Conservative D133E Mutation of Calmodulin Site IV Drastically Alters Calcium Binding and Phosphodiesterase Regulation†

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ABSTRACT: Two calmodulin mutants, F92W and F92W/D133E, were prepared using site-specific cassette-mediated mutagenesis to examine the structure/calcium affinity relationships of cation chelating residues in calcium binding sites III and IV. The mutant, F92W, was prepared to produce a strong fluorescent label to follow the calcium-induced structural changes in the C-terminal domain of the protein. A second mutant, F92W/D133E, was prepared to destroy the calcium binding to site IV and thereby eliminate cooperativity between sites III and IV. The macroscopic calcium dissociation constants of the two sites in the C-terminal domain were derived from the calcium titration data that had been fitted to a two-site Hill equation. The calcium dissociation constants of site III and site IV in the F92W/D133E mutant were 335 μM and 2.76 mM, respectively. These values were significantly greater than the values of 14 and 1 μM for site III and site IV in F92W calmodulin, respectively. These results suggested that a very conservative D133E mutation in the $+Z$ position of the site IV Ca^{2+}-binding loop drastically decreased the calcium binding affinity of the site (2760-fold) and also significantly reduced that of site III in the same domain (24-fold). The D/E calmodulin mutant also had a 3-fold lower phosphodiesterase activation activity with a 25-fold lower affinity for this enzyme than that of F92W calmodulin in the presence of low calcium concentration (50 μM). However, the maximum phosphodiesterase activation activity of the F92W/D133E mutant and the affinity of this mutant for the enzyme were similar to those of F92W calmodulin in the presence of high calcium concentration (15 mM), suggesting that the D133E mutation altered calcium regulation of calmodulin mediated phosphodiesterase activity without affecting calmodulin interaction with the enzyme.

Calmodulin (CaM)[1] is a major intracellular Ca^{2+}-binding protein consisting of a single, 148-residue polypeptide chain. It is present in all eukaryotic cells and mediates a variety of physiological processes in a Ca^{2+}-dependent manner [reviewed in Klee and Vanaman (1982)]. Both the crystal and solution structures of Ca^{2+}-bound CaM resemble a dumbbell, in which two structurally similar globular domains, the N- and C-terminal domains, are linked by a central helix (Babu et al., 1988; Seaton, et al., 1985; Heidorn & Trewhella, 1988; Matsushima et al., 1989; Ikura et al., 1991; Barbato et al., 1992). Each domain consists of a pair of calcium binding sites that have a helix-loop-helix (hlh) conformation, similar to other intracellular calcium binding proteins including parvalbumin and troponin C (Kretsinger & Nockolds, 1973; Herzberg & James, 1985). The solution structure of apocalmodulin also shows two globular domains similar to those observed in the Ca^{2+}-bound form (Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). However, the structure of the Ca^{2+}-bound form is different from that of apo-CaM in terms of the interhelical angles and the solvent-accessible hydrophobic areas (Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). Binding of calcium induces a change in the interhelical angles leading to the exposure of the hydrophobic core in each globular domain, which enables CaM to interact with a target enzyme, thereby regulating enzyme activity (Ikura et al, 1992; Meador et al., 1992).

The four hlh calcium binding sites are numbered from I to IV from the N-terminus. Sites I and II are paired and form the N-terminal domain and sites III and IV are paired as the C-terminal domain as shown for VU-1 CaM in Figure 1. Six residues in the loop region of each site are involved either directly or indirectly in calcium chelation. The six chelating residues are sequentially arranged in the $+X$, $+Y$, $+Z$, $-Y$, $-X$, and $-Z$ positions of a near-octahedral coordination sphere. While the $+X$ and $-Z$ positions are always occupied by Asp and Glu, respectively, the other positions can be occupied by a variety of amino acids (Falke et al., 1994).

The overall objective of our study is to examine the molecular mechanisms by which calcium binds to hlh calcium binding proteins such as CaM. Since most of the calcium binding proteins contain multiple hlh calcium

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[1] Abbreviations: CaM, calmodulin; hlh, helix-loop-helix; APH, acid-pair hypothesis; VU-1 CaM, a recombinant calmodulin encoded by a synthetic calmodulin gene; F92W CaM, a VU-1 calmodulin mutant in which phenylalanine is replaced by tryptophan at position 92; F92W/D133E CaM, a VU-1 calmodulin mutant in which phenylalanine is replaced by tryptophan at position 92 and aspartate replaced by glutamate at position 133; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PDE, phosphodiesterase; TFA, trifluoroacetic acid; Tris, trishydroxymethylaminomethane.

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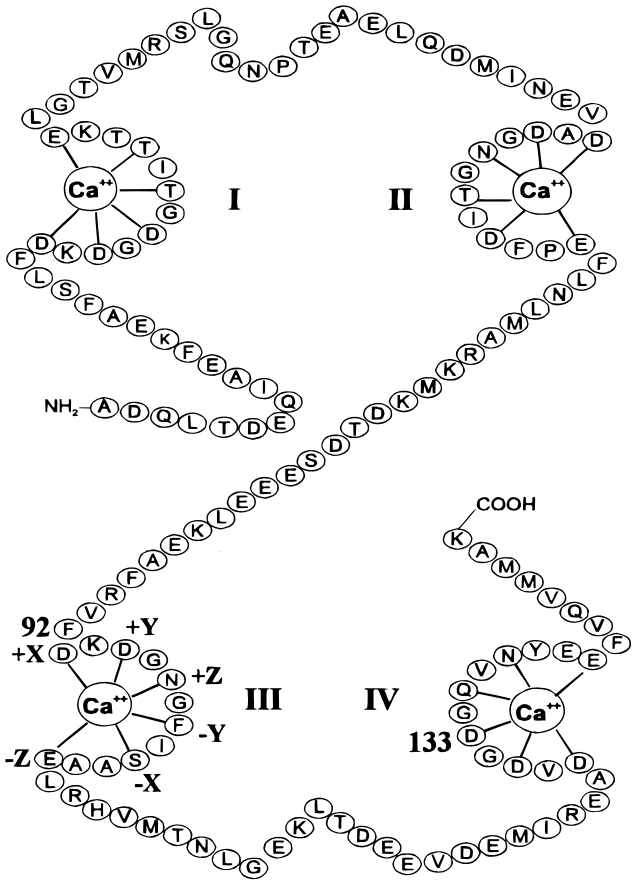

