Calmodulin Binds to Caldesmon in an Antiparallel Manner†

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ABSTRACT: Two of the five tryptophan residues (W659 and W692) in chicken gizzard smooth muscle caldesmon (CaD) are located within the calmodulin (CaM) binding sites in the C-terminal region of the molecule. When these Trp residues are replaced with Gly in either recombinant fragments or synthetic peptides of CaD, the affinity for CaM is decreased by at least 10-fold, suggesting that both of these residues are important for the interaction of CaD with CaM. To gain information about the topography of the CaM−CaD complex, we have carried out fluorescence titrations of CaM with Tb^{3+} as a substitute for Ca^{2+} in the presence of wild-type or mutated CaD variants. By exciting Trp residues of CaD fragments or peptides while monitoring the enhanced luminescence of CaM-bound Tb^{3+} ions via resonance energy transfer, we were able to estimate the relative proximity between the bound metal ions in the two domains of CaM and the Trp residues of CaD. Our results suggest that in the CaM−CaD complex the metal-binding sites III and IV in the C-terminal domain of CaM are very close to W659 of CaD; the N-terminal domain of CaM appears associated with the region of CaD in the vicinity of W692, although sites I and II are relatively far away from this Trp residue. These findings are consistent with a model in which CaM binds to CaD in an antiparallel manner. Such a binding mode, however, may be flexible enough to accommodate alternative spatial arrangements when the preferred binding sites are either altered or rendered unavailable.

Smooth muscle caldesmon (CaD)[1] is thought to play a regulatory role in smooth muscle contraction [for reviews, see Marston and Huber (1996), Marston and Redwood (1991), Matsumura and Yamashiro (1993), Sobue and Sellers (1991)]. *In vitro*, CaD binds to F-actin and attenuates actomyosin ATPase activity (Marston & Lehman, 1985; Ngai & Walsh, 1984). The inhibitory effect is reversed upon binding to CaM in the presence of Ca^{2+} (Horiuchi et al., 1986; Smith & Marston, 1985). This thin filament-based modulatory effect may provide additional "fine-tuning" to the well-established, myosin light chain phosphorylation-dependent, thick filament-based regulation (Adelstein & Eisenberg, 1980). Although other mechanisms, such as phosphorylation of CaD (Adam & Hathaway, 1993; Ngai & Walsh, 1987; Yamashiro et al., 1990), have also been suggested to cause the reversal of inhibition, pertinent evidence has not been documented. Thus, CaM (or another Ca^{2+}-binding protein) remains a prime candidate for switching on and off CaD's putative function both *in vitro* and *in vivo*.

Earlier data showed that CaM weakens the binding of CaD to F-actin, leading to the simple "flip-flop" model (Sobue et al., 1982). Although domain mapping studies revealed that the actin-binding site is indeed sufficiently close to the CaM-binding site in the C-terminal region of CaD (Fujii et al., 1987; Szpacenko & Dabrowska, 1986; Wang et al., 1991; Wang & Chacko, 1996), later experiments indicated that dissociation of CaD from F-actin is not prerequisite for the reversal of ATPase inhibition (Pritchard & Marston, 1989; Smith et al., 1987). It is thus possible that CaM, CaD, and actin-tropomyosin form a quarternary complex that does not inhibit the actomyosin ATPase activity (Pritchard & Marston, 1989).

We have previously localized the CaM-binding site to a nine-residue stretch of the CaD sequence from Met-658 to Ser-666 (referred to as site A). A synthetic peptide, GS17C (Gly-651 to Ser-667), comprising this segment indeed binds CaM with an affinity comparable to that of CaD (Zhan et al., 1991). Subsequently, another CaM-binding site (site B) located downstream of the GS17C sequence from Ser-687 to Lys-695 has been reported (Marston et al., 1994; Mezgueldi et al., 1994). The relations between these two sites and their respective roles in the regulatory function have been the subjects of active discussion, because the results obtained from various CaD fragments and/or peptides lead to conflicting models (Marston et al., 1994; Zhuang et al., 1995). For example, a fragment (H9) that contains only site B was shown to inhibit the acto-HMM ATPase activity, an effect that could be reversed by CaM (Marston et al., 1994); whereas a peptide (GS17C) that contains site A only was

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[1] Abbreviations used: CaD, chicken gizzard caldesmon; GS17C, the CaM-binding, site A-containing peptide of CaD (from residue 651 to 667); GS17XC, the variant peptide of GS17C that carries a W659G mutation; VG29C, the CaM-binding, site B-containing peptide of CaD (from residue 685 to 713); VG29XC, the variant of VG29C that carries a W692G mutation; 22K, the wild-type C-terminal, 22 kDa fragment of CaD (from residue 579 to 756); $22K_{A^-}$, the mutant fragment of 22K that contains a W659G replacement; $22K_{B^-}$, the mutant fragment of 22K that contains a W692G replacement; CaM, calmodulin; CaM41/75, the CaM mutant (Q41C,K75C) whose N-terminal domain is locked by a disulfide bond between residues 41 and 75; CaM85/112, the CaM mutant (L85C, I112C) whose C-terminal domain is locked by a disulfide bond between residues 85 and 112; DTT, dithiothreitol; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′- tetraacetic acid; F_{moc}, *N*-(9-fluorenyl)methoxy-carbonyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IANBD, iodoacetamido-4-nitrobenz-2-oxa-1,3-diazole; MLCK, myosin light chain kinase; NBD, the fluorescent moiety of IANBD; NTA, nitrilotriacetic acid; PCR, polymerase chain reaction; PIPES, 1,4-piperazine-diethanesulfonic acid.

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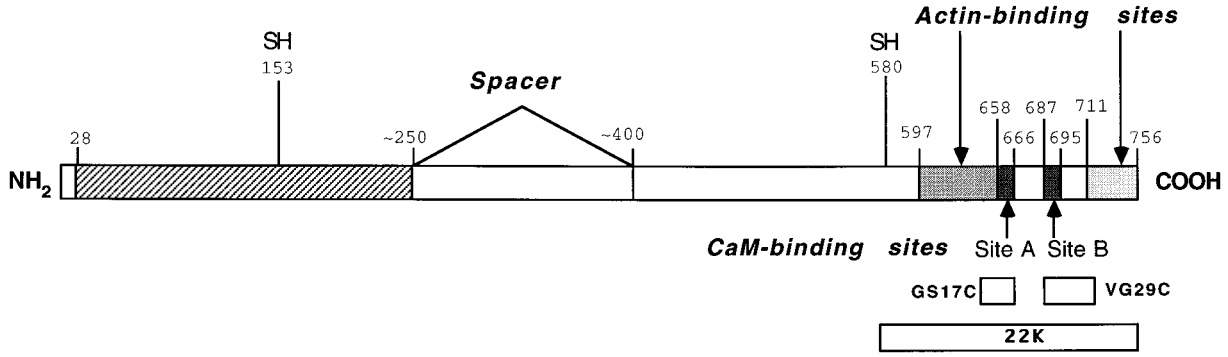
Scheme 1. Positions of CaD Peptides and Fragments Used in this Study



able to displace CaM from its complex with CaD and to restore inhibition, indicating that site A may also play a functional role (Zhuang et al., 1995). Scheme 1 shows the positions of GS17C and other CaD fragments/peptides used in this study.

