Drabikowski et al., 1977, 1982; Walsh et al., 1977) as well as $^1H$ NMR studies (our unpublished results) with the proteolytic fragment $TR_2C$ show that very similar, if not identical, perturbations for the tyrosines are observed upon binding of $Ca^{2+}$ ions, thus providing further evidence for this notion. However, in strong contrast to the above, flow dialysis experiments (Haiech et al., 1981), $^{23}Na$ NMR measurements (Delville et al., 1980), and most particularly terbium luminescence transfer experiments (Kilhoffer et al., 1980a,b; Wang et al., 1982; Wallace et al., 1982) provided evidence for another sequence in which the four sites are filled. It should be recalled that in these studies monovalent ions (Haiech et al., 1981; Delville et al., 1980) or trivalent metal ions were used as probes, and thus it has been alleged that these may behave differently from $Ca^{2+}$ (Seamon & Kretsinger, 1983). Indeed, monovalent cations are incapable of inducing $Ca^{2+}$-like conformational changes in the protein (Klee, 1980), and thus the affinity for these does not necessarily have to parallel that of $Ca^{2+}$. Moreover, the suggestion that $K^+$ will bind strongest to the site with the most carboxylate groups is not necessarily correct (Haiech et al., 1981). Since $Tb^{3+}$ is generally considered to be a reasonable replacement for $Ca^{2+}$ (Martin & Richardson, 1979), it is more difficult to rationalize why the results obtained with $Tb^{3+}$ luminescence transfer are out of line with the evidence dis-

[5] The metal-exchange rates are only a valid relative estimate of the dissociation constants when the metal on-rates are the same for all four sites. This seems a valid assumption since this rate is very close to the diffusion-limited on-rate (Andersson et al., 1982).

cussed above. This is perhaps related to an unexpected orientation of the tyrosines with respect to the $Ca^{2+}$ binding sites in CAM, which is difficult to assess at present in the absence of a crystal structure for the protein.

In conclusion, we feel that, in line with a large body of evidence, our studies provide strong evidence that domains III and IV are the strong $Ca^{2+}$ binding sites in CAM and that they bind in a cooperative fashion. Furthermore, the outcome of these studies emphasizes the notions that the structural relationship between a pair of calcium binding domains needs to be preserved to attain high affinity (Seamon & Kretsinger, 1983) and that $\alpha$-helices surrounding the calcium binding loop stabilize $Ca^{2+}$ binding (Reid et al., 1981).

Acknowledgments

We are indebted to Dr. Torbjörn Drakenberg for helpful comments and to Dr. Claude B. Klee and A. Aulabaugh for advice on the isolation of the proteolytic peptides.

**Registry No.** Calcium, 7440-70-2; cadmium-113, 14336-66-4.

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