FIGURE 1: Amino acid sequence of VU-1 CaM. The 12-residue loop region of the four calcium binding sites is illustrated and marked by site numbered I−IV from the N-terminus. Calcium chelating positions in site III are indicated as $+X$, $+Y$, $+Z$, $-Y$, $-X$ and $-Z$. Position 92 for Trp substitution and position 133 for Glu substitution are also indicated.

binding sites, the structure/calcium binding affinity relationships of individual hlh calcium binding motifs are complicated not only by the molecular properties of chelating and non-chelating residues in the helices and calcium binding loop but also by cooperative interactions between the paired hlh motifs in the same domain. This study is aimed at creating a CaM mutant inactivated at site IV with respect to calcium binding capacity. This mutant is designed to allow examination of the effect of structural changes on the calcium binding properties of site III independent of cooperative interactions between sites III and IV.

Previous studies have shown that replacement of the invariant Glu at the $-Z$ position of the calcium binding loop region to Ala decreases the calcium binding affinity of the two sites in the corresponding domain in CaM by 100−300-fold (Haiech et al., 1991). Mutation of this conserved Glu to Gln in the same position also dramatically reduces calcium binding to the corresponding site in the absence of magnesium. Also, mutation of Glu ($-Z$) to either Gln or Lys eliminates calcium binding to the mutated site in the presence of magnesium (Maune et al., 1992). The changes from Glu ($-Z$) to either Ala, Gln, or Lys, which include charge changes and elimination of one or both of the oxygen dentates, are drastic, and therefore the effects of these changes on calcium affinity are open to a number of explanations. Recent studies on 33 amino acid residue peptide analogs of the hlh motif have shown that mutation of Asp to Glu in the $+Z$ position of the loop region eliminates both calcium and magnesium binding to the peptide (Reid & Procyshyn, 1995). Compared to E/A, E/Q, or E/K mutations, this D/E mutation is much more conservative and consists of increasing the length of the side chain in the $+Z$ position by one methylene group. However, it is unknown if the Asp to Glu mutation in the $+Z$ position in one of the calcium binding sites of CaM would have the same effect on calcium binding in the whole protein as it did in the single-site model peptide.

In the present study, the VU-1 CaM expression vector, pVUCH-1, is used to prepare a CaM mutant in which Asp in the $+Z$ position of site IV is replaced by Glu (Roberts et al., 1985; Lukas et al., 1987). D/E mutation at the $+Z$ position was chosen over that of E/A, E/Q, or E/K at the $-Z$ position because the former change maintains the overall charges of the site in the apopeptide. It is anticipated that this conservative D/E mutation would eliminate calcium binding to site IV in CaM as it did in the single site hlh model peptide (Reid & Procyshyn, 1995). A Trp residue is also introduced into position 92 to replace Phe in order to have a fluorescent probe to monitor the calcium-induced conformational changes in the C-terminal domain. Results from the present study show that this very conservative D/E single-site mutation decreases the calcium affinity of site IV approximately 2760-fold and reduces that of the partner site III approximately 24-fold. The D133E mutant has a significantly reduced affinity for and maximal activation of phosphodiesterase (PDE) which is restored to normal levels at high calcium concentrations. This study provides the first evidence of a correlation between results from the single-site hlh model peptides and a whole protein model.

EXPERIMENTAL PROCEDURES

Construction of Calmodulin Mutants. The plasmid pVUCH-1 and *Escherichia coli* strain K12 UT481 were generously provided by Dr. T. Lukas. Mutant plasmids were constructed from the plasmid pVUCH-1 using site-specific cassette-mediated mutagenesis as described previously (Lukas et al., 1987; Craig et al., 1987). For preparation of the F92W mutant, the plasmid pVUCH-1 was digested with *Stu*I and *Ecl*XI. The digestion mixture was separated on a 0.8% agarose gel, and the double-digested plasmid was extracted from the gel using the GeneClean II kit from Bio 101. The purified double-digested plasmid was dephosphorylated with alkaline phosphatase and ligated with a phosphorylated double-stranded synthetic DNA cassette (44 bp) bearing 3′ and 5′ ends compatible with the plasmid. The inserted DNA cassette contained a codon, TGG, for Trp in place of the codon, TTC, for Phe. The ligated plasmid was introduced into the *E. coli* K12 UT481 by conventional Ca^{2+}/heat treatment (Sambrook et al., 1989). A positive clone was identified by restriction enzyme digestion and automated DNA sequencing on an ABI 373A DNA sequencer. For preparation of the F92W/D133E mutant CaM, the plasmid for F92W CaM was digested with *Hin*dIII and *Hpa*I, followed by the aforementioned procedures. The DNA cassette (65 bp) for the F92W/D133E mutant contained a codon, GAG, for Glu in place of the codon, GAC, for Asp. Again, a positive F92W/D133E CaM clone was identified by restriction enzyme digestion and DNA sequencing. All enzymes were purchased from Boehringer Mannheim. Oligonucleotides used for DNA cassettes were synthesized on an Applied Biosystems DNA synthesizer.