The facts that CaD contains more than one CaM- and actin-binding site in a relatively short polypeptide segment (~100 amino acid residues) and that the C-terminal domain has a considerably compact 3D structure (Levine et al., 1990; Mabuchi & Wang, 1991) suggest that the tertiary folding of CaD in this region must facilitate multiple contacts among these protein components (Huber et al., 1996; Zhuang et al., 1995). In order to better understand the regulation of CaD by CaM, one wishes to know the spatial relationship between these proteins. In the absence of high-resolution structural information, we have addressed this problem by fluorescence energy transfer techniques. Our data indicate that CaM binds to CaD in an antiparallel fashion and provide a model by which some of the previous controversies may be reconciled.

MATERIALS AND METHODS

Synthetic Peptides. Four synthetic peptides were used in this study:

GS17C: NH_2-^{651}GVRNIKSM**W**EKGNVFSS^{667}C-$CONH_2$

GS17XC: NH_2-^{651}GVRNIKSM**G**EKGNVFSS^{667}C-$CONH_2$

VG29C: NH_2-^{685}VSSRINE**W**LTKTPEGNKSPAP-KPSDLRPG^{713}C-$CONH_2$

VG29XC: NH_2-^{685}VSSRINE**G**LTKTPEGNKSPAP-KPSDLRPG^{713}C-$CONH_2$

GS17C and VG29C each contain one of the two putative CaM-binding sites: site A [singly underlined, Met-658 to Ser-666, see Zhan et al. (1991)] or site B [doubly underlined, Ser-687 to Lys-695, see Marston et al. (1994) and Mezgueldi et al. (1994)]. In the two variants, GS17XC and VG29XC, the Trp residue in site A or site B was replaced with Gly. All peptides were synthesized on an automated peptide synthesizer (ABI Model 431A) using Rink resin (Advanced ChemTech, Louisville, KY) and the "FastMoc" method. After synthesis, the peptides were cleaved from the resin according to a trifluoroacetic acid-based protocol and purified by reversed phase HPLC using a semipreparative C-8 column (Phenomenex, Torrance, CA), as described previously (Wang & Wang, 1996; Zhuang et al., 1995). All peptides thus purified were analyzed by determination of the amino acid composition, partial sequencing, and by MALDI-TOF mass spectrometry (PerSeptive Biosystems, Voyager RP) to ascertain the purity and complete removal of protecting groups. Concentrations of the Trp-containing peptides were determined spectrometrically using an extinction coefficient at 280 nm of 5600 cm^{-1} M^{-1} (Bailey, 1966). For the mutated peptides which do not contain Trp, an extrinsic fluorescent probe was used: we have attached a thiol-directed fluorophor, iodoacetamido-4-nitrobenz-2-oxa-diazole (IANBD) to the C-terminal Cys residue of all four peptides. The freshly reduced peptides were reacted with the probe (peptide: IANBD = 1:10) for 5 h at room temperature; the reaction was terminated by addition of excess DTT, followed by exhaustive dialysis using M_r 1000 cut-off dialysis tubings (Spectrum Medical Industries, Inc.). A molar extinction coefficient at 480 nm of 25 000 cm^{-1} M^{-1} was used for IANBD (Rosenfeld & Taylor, 1985).

Preparation of Wild-Type and Mutated Recombinant CaD Fragments. The wild-type 22 kDa C-terminal fragment of chicken gizzard CaD, 22K (from Lys-579 to Pro-756), was expressed in *Escherichia coli* using a T7 RNA polymerase-promoter based expression vector pAED4 (Doering & Matsudaira, 1996) and purified first by passing through a DE-52 column to remove nucleic acids and then by CaM-Sepharose affinity column chromatography as previously described (Wang et al., 1991). Mutagenesis of 22K was carried out by the overlap extension PCR technique (Ho et al., 1989). The wild-type plasmid, pAED4CaD22K, was used as a template. Primers having the following sequences were used:

5′-ATCAAGAGCATG**GGG**GAGAAAGGGAAT-3′

5′-ATTCCCTTTCTC**CCC**CATGCTCTTGAT-3′ for $22K_{A^-}$

5′-CGTATCAACGAA**GGG**CTAACCAAGACC-3′

5′-GGTCTTGGTTAG**CCC**TTCGTTGATACG-3′ for $22K_{B^-}$

where the underlined nucleotides denote changes of tryptophan to glycine at positions 659 and 692, respectively. The PCR products were subcloned into pAED4 at *Nde*I and *Xho*I sites. Mutations were confirmed by DNA sequencing over the region of interest (Sanger et al., 1977). Both mutants

were expressed in BL21(DE3) cells and purified as the wild-type 22K under low-salt (50 mM KCl) conditions (Wang et al., 1991).

Other Proteins. CaD was isolated from chicken gizzards as previously described (Bretscher, 1984; Wang, 1988). Recombinant chicken brain wild-type CaM (a gift of Dr. A. R. Means) was overexpressed in *E. coli* using the pAED4 vector and purified by phenyl-Sepharose column chromatography (Dedman & Kaetzel, 1983). CaM mutants (CaM41/75 and CaM85/112) were prepared as described previously (Tan et al., 1996).

Tb^{3+}-Titration. The trivalent lanthanide ion, Tb^{3+}, binds to CaM at the 4 Ca^{2+}-binding sites with an affinity 10−100-fold higher than that of Ca^{2+}, but with a reversed preference: Ca^{2+} fills up sites III and IV in the C-terminal domain of CaM before binding to sites I and II in the N-terminal domain, whereas Tb^{3+} prefers binding to sites I and II (Mulqueen et al., 1985; Wang et al., 1982; Wang et al., 1984). When bound to sites III and IV, Tb^{3+} can be excited via resonance energy transfer from nearby Tyr residues (Tyr-99 and Tyr-138) resulting in enhanced luminescence at $\lambda = 543$ nm (Kilhoffer et al., 1980; Wallace et al., 1982; Wang et al., 1982). The concentration of the $TbCl_3$ stock solution was determined by titrating it into a CaM solution of known concentration (Wang et al., 1982; Wang, 1989). The CaM concentration was in turn determined spectrophotometrically using an extinction coefficient at 277 nm of 3300 cm^{-1} M^{-1} (Klee, 1977). As Tb^{3+} tends to form hydroxide precipitates in basic solutions, titrations with $TbCl_3$ were carried out in a buffer containing 25 mM PIPES (pH 6.9) and 50 mM KCl. For all stoichiometric titrations, CaM was rendered Ca^{2+}-free by first dialyzing the stock solution (360 μM) against a buffer containing 30 μM EDTA, followed by dilution to the desired concentration (~7 μM) with a Chelex-treated buffer; the final CaM solution contained less than 1 μM EDTA. The absence of Ca^{2+} was further confirmed by titrations with $CaCl_2$, which resulted in an increase in the intensity of either the Tyr fluorescence (for CaM alone) or the Trp fluorescence (when CaD peptide was present; see Results).