Protein Expression and Purification. All recombinant proteins were purified from 20 L cultures of positive clones by a modification of the method of Roberts et al. (1985). Fermentation was carried out using a Chemap fermenter (Volketswil, Switzerland). IPTG was added to a final concentration of 1 mM when OD_{600} was 0.2, and the culture was grown overnight after the addition of IPTG. Ultrasonication with 3 × 30 s bursts on a Vibra-Cell sonicator (Fisher) was carried out after lysis with lysozyme (0.2 mg/mL). The ammonium sulfate and isoelectric precipitation steps were omitted, and heat-treatment in the presence of 0.2 mM PMSF as a protease inhibitor was added as described previously (Dedman & Kaetzel, 1983). Proteins were purified by a phenyl-Sepharose (Pharmacia) column with a bed volume of 10 mL by the method of Roberts et al. (1985). The eluants from the column were dialyzed extensively against deionized water. The dialyzates were lyophilized, and the dried proteins were stored at −20 °C.

Analysis of Proteins. The homogeneity of the recombinant proteins was evaluated by SDS−polyacrylamide gel electrophoresis (Sambrook et al., 1989). The amino acid composition analysis was performed using a precolumn derivatization HPLC method on a 420A Derivatizer, a 130A HPLC separation system, and a 920A data analysis module from Applied Biosystems, Perkin Elmer. The molecular weight of each purified protein was determined by electrospray mass spectrometry on a VG Quattro Quadrupol mass spectrometer (Fisons, Altrincham, England). Protein concentrations were based on the protein extinction coefficients, $\epsilon_{278} = 1500\ M^{-1}\ cm^{-1}$ for VU-1 CaM from Yazawa et al. (1980) and $\epsilon_{280} = 8223\ M^{-1}\ cm^{-1}$ for F92W and F92W/D133E calmodulins that were determined through the amino acid composition analysis.

Preparation of the CaM Binding Peptide W4I-M13. A 26-residue peptide analog corresponding to the CaM binding site of the skeletal muscle myosin light chain kinase (Ikura et al., 1990) was synthesized on a 432A peptide synthesizer from Applied Biosystems. The Trp residue in position 4 in the peptide was replaced by Ile, a nonfluorescent residue, to eliminate interference with the Trp fluorescence of the Trp-labeled CaM. The peptide, W4I-M13 (KRRIKKNFIAVSAANRFKKISSSGAL), was purified by preparative reverse-phase HPLC on a C18 column using 0.1% TFA-acetonitrile (A)/0.1% TFA-water (B) as the mobile phase. The column was washed with a linear gradient from 0:100 (A:B) to 60:40 (A:B) over 40 min. The major peak monitored at 214 nm was collected, dialyzed against deionized water, and lyophilized. The identity of the W4I-M13 peptide was verified by amino acid composition analysis and mass spectrometry.

Far-UV Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded on a Jasco J720 spectropolarimeter at ambient temperature. Each purified protein was dissolved in buffer (MOPS, 50 mM; KCl, 100 mM; EGTA, 1 mM; pH 7.20) at concentrations of 15−30 μM, and 0.9 mL of the protein solution was used for each measurement. The water used for preparing the buffers was deionized water treated with Chelex 100 resin (Bio-Rad). CD spectra were performed before and after addition of calcium chloride solution to saturation.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Shimadzu RF540 spectrofluorophotometer at ambient temperature. Spectra and titrations were performed on samples (2 mL) at concentrations of 40 μM for the VU-1 CaM and 15 μM for the F92W and F92W/D133E CaM mutants, in the same buffer used for the CD spectral measurements. Calcium titration of VU-1 CaM was monitored by following the tyrosine fluorescence at the excitation wavelength of 278 nm and emission wavelength of 309 nm. The slit width for both excitation and emission wavelengths was 5 nm. Calcium titrations of F92W and F92W/D133E calmodulins were monitored by following the tryptophan fluorescence at the excitation wavelength of 282 nm and emission wavelength of 340 nm. The slit width for excitation wavelength was 5 nm, and that for the emission wavelength was 2 nm. Standard $CaCl_2$ solution was obtained from Orion Research Inc. (Boston, MA).

Effect of W4I-M13 CaM Binding Peptide on the Calcium Binding Affinity of Calmodulins. Calcium titrations of VU-1, F92W, and F92W/D133E calmodulins were carried out in the presence of the CaM binding peptide, W4I-M13. The protein concentrations were 30 μM for VU-1 and 10 μM for both F92W and F92W/D133E mutants, respectively, in the same buffer used for CD spectral measurements. The protein to peptide ratios were 1:1.5, 1:4, and 1:4 for VU-1, F92W, and F92W/D133E calmodulins, respectively. The calcium titrations of these proteins were monitored under the same conditions as described previously in this section.

Calcium Titration Data Analysis. Free Ca^{2+} concentrations were calculated from total Ca^{2+} concentrations in the EGTA buffer using the EQCAL program from Biosoft. Each set of the fluorescence titration data was fitted to the following one-site (eq 1) and two-site (eq 2) Hill equations using SlideWrite*plus* for Windows from Advanced Graphics Software, Inc. (Carlsbad, CA).

$$f = \frac{[Ca^{2+}]^n}{K^n + [Ca^{2+}]^n} \quad (1)$$

f is the fraction of fluorescence intensity change expressed as the fluorescence intensity change at a given free Ca^{2+} concentration from that of apo-CaM over the maximum change during the titration, K is the average of the apparent calcium dissociation constants of the two sites in the C-terminal domain of CaM, and n is the slope factor of the titration curve.

$$f = f_1\frac{[Ca^{2+}]^{n_1}}{K_1^{n_1} + [Ca^{2+}]^{n_1}} + (1 - f_1)\frac{[Ca^{2+}]^{n_2}}{K_2^{n_2} + [Ca^{2+}]^{n_2}} \quad (2)$$

f_1 is the fraction of the fluorescence intensity change attributed to one site in the C-terminal domain, K_1 and K_2 are the apparent dissociation constants or the macroscopic dissociation constants of the two sites in the C-terminal domain, respectively, and n_1 and n_2 are the slope factors of the two phases of the titration curve, respectively.

Phosphodiesterase Activation Assay. Bovine brain CaM (Sigma) and the three recombinant proteins were tested for their regulation of CaM-dependent PDE activity in the presence of calcium. The initial cAMP hydrolysis rate was determined as a function of CaM concentration in an assay buffer containing 0.2 unit/mL of bovine heart PDE (Sigma), 2 mM [^{3}H]cAMP (ICN), 50 μM $CaCl_2$, 3 mM $MgSO_4$, 100 mM KCl, 40 mM Tris-HCl, pH 7.5, in a volume of 100 mL

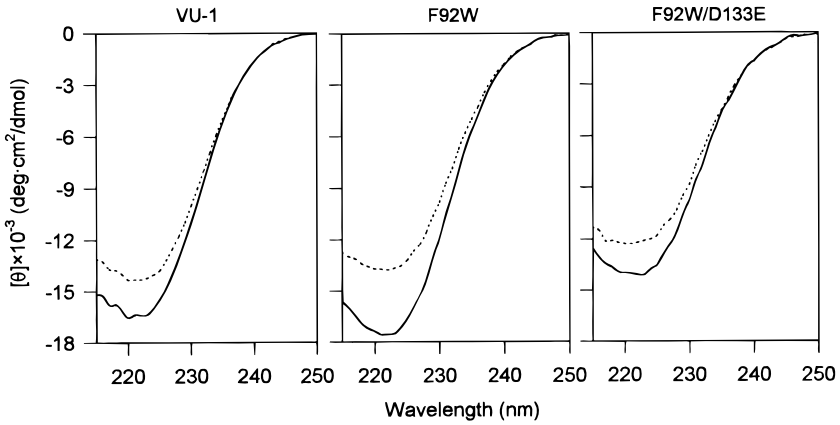

FIGURE 2: Far-UV circular dichroism spectra. Spectra were recorded at the protein concentrations of 28.5 μM VU-1, 24 μM F92W, and 15 μM F92W/D133E in the EGTA buffer (MOPS, 50 mM; KCl, 100 mM; EGTA, 1 mM; pH 7.20) before and after the addition of calcium chloride to saturation (free calcium concentration: 7 mM for VU-1 and F92W and 35 mM for F92W/D133E). The solid lines represent the spectra in the presence of calcium, and the dashed lines represent the spectra in the absence of calcium.

as described previously (Wallace et al., 1983). PDE activation by F92W and F92W/D133E was also assayed in the same buffer at higher calcium concentrations (15 mM). The assay was carried out at 30 °C for 10 min and terminated in a boiling water bath for 1 min. Snake venom (*Crotalus atrox*, Sigma), as a source of nucleotidase, was added at a concentration of 167 μg/mL, and the reaction continued at 30 °C for 15 min. Bio-Rad AG1-X2 anion exchange resin slurry (30%, g/mL) was added to the reaction mixture to adsorb the un-hydrolyzed cAMP, and an aliquot of the supernatant was counted in Cytoscint scintillation fluid (ICN) on a Beckman LS 6000 TA liquid scintillation counter. All assays were performed in triplicate, and the PDE activation activity was expressed as picomoles of cAMP hydrolyzed per unit of PDE per min (pmol/unit/min). Data were fitted to the following Hill equation using Sigmaplot:

$$v = \frac{V_{max}C}{K_{50} + C}$$

where v is the activated PDE activity at a given concentration, C, of CaM, V_{max} is the maximum activated PDE activity, and K_{50} is the concentration of CaM that is able to produce one-half of the maximum activated PDE activity.

Statistical analysis was carried out by unpaired Student's *t*-test. A probability p of less than 0.05 was considered significant.

RESULTS

Each of the three purified proteins migrated as a single band on the SDS−polyacrylamide gel in the presence of 1 mM $CaCl_2$ or 1 mM EGTA (data not shown). Amino acid composition analyses of the three proteins give the expected results within experimental error (data not shown). The molecular weight of each purified protein determined by mass spectrometry matches the calculated molecular weight (data not shown).

Far-UV Circular Dichroism Spectra. The far-UV CD spectra of VU-1, F92W, and F92W/D133E calmodulins in the absence and presence of calcium are shown in Figure 2. The corresponding mean residue ellipticity values and changes in ellipticity at 222 nm are presented in Table 1. Calcium was observed to induce an increase in the magnitude of the negative ellipticities of VU-1, F92W, and F92W/D133E calmodulins. However, not only do the absolute ellipticities at 222 nm of each protein vary, but the relative changes from the Ca^{2+}-free state to the Ca^{2+}-bound state of these proteins are also different (Table 1). It appears that the Trp and Glu substitutions affect the overall structure of CaM.