Ca^{2+} Titrations. Interactions between CaM (wild-type or mutants) and synthetic peptides or recombinant fragments of CaD were studied by adding aliquots of the CaM stock solution to 0.6 mL of a CaD peptide/fragment-containing solution (50 mM KCl, 1 mM $CaCl_2$, and 20 mM HEPES, pH 7.5) and monitoring either the tryptophan emission (λ_{exc} = 295 nm; λ_{em} = 320 nm) of the fragments or the NBD fluorescence (λ_{exc} = 490 nm; λ_{em} = 540 nm) of the labeled peptides. The titration data were analyzed by fitting with the equation described by Morris and Lehrer (1984): $AKv^2 - (1 + nKA + KB)v + nKB = 0$, where A and B are the total concentrations of the CaD peptide/fragment and CaM, respectively, v is the fractional saturation of the binding sites measured by the fluorescence enhancement, K is the binding constant, and n is the apparent stoichiometry of the complex, AB_n. The curve fitting was carried out using a nonweighted, nonlinear least-squares method.

Binding was also monitored as a function of free Ca^{2+} concentrations. Solutions containing CaM (12.7 μM) with or without GS17C or VG29C (10 μM) in 50 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM EGTA, and 1 mM nitrilotriacetic acid (NTA) were titrated with 50 mM $CaCl_2$, while both the pH and the fluorescence intensity (λ_{exc} = 295 nm, λ_{em} = 325 nm for the CaM-peptide mixtures, and λ_{exc} = 280 nm, λ_{em} = 308 nm for CaM alone) were monitored. $[Ca^{2+}]_{free}$ was calculated with a computer program (Perrin & Sayce, 1967) using the measured pH and published values for the binding constants (Sillen & Martel, 1964). Data points, after correction for dilution, were fitted with the Hill equation

$$F = F_0 + \Delta F\,(K_a[Ca^{2+}])^n/(1 + (K_a[Ca^{2+}])^n)$$

where F_0 and ΔF are the initial fluorescence and maximal change in fluorescence, respectively; K_a is the apparent binding constant, and n is the Hill coefficient.

Table 1: Summary of Binding Parameters of CaM for Synthetic Peptides and Recombinant Fragments of CaD and Their Variants[a]

peptide/fragment	K_a (M^{-1})	n	F_∞/F_o
$GS17C_{NBD}$	$(5.22 \pm 0.38) \times 10^6$	0.88 ± 0.06	6.52 ± 0.06
$GS17XC_{NBD}$	ND[b]	ND[b]	ND[b]
$VG29C_{NBD}$	$(0.97 \pm 0.14) \times 10^6$	1.19 ± 0.19	2.98 ± 0.05
$VG29XC_{NBD}$	ND[b]	ND[b]	ND[b]
22K	$(8.55 \pm 1.68) \times 10^6$	0.92 ± 0.04	3.81 ± 0.06
$22K_{A^-}$	$(0.71 \pm 0.26) \times 10^6$	0.94 ± 0.47	3.36 ± 0.16
$22K_{B^-}$	$(0.75 \pm 0.31) \times 10^6$	1.19 ± 0.48	2.99 ± 0.19

[a] Solutions containing various peptides or fragments were titrated with CaM in the presence of Ca^{2+}; either the NBD (for the labeled synthetic peptides) or the Trp (for the recombinant fragments) fluorescence was monitored. The data were then analyzed by a nonlinear regression fitting program to determine the binding parameters, including the binding constant (K_a), apparent stoichiometry (n), and the overall fluorescence enhancement (F_∞/F_o). [b] Although there was a slight increase in fluorescence indicating some very weak binding, no reliable fit could be obtained from these data sets.

RESULTS AND DISCUSSION

Significance of Trp Residues for the CaD−CaM Interaction. The C-terminal region of CaD contains two CaM-binding sites, each containing a Trp residue: W659 in site A and W692 in site B. To test whether these Trp residues contribute to the interaction with CaM, we have synthesized two peptides: GS17C and VG29C, corresponding to the two CaM-binding sites, and their respective variants, GS17XC and VG29XC, in which the Trp residues were replaced by Gly. Binding of CaM to these peptides was examined by fluorescence titration, with monitoring of the emission intensity of either Trp fluorescence or the fluorescence of a probe (IANBD) attached to the C-terminal Cys residues. For the NBD-labeled wild-type peptides, simultaneous measurements of both Trp and NBD fluorescence changes yielded the same binding parameters (data not shown), thus, indicating that the two spectral changes are reporting the same binding event. Both $GS17C_{NBD}$ and $VG29C_{NBD}$ exhibited relatively strong binding to CaM in the presence of Ca^{2+} (Table 1; Figure 1, closed symbols), which can be reversed by EGTA, as observed previously for the unlabeled wild-type peptides (Zhuang et al., 1995). The Trp-to-Gly mutants, on the other hand, showed little binding under the same conditions (Figure 1, open symbols), suggesting that Trp residues are important for CaM binding to CaD.

This conclusion was further tested by using bacterially expressed C-terminal CaD fragment, 22K (from K579 to P756) and its two mutants, $22K_{A^-}$ and $22K_{B^-}$, having, like the peptides, a Trp-to-Gly replacement in site A (W659G) and site B (W692G), respectively. Binding of these fragments to CaM was investigated by stoichiometric titration

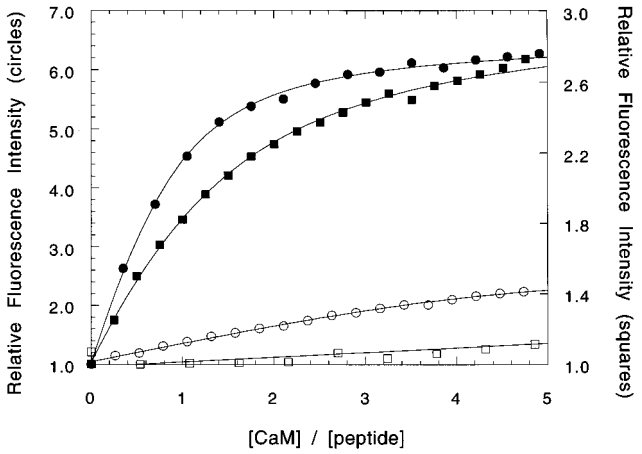


FIGURE 1: Fluorescence titration of NBD-labeled CaD peptides GS17C, VG29C, and their variants, GS17XC and VG29XC with CaM. Binding of CaM to $GS17C_{NBD}$ (closed circles) and $VG29C_{NBD}$ (closed squares), and the corresponding variants, $GS17XC_{NBD}$, (open circles) and $VG29XC_{NBD}$ (open squares), was monitored by the NBD fluorescence (λ_{exc} = 490 nm; λ_{em} = 530 nm). Conditions: 1.0 μM of NBD-labeled peptides in 20 mM HEPES (pH 7.5), 50 mM KCl, and 1 mM $CaCl_2$ were titrated with a stock solution (360 μM) of CaM. The curves were calculated based on the best fit parameters (Table 1).