Table 1: Molar Ellipticity of Calmodulin Mutants[a]

protein	$[\theta]_{222} \times 10^{-3} \pm$ SE (deg cm^2/dmol)		
	$-$ Ca^{2+}	$+$ Ca^{2+}	$\Delta[\theta]_{222}$
VU-1	14.31 ± 0.08	16.38 ± 0.06	2.07
F92W	13.76 ± 0.18	17.54 ± 0.03	3.78
F92W/D133E	12.20 ± 0.02	14.09 ± 0.04	1.89

[a] Data are expressed as the mean ± standard error of three separate measurements.

Fluorescence Spectra. The fluorescence excitation spectra of VU-1, F92W, and F92W/D133E calmodulins were recorded at emission wavelengths of 309 nm (VU-1 CaM) and 340 nm (F92W and F92W/D133E), respectively (data not shown). The maximum excitation wavelength was observed at 278 nm for VU-1 and at 282 nm for both F92W and F92W/D133E calmodulins. Therefore, calcium titrations of VU-1 CaM were performed at the excitation wavelength of 278 nm, and those for F92W and F92W/D133E mutants were performed at 282 nm.

Tyrosine fluorescence emission spectra of VU-1 and tryptophan fluorescence emission spectra of F92W and F92W/D133E calmodulins recorded before and after addition of calcium are shown in Figure 3. Calcium was observed to induce a 3-fold increase in the fluorescence intensity of VU-1 CaM, a 2-fold increase in F92W CaM, and a 1.6-fold increase in F92W/D133E CaM at the corresponding maximum emission wavelength. There appears to be no shift in the maximum emission wavelength for VU-1 CaM in the absence and presence of calcium. Therefore the calcium titration of VU-1 CaM was monitored at the maximum emission wavelength of 309 nm. A small blue shift in the emission maximum from 342 nm in the absence of calcium to 340 nm in the presence of calcium for F92W CaM was observed. A blue shift in the emission maximum from 342 nm in the absence of calcium to 338 nm in the presence of calcium was observed for F92W/D133E CaM. Therefore, the calcium titrations of F92W and F92W/D133E CaM mutants were monitored at 340 nm where the largest

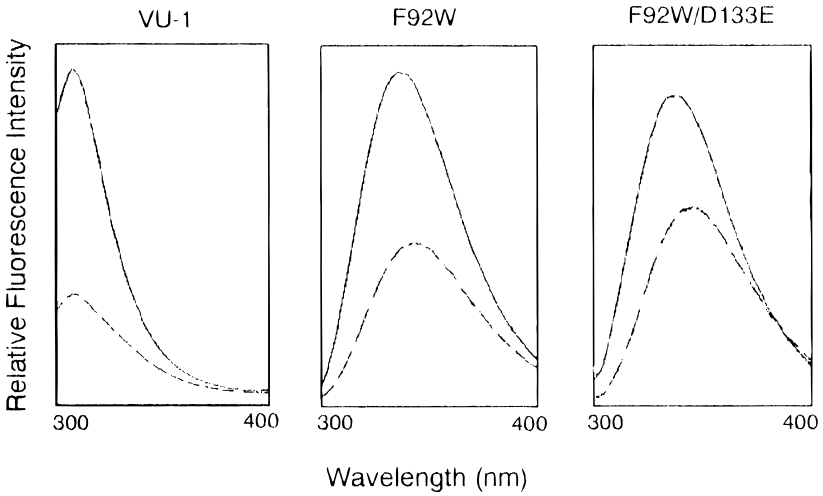

FIGURE 3: Fluorescence emission spectra. Relative fluorescence intensities were recorded as a function of emission wavelength at the tyrosine excitation wavelength of 278 nm for VU-1, and the tryptophan excitation wavelength of 282 nm for F92W and F92W/D133E calmodulins, respectively. The fluorescence emission spectra of 40 μM VU-1, 15 μM F92W, and 15 μM F92W/D133E in the EGTA buffer as specified in Figure 2 before (dashed lines) and after (solid lines) the addition of calcium chloride to a final free calcium concentration of 1 mM (VU-1 and F92W calmodulins) or 8 mM (F92W/D133E calmodulin). The slit width of excitation is 5 nm. The slit width of emission is 5 nm for VU-1 CaM and 2 nm for both F92W and F92W/D133E CaM mutants, respectively.

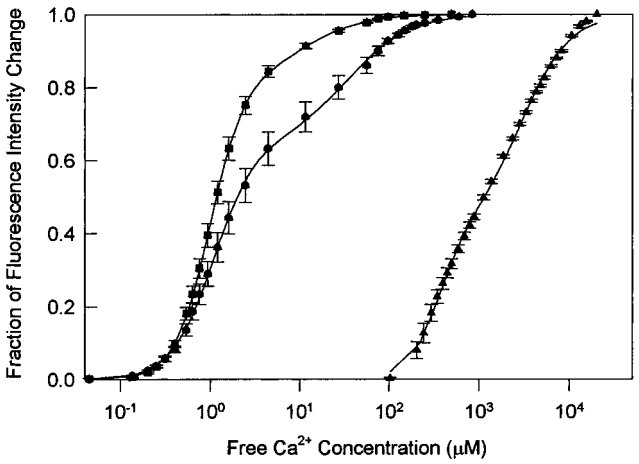

FIGURE 4: Calcium titrations of CaM mutants monitored by fluorescence intensity changes. The fluorescence intensity changes were monitored at the corresponding emission wavelengths of 309 nm (tyrosine fluorescence) for VU-1 CaM (solid circle) and 340 (tryptophan fluorescence) for both F92W (solid square) and F92W/D133E CaM (solid triangle) mutants, respectively. Each data point represents the average of six (VU-1 and F92W) or nine (F92W) separate titrations, and the standard error is shown as the error bar. Conditions are identical to those described in Figure 2.