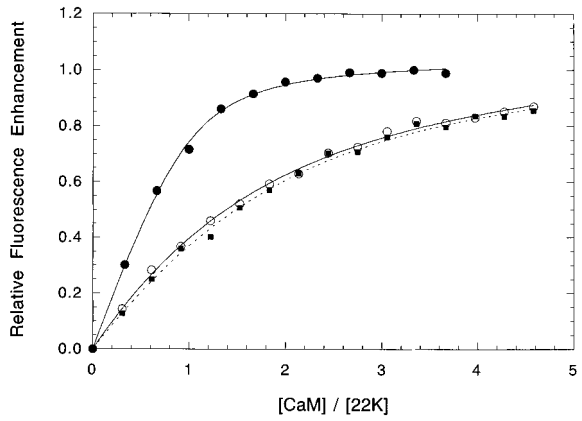


FIGURE 2: Fluorescence titration of the 22K recombinant fragment of CaD and its $22K_{A^-}$ and $22K_{B^-}$ variants with CaM. Mutation of the Trp residue into Gly in either site A (W659G, open circles) or site B (W692G, closed squares) resulted in lower affinities toward CaM as compared with the wild-type 22K (closed circles). Aliquots of CaM (360 μM) were added to solutions containing 1.4 μM 22K, $22K_{A^-}$, or $22K_{B^-}$. The relative enhancement of Trp emission (λ_{exc} = 295 nm; λ_{em} = 320 nm) was monitored. Other conditions are the same as in Figure 1.

monitored by the Trp fluorescence. The wild-type 22K fragment bound CaM with an affinity similar to that of intact CaD; however, both mutated fragments bound CaM with association constants approximately 10-fold lower (Figure 2 and Table 1). As in the case of the synthetic peptides, the loss of Trp residue in either site A or site B in the 22K fragment had a detrimental effect on CaM binding. The same conclusion was also reached recently by Graether et al.(1997), who mutated a similar CaD fragment by replacing the same Trp residues (W659 and W692) with Ala and found that the doubly mutated fragment exhibited a 50-fold decrease in the affinity for CaM.

The experiments in Figure 2 also indicate that (i) the CaM affinities of both $22K_{A^-}$ and $22K_{B^-}$ are relatively high, as compared to the corresponding mutated synthetic peptides and (ii) there is little difference between the apparent CaM affinities of $22K_{A^-}$ and $22K_{B^-}$, although the shorter, site A-containing peptide (GS17C) binds CaM 3−5 times more strongly than does the site B-containing peptide [VG29C; see Zhuang et al. (1995)]. These observations are consistent with the idea that in each of the 22K mutants the unaltered site is still able to interact with CaM, and they suggest that, even in the absence of Trp, there are some weak interactions within each CaM-binding site.

Relative Orientation of CaM with Respect to CaD in the Complex: (a) Ca^{2+} Binding. We have used the Trp fluorescence change to monitor the Ca^{2+} dependence of the interaction between CaM and CaD fragments. Upon addition of Ca^{2+} to the mixtures of CaM and CaD peptides, the Trp fluorescence of both GS17C and VG29C increased, confirming that complexes between Ca^{2+}−CaM and the peptides were formed (Figure 3A). The end points for the two titrations, however, were different: the fluorescence increase of GS17C reached a plateau at a stoichiometry of ∼2 Ca^{2+} per CaM, whereas that of VG29C continued until 4 Ca^{2+} per CaM were added. Thus, Ca^{2+} binding to only one domain of CaM is sufficient for the interaction with the GS17C peptide. Once the complex is formed, additional Ca^{2+} binding to the other domain has no effect on the Trp fluorescence. In view of the preferential binding of Ca^{2+} to sites III and IV of CaM, the C-terminal domain of CaM is likely the site that interacts with GS17C. It is less clear why the Trp fluorescence of VG29C is affected by Ca^{2+} binding to all four sites of CaM.

We have repeated the titrations in an EGTA-NTA Ca^{2+}-buffering solution and plotted the fluorescence changes as a function of free [Ca^{2+}]. In a mixture of CaM and the CaD peptide, complex formation was monitored by the Ca^{2+}-dependent Trp fluorescence change, whereas for CaM alone, binding of Ca^{2+} to the C-terminal domain was reported by the Tyr fluorescence change (Figure 3B). Compared to the binding isotherm of free CaM, the binding curve of CaM-GS17C was clearly shifted to a lower [Ca^{2+}]. This is consistent with the idea that binding of GS17C to CaM enhances the affinity of the C-terminal sites for Ca^{2+} and that Ca^{2+} binding to sites III and IV is sufficient for the complex formation. In contrast, the binding isotherm for the CaM−VG29C mixture is at a slightly higher [Ca^{2+}] than that for CaM alone. This indicates that binding of VG29C to CaM is not associated with Ca^{2+} binding to the high-affinity sites, but rather with that to the low-affinity sites (I and II). The fact that the midpoints of the titration curves for free CaM and CaM−VG29C are similar indicates that the interaction with the CaD peptide raises the Ca^{2+} affinity for the N-terminal sites, which in free CaM is about 5 times weaker (Linse et al., 1991), to approximately the same as that for the C-terminal sites of free CaM. Thus, in the stoichiometric titration for VG29C in Figure 3A, both classes of sites are being titrated simultaneously, resulting in an apparent end point of 4.

(b) Tb^{3+} Binding. In order to characterize the proximity between the Trp residues of CaD and the metal binding sites of CaM, we have used a fluorescent trivalent rare earth ion, Tb^{3+}, as a substitute for Ca^{2+}. We found that at saturating concentrations of Tb^{3+}, CaM had a similar affinity for CaD peptides as in the presence of Ca^{2+} (data not shown). Thus, it is reasonable to assume that the information obtained with Tb^{3+} would be applicable to that obtained in the presence of Ca^{2+}. A notable difference is that the binding preference