Table 2: Calcium Dissociation Constants of CaM and CaM Mutants[a]

protein	K_1 (μM)	K_2 (μM)
VU-1	1.1 ± 0.1	32 ± 5.7
F92W	1.0 ± 0.1	14 ± 2.4
F92W/D133E	335 ± 21	2759 ± 45
TR_2C[b]	0.4	10
F34[c]	0.6	18.4
F34[d]	1	23.6

[a] Subscripts 1 and 2 refer to the high and low affinity sites in the C-terminal domain of each protein or CaM fragment, respectively. Each value is expressed as the mean $\pm$ SE of six (VU-1 and F92W mutants) and nine (F92W/D133E mutant) separate titrations. [b] Data for the CaM fragment of the C-terminal domain from Linse et al. (1991). [c] Data for the CaM fragment of the C-terminal domain. Flow dialysis was used for binding measurement. Data from Minowa and Yagi (1984). [d] Data for the CaM fragment of the C-terminal domain. Equilibrium dialysis was used for binding measurement. Data from Minowa and Yagi (1984).

difference in the fluorescence intensity of the apoprotein and Ca^{2+}-saturated protein occurred.

Calcium Titration. Each set of titration data was fitted to one-site (eq 1) and two-site (equation 2) models. The two-site model is more appropriate to the data as judged by the fitting coefficients (data not shown), the residuals between the calculated value based on the model and the experimental value of each data point (data not shown), and visual observation (Figure 4). Accordingly, the macroscopic calcium dissociation constants, K_1 and K_2, of the two sites in the C-terminal domain of all three calmodulins are calculated from each fitted two-site equation (Table 2). In all three calmodulins, K_1 is approximately 1 order of magnitude lower than K_2, suggesting that one site has approximately 1 order of magnitude higher calcium affinity than the other in the C-terminal domains of these proteins. K_1 of F92W CaM (1 μM) is similar to that of VU-1 CaM (1.1 μM), whereas K_2 of F92W CaM (14 μM) is significantly lower than that of VU-1 CaM (32 μM) (Table 2). These data indicate that the higher affinity site in the C-terminal domain of F92W CaM has similar calcium affinity to that of VU-1 CaM. However, the lower affinity site in the C-terminal domain of F92W CaM has approximately 2.3-fold higher calcium affinity than that of VU-1 CaM. The K_1 and K_2 of F92W/D133E CaM are estimated as 335 and 2760 μM, respectively, which are significantly greater than that of F92W CaM (Table 2). These data indicate that the two sites in the C-terminal domain of F92W/D133E CaM mutant have much lower binding affinities for calcium than those of F92W CaM.

Effect of W4I-M13 CaM Binding Peptide on the Calcium Affinity of the Mutant Calmodulins. It was observed that W4I-M13 CaM binding peptide did not affect the fluorescence emission spectra of all three recombinant calmodulins in the absence of calcium but caused a blue shift in the Trp fluorescence emission spectra of F92W and F92W/D133E calmodulins in the presence of calcium (data not shown). The maximum emission wavelength of F92W in the presence of calcium was shifted from 340 nm in the absence of W4I-

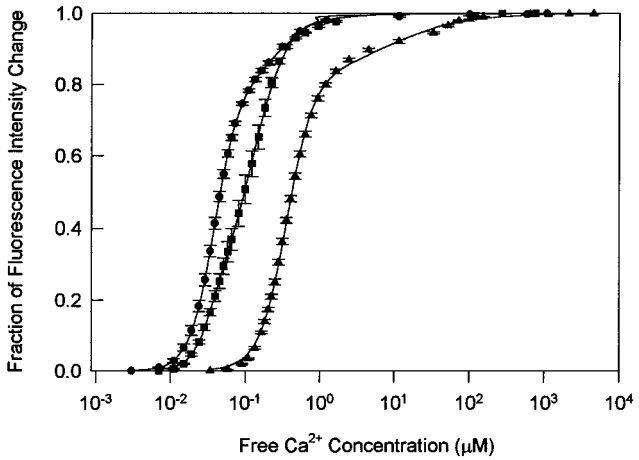

FIGURE 5: Effect of W4I-M13 CaM binding peptide on the calcium affinity of calmodulins. The fraction change in tyrosine (VU-1) and tryptophan (F92W and F92W/D133E) fluorescence intensity is plotted as a function of free calcium concentration in the presence of W4I-M13 CaM binding peptide. The protein concentrations were 30 μM for VU-1 (solid circle) and 10 μM for both F92W (solid square) and F92W/D133E (solid triangle) calmodulins, respectively. The protein to peptide ratios were 1:1.5 for VU-1 and 1:4 for both F92W and F92W/D133E calmodulins, respectively. Each data point represents the average of three (VU-1) or six (F92W and F92W/D133E) separate titrations, and the standard error is shown as the error bar.

Table 3: Effect of W4I-M13 CaM-Binding Peptide on the Calcium Dissociation Constants of CaM and CaM Mutants[a]

protein	K'_1 (μM)	K'_2 (μM)
VU-1	0.038 ± 0.002	0.25 ± 0.012
F92W	0.037 ± 0.003	0.143 ± 0.003
F92W/D133E	0.358 ± 0.005	8.13 ± 0.75

[a] Calcium titrations of CaM and CaM mutants were carried out in the presence of the CaM binding peptide, W4I-M13. Each value is expressed as the mean ± SE of three (VU-1) and six (F92W and F92WD133E mutants) separate titrations, respectively.