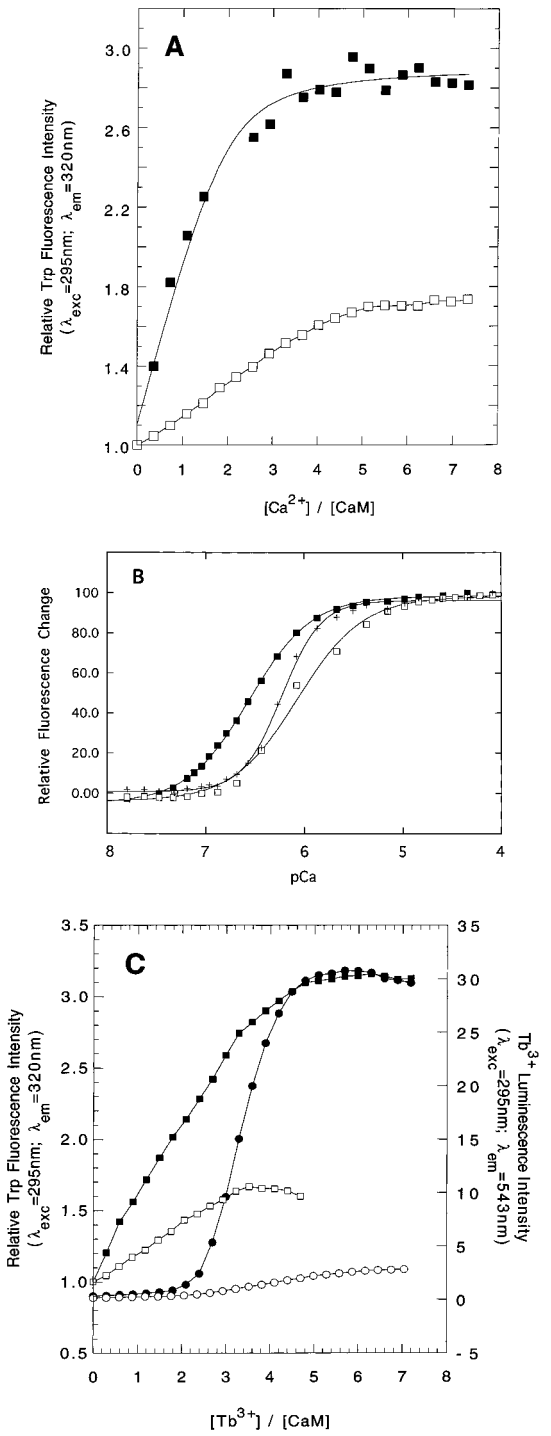


FIGURE 3: Fluorescence titration of CaM complexed with CaD peptides with $CaCl_2$ or $TbCl_3$. Mixtures of CaM (9−12 μM) and approximately equimolar GS17C (closed symbols) or VG29C (open symbols) were titrated with either $CaCl_2$ (A and B) or $TbCl_3$ (C), while the tryptophan emission (λ_{exc} = 295 nm; λ_{em} = 320 nm; squares) and/or tryptophan-sensitized Tb^{3+} luminescence (λ_{exc} = 295 nm; λ_{em} = 543 nm; circles) were monitored. In panel B, binding of Ca^{2+} to CaM alone was used as a reference, in which case the Tyr emission (λ_{exc} = 280 nm; λ_{em} = 308 nm; crosses) was monitored; the data were then fitted with Hill equation (see Materials and Methods). The calculated binding parameters are: $K_a = (3.63 \pm 0.07) \times 10^6$ M^{-1} and $n = 1.36 \pm 0.03$ for the GS17C−CaM complex; $K_a = (1.17 \pm 0.07) \times 10^6$ M^{-1} and $n = 1.32 \pm 0.08$ for VG29C−CaM; and $K_a = (1.69 \pm 0.04) \times 10^6$ M^{-1} and $n = 2.18 \pm 0.10$ for CaM alone. Solution conditions: 25 mM PIPES (pH 6.9), 50 mM KCl.

of Tb^{3+} for the N-terminal sites I and II is opposite to that of Ca^{2+} (Wang et al., 1984). Tb^{3+} emits luminescence that can be sensitized via resonance energy transfer from aromatic groups such as Tyr or Trp if they are located within a short distance (Horrocks & Sudnick, 1981). If CaM-bound Tb^{3+} ions in the complex are sufficiently close to the Trp residues of CaD, which, as shown above, form an integral part of the binding site, one would expect to detect resonance energy transfer from these residues (R_o for the Trp-Tb^{3+} pair is 3.35 Å; Horrocks & Sudnick, 1981). To explore this possibility, we have carried out titrations of $TbCl_3$ into the mixtures of CaM and CaD fragments or peptides comprising CaM-binding sites and monitored the Tb^{3+} luminescence while exciting the Trp residues at 295 nm.

When a 1:1 mixture of VG29C and CaM was titrated with $TbCl_3$, Trp fluorescence exhibited an approximately 60% increase (Figure 3C; open squares), but very little Trp-enhanced Tb^{3+} luminescence was detected throughout the titration (Figure 3C; open circles). It should be pointed out that Tb^{3+} is considered a semi-isotropic center for Föster-type energy transfer because of its degenerate energy levels (Horrocks, 1993). The uncertainty in the value of the orientation factor, κ^2, is substantially reduced from a range of 0−4 for most chromophores to a range between $^1/_3$ and $^4/_3$ (Dale & Eisinger, 1974), which corresponds to a maximal uncertainty in the distance measurement of ~25%. The lack of energy transfer must therefore indicate a large separation between the metal ion and the fluorophor, rather than an unfavorable orientation between them. Thus, our observation indicates that this site B-containing CaD peptide binds in such a way that its Trp residue (W692) is located relatively far from all CaM-bound Tb^{3+} ions.

When GS17C was used, a large Tb^{3+} emission sensitized by Trp excitation was observed upon addition of the second, but not the first, pair of Tb^{3+} ions (Figure 3C; closed circles). The initial lack of energy transfer suggests that the first pair of Tb^{3+} ions (at sites I and II) are far away from the Trp residue of GS17C. Conversely, the fact that the second pair of CaM-bound Tb^{3+} ions were sensitized by Trp indicates that they must be sufficiently close to W659 of GS17C. These observations indicate that GS17C binds to CaM at a site near the Ca^{2+}-binding sites III and IV, but farther away from sites I and II, consistent with our conclusions based on the Ca^{2+} titrations (Figure 3B).

It is noteworthy that binding of Tb^{3+} to sites I and II in the N-terminal domain of CaM allows binding of GS17C, as indicated by the Trp fluorescence increase at Tb^{3+}/CaM ≤ 2 (Figure 3C, closed squares). If, as concluded above, GS17C binds to the C-terminal domain of CaM, there would have to be a long-range effect, i.e., binding of Tb^{3+} ions to sites in the N-terminal domain enables CaM to interact with the peptide at the C-terminal domain. Alternatively, at low Tb^{3+}:CaM ratios, GS17C may bind to CaM at the N-terminal domain in such a position that no Trp-to-Tb^{3+} energy transfer can occur and *move* to the C-terminal domain when the second pair of Tb^{3+} bind to sites III and IV. Although the former interpretation appears simpler, the latter is more compatible with current knowledge about the Ca^{2+}-induced transitions from a closed to an open conformation in the two domains of CaM (Tan et al., 1996; Zhang et al., 1995). To distinguish between the two possibilities, one would need to assess the specificity of the peptide−CaM interaction. We have tested this by using CaM mutants having the N- or the C-terminal domain locked with a disulfide bond, making it inaccessible for a target peptide to bind (see below).

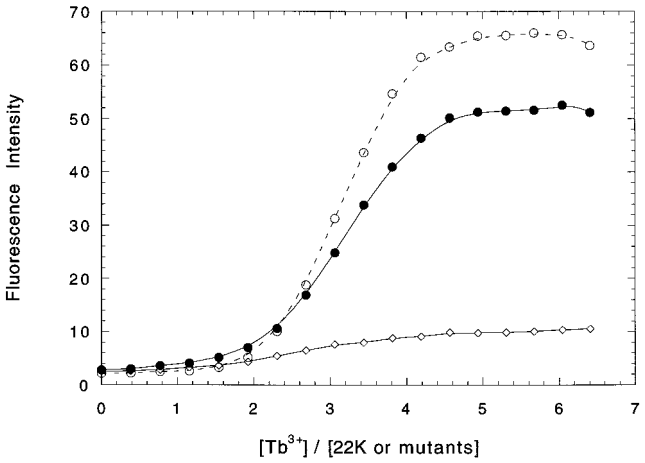


FIGURE 4: Sensitization of CaM-bound Tb^{3+} ions by tryptophan residues of wild-type and mutant CaD fragments. Mixtures of 3.5 μM CaM with equimolar wild-type 22K (closed circles), $22K_{A^-}$ (open diamonds) or $22K_{B^-}$ (open circles) were titrated with $TbCl_3$ and the tryptophan-sensitized Tb^{3+} luminescence ($\lambda_{exc} = 295$ nm; $\lambda_{em} = 543$ nm) was monitored. Other conditions are the same as in Figure 3.

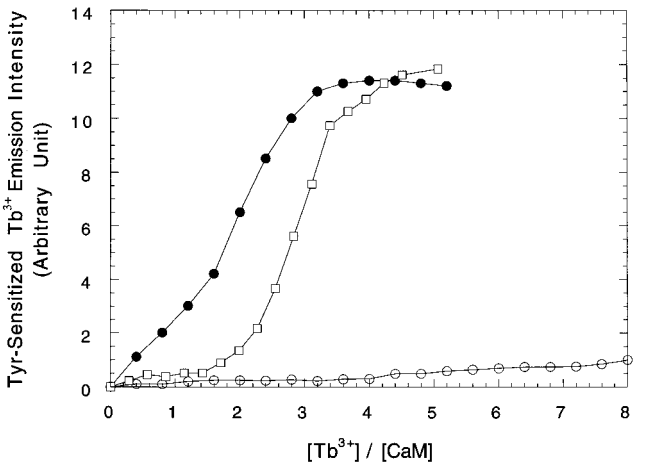


FIGURE 5: Sensitization of Tb^{3+} ions bound to CaM or its mutants by Tyr residues. N-locked (CaM41/75; closed circles), C-locked (CaM85/112; open circles) mutants, or wild-type (open squares) CaM at 3 μM were titrated with $TbCl_3$ and the tyrosine-sensitized Tb^{3+} luminescence ($\lambda_{exc} = 280$ nm; $\lambda_{em} = 543$ nm) was monitored. Other conditions are the same as in Figure 3.

We then extended these experiments by using the recombinant C-terminal fragments of CaD: 22K, $22K_{A^-}$, and $22K_{B^-}$. As in the case of the synthetic peptides, addition of the first two Tb^{3+} ions did not result in energy transfer from the Trp residues of the wild-type 22K, although complex formation was clearly evident from the increase of Trp fluorescence (data not shown). The second pair of Tb^{3+} ions exhibited strong Trp-sensitized emission (Figure 4). Similar results were obtained with $22K_{B^-}$, except that the relative enhancement of Trp-sensitized Tb^{3+} luminescence was about 20% higher than that of the wild-type 22K. In contrast, Tb^{3+} titration of the mixture of CaM and $22K_{A^-}$, in which site A is impaired, showed very little Trp-induced Tb^{3+} emission (Figure 4). These data indicate that W692 in site B does not contribute to the Tb^{3+} sensitization and the only contribution comes from W659. Our experiments with both short peptides and longer CaD fragments lead to the same conclusion: CaM binds to CaD fragments in such a manner that at least one of the Tb^{3+} ions bound at the C-terminal domain is sufficiently close to W659 in site A and the two Tb^{3+} ions bound to the N-terminal domain of CaM are too far from any of the CaD Trp residues for efficient resonance energy transfer.

In an attempt to estimate the distance between W659 of CaD and Tb^{3+} at sites III and IV of CaM, we have used the complex of CaM and a synthetic peptide of the CaM-binding site of myosin light chain kinase (MLCK) as a reference. The distance from the Trp residue of the MLCK peptide to Ca^{2+} at sites III and IV is approximately 12 Å (Ikura et al., 1992; Protein Data Bank File 2bbm). It is known that a separation of 12 Å between Trp and Tb^{3+} in parvalbumin results in observable sensitized luminescence of Tb^{3+} [with a transfer efficiency of 5.32×10^{-4}, see Horrocks and Sudnick (1981)]. In fact, energy transfer from Trp to Tb^{3+} in the CaM−MLCK peptide complex was indeed reported (Chabbert et al., 1995). Thus, the fact that we also detected Trp-to-Tb^{3+} energy transfer indicates that it is possible that W659 of GS17C is in a similar position with respect to CaM as the Trp of the MLCK peptide. A distance of 25 Å would correspond to a 100-fold reduction and, therefore, practically undetectable, energy transfer efficiency. These two distances may thus serve as a reference for the estimated distances between the two Trp residues of CaD and the Ca^{2+}-binding sites of CaM: W659 is 12 Å or less from sites III and IV of CaM and W692 is probably 25 Å or more away from all Ca^{2+}-binding sites of CaM.

Effect of Intradomain Cross-linking of CaM on Its Interaction with CaD. We have further tested the conclusion regarding the relative orientation of CaD and CaM in the complex by using CaM mutants that have either the N-terminal (CaM41/75) or the C-terminal domain (CaM85/112) locked with a disulfide bond in the Ca^{2+}-free, closed conformation (Tan et al., 1996). It has been shown that the Ca^{2+}-binding ability of the metal-binding sites within the locked domain of these mutants is impaired (Tan et al., 1996). Tb^{3+}-binding was also affected in a similar manner: for the N-terminal domain locked mutant (CaM41/75), addition of Tb^{3+} resulted in Tyr-sensitized luminescence without a lag (Figure 5). This is consistent with the idea that once the original high-affinity sites (I and II) in the N-terminal domain are blocked, sites III and IV in the C-terminal domain are being filled upon addition of the first pair of Tb^{3+} ions. The stoichiometry, however, exceeded 2, indicating that binding of Tb^{3+} to the locked domain is not completely abolished. From the shape of the titration curve, it was estimated that the Tb^{3+} affinity of the locked sites I and II in CaM41/75 was lowered from 10^7-10^8 M^{-1} (Wang et al., 1984) to about 10^5-10^6 M^{-1}, which is still higher than that of the intact sites III and IV (1.5×10^5 M^{-1}; Wang et al., 1984). As for the C-terminal locked mutant (CaM85/112), the unaffected N-terminal sites do not contain Tyr residues, and the affinity for the C-terminal sites is even weaker owing to the cross-linking. Accordingly, addition of Tb^{3+} to CaM85/112 did not result in any Tyr-sensitized luminescence (Figure 5) except at very high Tb^{3+}:CaM ratios.

When the wild-type 22K fragment was titrated with either CaM41/75 or CaM85/112 in the presence of Ca^{2+}, the Trp fluorescence increased (Figure 6), again, indicating complex formation. The titration data yielded an association constant of $(3.0 \pm 0.2) \times 10^5$ M^{-1} and $(2.7 \pm 0.4) \times 10^5$ M^{-1} for the N- and the C-terminal locked CaM mutant, respectively. Thus, both CaM mutants, despite each having only one functional domain, were capable of interacting with the 22K

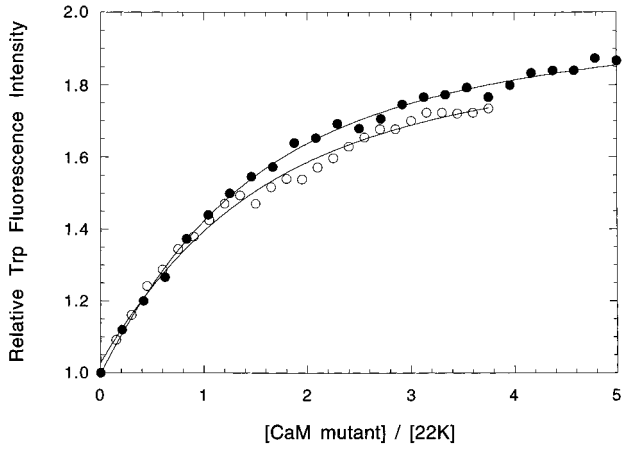


FIGURE 6: Fluorescence titrations of wild-type 22K with CaM mutants. The wild-type CaD fragment 22K (1.08 μM) was titrated with the N-locked (CaM41/75; closed circles) or C-locked (CaM85/112; open circles) CaM mutants and the tryptophan fluorescence intensity (λ_{exc} = 295 nm; λ_{em} = 320 nm) was monitored. The data were fitted with a binding equation with the stoichiometry fixed at 1.0; the binding constants were calculated to be 3.0×10^5 M^{-1} for CaM41/75 and 2.7×10^5 M^{-1} for CaM85/112. Conditions: 20 mM HEPES (pH 7.5), 50 mM KCl, and 1 mM $CaCl_2$.