M13 to 326−328 nm in the presence of the peptide. For F92W/D133E CaM, this shift occurred from 338 to 326−328 nm. Calcium titrations of F92W and F92W/D133E mutants were monitored at the emission wavelengths of 340 and 330 nm. Although the absolute fluorescence intensity monitored at the two wavelengths was different, the fraction of fluorescence intensity change was not significantly affected (data not shown). Figure 5 shows the calcium titration curves of VU-1, F92W and F92W/D133E calmodulins in the presence of the CaM binding peptide, W4I-M13. Again the two-site model is more appropriate than the one-site model for these titration curves (data not shown). The macroscopic calcium dissociation constants, K'_1 and K'_2, of all three calmodulins are shown in Table 3. Compared to the values of K_1 and K_2, K'_1 and K'_2 are significantly lower ($p < 0.05$), suggesting that the calcium affinity of all three calmodulins are higher in the presence of the CaM binding peptide than in the absence of the peptide.

Phosphodiesterase Activation Assay. PDE activation parameters, V_{max} and K_{50}, of the bovine brain CaM and the three recombinant calmodulins are presented in Table 4. The PDE activation curves of F92W and F92W/D133E calmodulins are depicted in Figure 6. At low calcium concentration (50 μM), both VU-1 and F92W calmodulins activated PDE to a maximum level similar to that obtained with bovine brain CaM ($p > 0.05$). While the K_{50} of VU-1 CaM is

Table 4: Phosphodiesterase Activation Activity of Calmodulins

	50 μM Ca^{2+}		15 mM Ca^{2+}	
protein	V_{max} (pmol/unit/min)	K_{50} (nM)	V_{max} (pmol/unit/min)	K_{50} (nM)
CaM	176.9 ± 7.5	10.9 ± 0.9		
VU-1	171.6 ± 4.6	10.8 ± 1.0		
F92W	169.4 ± 4.8	7.4 ± 0.7	166 ± 8	43 ± 6
F92W/D133E	63.2 ± 2.5	185.2 ± 21.1	186 ± 9	41 ± 4

[a] Each value is presented as the mean ± S. E. of triplicate measurements. V_{max} is the maximum activation activity, and K_{50} is CaM concentration required for half-maximum PDE activation activity.

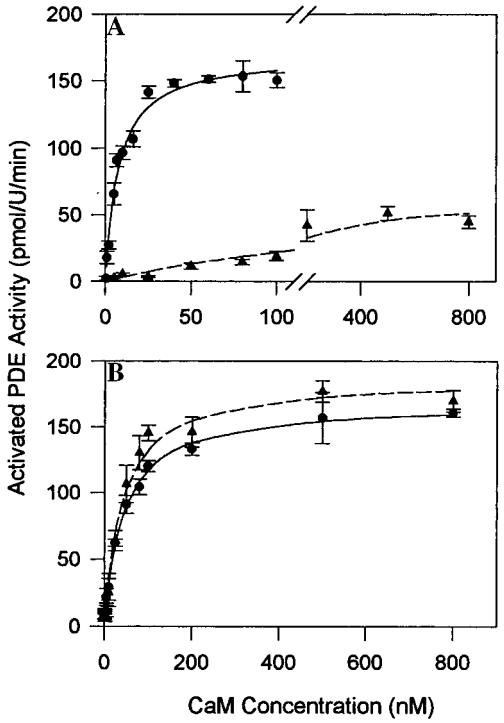

FIGURE 6: Phosphodiesterase activation curves. The initial cAMP hydrolysis rate is plotted as a function of the concentration F92W (solid circle) and F92W/D133E (solid triangle) calmodulins. The assay was carried out in a buffer containing 0.2 unit/mL of PDE, 2 mM [^{3}H]cAMP, 3 mM $MgCl_2$, 100 mM KCl, 40 mM Tris-HCl, 50 μM $CaCl_2$ (A) or 15 mM $CaCl_2$ (B), pH 7.5, in a volume of 100 mL at 30 °C. Each data point represents the average of triplicate measurements, and the standard error is shown as the error bar.

similar to that of bovine brain CaM ($p > 0.05$), the K_{50} of F92W CaM is significantly lower than that of VU-1 CaM or bovine brain CaM (Table 4). These data suggest that VU-1 CaM and bovine brain CaM have similar affinity for the bovine heart PDE, whereas F92W CaM has a higher affinity for the enzyme. The V_{max} of F92W/D133E CaM is approximately 3-fold lower than that of F92W CaM, and the K_{50} of F92W/D133E for PDE is 25-fold greater than that of F92W CaM in the presence of 50 μM calcium. These data suggest that F92W/D133E CaM has a significantly lower PDE activation activity with a significantly lower affinity for PDE than F92W CaM. However, the V_{max} and K_{50} of F92W/D133E CaM are similar to those of F92W CaM at higher calcium concentration (15 mM) ($p > 0.05$), suggesting that the PDE regulation activity of F92W/D133E CaM is restored to that of F92W CaM by a high concentration of calcium. The K_{50} of F92W for the enzyme is 6-fold greater at the higher calcium concentration which may

indicate an ionic effect of the high calcium concentration on calmodulin interaction with the enzyme.

DISCUSSION

Binding constants for calcium in Ca^{2+}-binding peptides and proteins have been estimated by a variety of methods such as equilibrium and flow dialysis, CD-, NMR-, and fluorescence-monitored calcium titration. In the present study, fluorescence is used to monitor the calcium titrations of VU-1, F92W, and F92W/D133E calmodulins. Although the only Tyr residue in site IV of VU-1 CaM allows us to titrate these proteins by monitoring the Tyr fluorescence intensity change, a Trp residue is introduced into site III of VU-1 CaM because we intend on having a spectral probe in site III at a position similar to that of F105W chicken troponin C (Trigo-Gonzalea et al., 1992; Pearlstone et al., 1992). It is anticipated that Trp substitution at this particular position will allow us to titrate the calcium-induced conformational transition in the C-terminal domain, and calcium binding to the N-terminal domain will not affect the Trp fluorescence as in the case of F105W troponin C (Trigo-Gonzalea et al., 1992; Pearlstone et al., 1992).