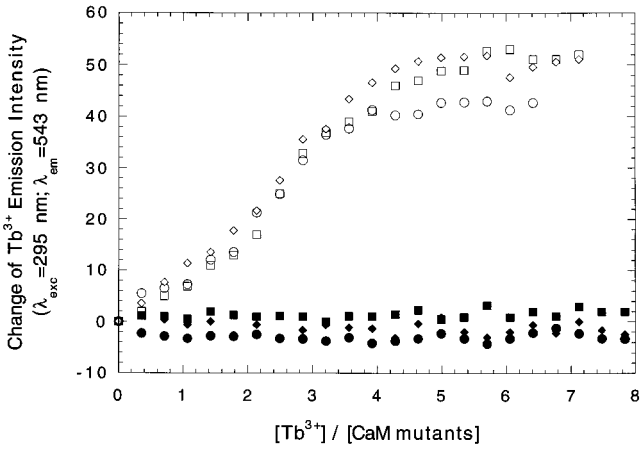


FIGURE 7: Tb^{3+} titrations of complexes formed between wild-type or mutant CaD fragments and CaM mutants. Mixtures of 4 μM of the N-locked (CaM41/75; open symbols) or C-locked (CaM85/112; closed symbols) CaM mutants with equimolar wild-type 22K (diamonds), $22K_{A^-}$ (circles), or $22K_{B^-}$ (squares) were titrated with $TbCl_3$ and the tryptophan-sensitized Tb^{3+} luminescence (λ_{exc} = 295 nm; λ_{em} = 543 nm) was monitored. Other conditions are the same as in Figure 3.

CaD fragment in a Ca^{2+}-dependent manner, although with decreased affinities.

We then added $TbCl_3$ to the mixture of the wild-type 22K CaD fragment or its Trp-to-Gly variants (W659G and W692G) with either CaM41/75 or CaM85/112 and monitored fluorescence energy transfer between CaD and CaM by measuring the Trp-sensitized Tb^{3+} luminescence. As expected, when the N-terminal locked CaM41/75 was used, Tb^{3+} emission was enhanced without a lag phase upon complex formation with wild-type 22K and $22K_{B^-}$, whereas the mixture of the C-terminal locked CaM85/112 with all three CaD fragments produced no Trp-sensitized Tb^{3+} luminescence (Figure 7). These observations further support the view that CaM binds to CaD in such a manner that its C-terminal domain is close to W659 of site A, and its N-terminal domain is relatively more distant from the Trp residues of both sites A and B.

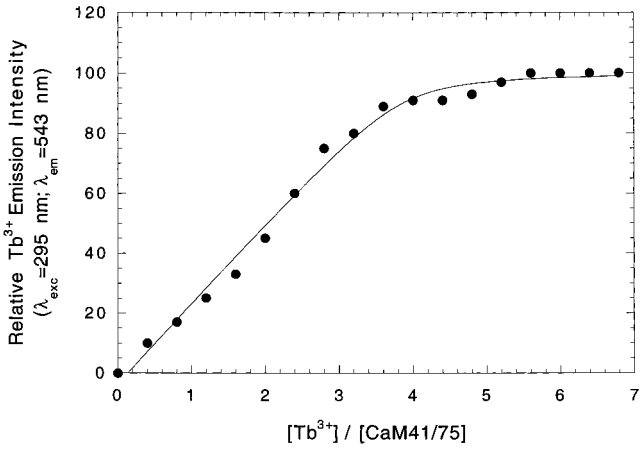


FIGURE 8: Titration of the complex formed between VG29C and CaM41/75 with $TbCl_3$. Mixture of 5 μM N-locked CaM41/75 with equimolar VG29C was titrated with $TbCl_3$ and the tryptophan-sensitized Tb^{3+} luminescence (λ_{exc} = 295 nm; λ_{em} = 543 nm) was monitored. Other conditions are the same as in Figure 3.

Surprisingly, we found that Tb^{3+} bound to the N-terminal locked CaM41/75 was also able to receive resonance energy from $22K_{A^-}$ where no Trp is present in site A (Figure 7; open circles). This result can be explained based on the assumption that in this complex the C-terminal domain of CaM41/75 may recognize W692 and bind to site B, resulting in energy transfer from this Trp to Tb^{3+} bound to sites III and IV.

We have further tested the postulated switch-over from a "preferred" (but unavailable) binding site to a "second best" site by using the site B-containing peptide, VG29C. As described above, VG29C binds to the wild-type CaM in such a way that W692 is relatively far away from all four metal-binding sites. When we titrated Tb^{3+} into a 1:1 mixture of VG29C and CaM41/75, however, an enhanced Tb^{3+} luminescence was obtained, similar to that for the $22K_{A^-}$ fragment (Figure 8). Clearly, locking the N-terminal domain of CaM in a closed conformation makes the specific binding site for VG29C unavailable and causes the peptide to bind to the C-terminal domain of CaM41/75, where it is able to transfer energy to the bound Tb^{3+} ions. These results, combined with the enhanced affinity for Ca^{2+} in the N-terminal domain, but not the C-terminal domain, of CaM upon interaction with VG29C (Figure 3B), provide strong evidence for the specific interaction between site B of CaD and the N-terminal domain of CaM. The topography at the N-terminal domain is, however, different from that at the C-terminal domain in that the Trp residue (W692) is farther away from the metal-binding sites.

CONCLUSION

Trivalent lanthanide ions are good Ca^{2+} analogs and, therefore, have been used to probe Ca^{2+}-binding proteins in spectroscopic studies (Horrocks & Sudnick, 1981; Martin & Richardson, 1979). Here, we report the successful use of Tb^{3+} by resonance energy transfer techniques to assess the proximity between a Ca^{2+}-binding protein and its target molecule. Since many Ca^{2+}-binding proteins, such as CaM, interact with their targets at a hydrophobic site which very often contains an aromatic residue suitable to act as an energy donor for the bound Tb^{3+} ions, the use of this approach may find a general application in other systems. For example, a

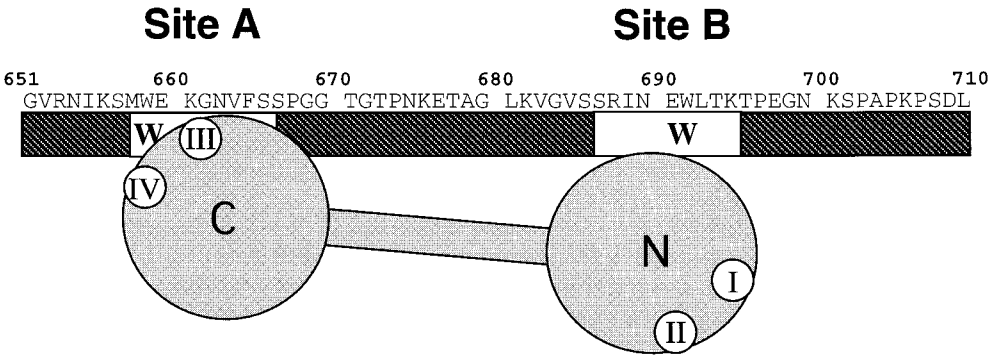


FIGURE 9: A schematic presentation of the CaD-CaM interaction in the C-terminal region of CaD. The model depicts an elongated CaM molecule with its C-terminal domain interacting with site A and the N-terminal domain interacting with site B of CaD. The two metal-binding sites of CaM in the C-terminal domain (sites III and IV) are close to W659 of site A and the other two sites (I and II) in the N-terminal domain of CaM are relatively far away from W692 of site B. The amino acid sequence of the CaD segment is also shown.

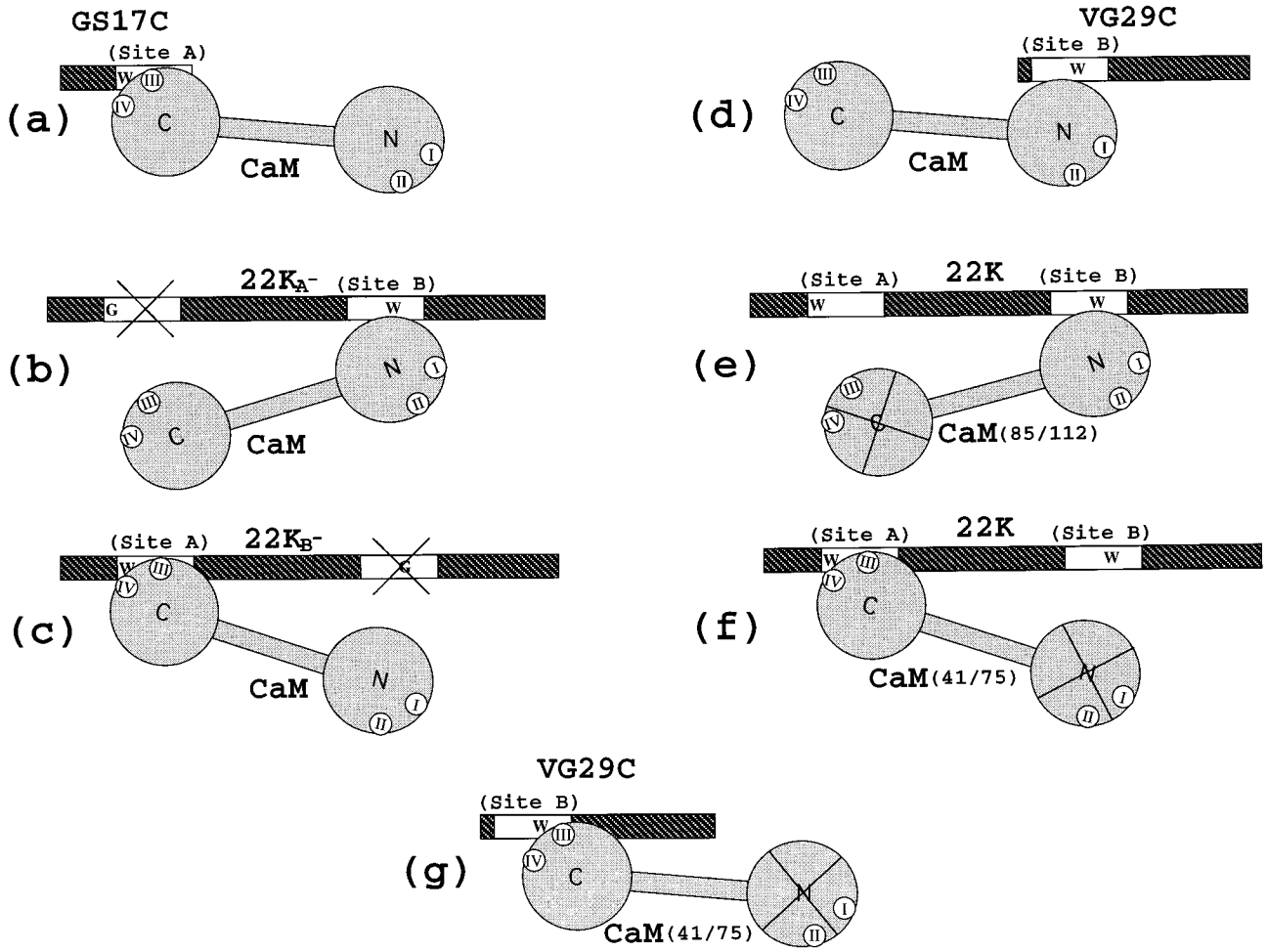


FIGURE 10: A schematic presentation of various CaD−CaM complexes studied in this work. (a) CaM binding to GS17C; (b) CaM binding to mutated (W659G) CaD fragment $22K_{A^-}$; (c) CaM binding to mutated (W692G) CaD fragment $22K_{B^-}$; (d) CaM binding to VG29C; (e) C-terminal locked CaM mutant binding to 22K CaD fragment; (f) N-terminal locked CaM mutant binding to 22K CaD fragment; (g) N-terminal locked CaM binding to VG29C, in which case the specific interaction site is not available and site B binds to the C-terminal domain of the CaM mutant.

similar method has recently been used to probe the distance between CaM and the MLCK peptide (Chabbert et al., 1995).

The results of this work indicate that hydrophobic side chains of Trp at positions 659 and 692 make an important contribution to the CaM−CaD interaction. The model emerging from our experimental data depicts an antiparallel orientation in the CaD−CaM complex, as illustrated schematically in Figure 9. In contrast to many intracellular high affinity CaM targets, CaD binds CaM with a moderate affinity ($K_a = 10^6-10^7$ M^{-1}) and renders CaM in an extended conformation (Mabuchi et al., 1995). In such a complex, the C-terminal domain of CaM is in close contact with site A centered around W659, whereas the N-terminal domain of CaM is associated with the downstream site B around W692 of CaD (Figure 9). Although both Trp residues make significant contributions to the interaction between CaD and CaM, their position in the respective binding sites differs considerably in that W659 is sufficiently close ($\leq$12 Å) to the metal binding sites (sites III and IV) for an efficient energy transfer when these sites are occupied with Tb^{3+},

whereas the distance between W692 and sites I and II is much longer (>25 Å).

This model is consistent with a number of observations: when site A is intact and the C-terminal domain of CaM is functional (Figure 10, panels a, c, and f), Tb^{3+} ions bound at sites III and IV are sensitized by W659, regardless whether site B is present or not. When site B is intact and the N-terminal domain of CaM is functional (Figure 10, panels b, d, and e), a complex is formed but no Trp-sensitized Tb^{3+} luminescence occurs. This optimal-binding mode may, however, be changed whenever the preferred sites are either absent or not functional. For example, when the N-terminal domain of CaM is locked by a disulfide bond (CaM41/75), and at the same time the Trp (W659) in site A of CaD is replaced with Gly, the interaction that actually occurs is that between the available C-terminal domain of CaM and the intact site B of CaD (Figure 7, open circles; Figure 8, and Figure 10g), indicating a relatively weak specificity of the interaction.

All these results suggest certain flexibility in the regulatory interactions between the two proteins. Such flexibility allows the complex to accommodate alternative spatial arrangements when the preferred binding sites are either altered or rendered unavailable. It also explains the previous observation that CaD fragments containing only either site B [such as H9; see Marston et al. (1994)] or site A [GS17C; see Zhuang et al. (1995)] express regulatory properties, and that both CaM41/75 and CaM85/112 were able to reverse CaD-induced inhibition of actomyosin ATPase activity (Huber et al., 1996).

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