The two macroscopic calcium dissociation constants, K_1 and K_2, are assumed to be the dissociation constants for site III and site IV in the C-terminal domain, although not necessarily in that order. The reason for this assumption is 3-fold. First, F29W and F105W point mutations in troponin C, a member of CaM superfamily, have been successfully used as spectral probes in monitoring the conformational transitions in the N- and C-terminal domains, respectively (Trigo-Gonzalea et al., 1992; Pearlstone et al., 1992). It has also been reported that changes in the environment of the only Tyr residue, Y138, analogous to Y138 in VU-1 CaM, reflect binding of calcium to the C-terminal domain of human calmodulin like protein (Durussel et al., 1993). Second, studies from the intact CaM and trypsin-digested fragments each containing two of the Ca^{2+}-binding sites suggest that the calcium binding to one domain does not affect calcium binding to the other (Linse et al., 1991; Minowa & Yagi, 1984). Although other studies suggest that interdomain interactions are evident (Seamon, 1980; Wang et al., 1984; Kilhoffer et al., 1992; Pedigo & Shea, 1995; Shea et al., 1996), it is unknown to what extent these interactions may affect the calcium binding affinity of each domain. We assume these interdomain interactions do not significantly affect the calcium affinity of each domain. Third, the calcium dissociation constants of F92W CaM obtained in this study (1 and 14 μM) are similar to those obtained from the CaM C-terminal domain fragments (K_1 ranges from 0.4 to 1 μM, and K_2 ranges from 10 to 23.6 μM, Table 2). These data again suggest that the Trp fluorescence changes in the whole CaM molecule reflect the calcium-induced conformational changes of the C-terminal domain in CaM.

To determine any influence by the F/W substitution, F92W CaM was compared to VU-1 CaM in terms of CD spectra, calcium affinity, and PDE activation activity. Although the overall pattern of the spectra of F92W CaM in the presence and absence of calcium is similar to that of VU-1 CaM, the calcium-induced ellipticity change observed for F92W CaM at 222 nm is approximately 2-fold greater than that for VU-1 CaM (Table 1). Although the higher affinity site in the C-terminal domain of F92W CaM has similar calcium affinity to that of VU-1 CaM, the lower affinity site in the C-terminal domain of F92W CaM has an approximately 2.3-fold higher calcium affinity than that of VU-1 CaM (Table 2). Both F92W and VU-1 calmodulin activate the bovine heart PDE to a similar maximum level; however, F92W CaM has an approximately 1.5-fold higher affinity for PDE than VU-1 CaM (Table 4). Altogether, these data suggest that the F/W mutation affects the overall structure of CaM so that calcium affinity of the lower affinity site in the C-terminal domain increases, and as a result, the affinity for PDE increases. NMR studies have shown that Phe92 of CaM becomes more buried in the presence of calcium because this residue undergoes a net decrease in exposed surface upon calcium binding (Finn et al., 1995). Substitution of a bulkier and more hydrophobic Trp residue for Phe at position 92 may alter the local structure of CaM in favor of the calcium-bound state of the local site (most likely site III as discussed in more detail later) resulting in an increase in the calcium-induced ellipticity change, calcium affinity, and overall affinity for PDE. It should be noted that this effect of the F92W mutation in the VU-1 CaM is contrary to the lack of effect observed for a similar change in troponin C (Trigo-Gonzalea et al., 1992; Pearlstone et al., 1992).

We have tentatively assigned the calcium dissociation constants, K_1 and K_2, to the two sites in the C-terminal domains of VU-1 and F92W calmodulins on the basis of the Acid Pair Hypothesis (Reid & Hodges, 1980). According to the APH, site IV should have a higher affinity for calcium than site III. This is because there are four acidic amino acid residues in chelating positions in site IV with two of them paired on the *Z* coordinating axis, whereas three acidic residues are located in chelating positions in site III with none of them paired on either the *X* or the *Z* axis. As a result, the calcium dissociation constant with a lower value (K_1) is tentatively assigned to site IV and that with a greater value (K_2) to site III. Site IV of F92W CaM is similar to site IV of VU-1 CaM in terms of calcium affinity. However, site III of F92W CaM has a higher calcium affinity than the same site in VU-1 CaM possibly due to the Trp substitution for Phe in position 92 at the C-terminal end of the first helix in site III as described earlier.

It is interesting to note that the APH would predict site IV of F92W/D133E to be a high-affinity calcium binding site because of the two acid pairs located on the *X* and *Z* axes. The fact that this site is very low affinity indicates that not only is location of acidic residues in the loop region critical to cation affinity but the type of acidic residues can greatly affect cation affinity. This aspect of the APH is currently under investigation in our laboratory.

In an attempt to eliminate the calcium binding affinity of site IV in CaM, the D133E CaM mutant is designed from the results of a study on a synthetic hlh peptide model of CaM site III in which the $+Z$ residue was changed to a Glu to produce a *Z* axis Glu−Glu acid pair (Reid & Procyshyn, 1995). The D/E mutation in the $+Z$ position caused the peptide to lose all calcium and magnesium binding capacity. Therefore, we expected that the D133E CaM mutant would have little or no calcium affinity in site IV. Since site III is unchanged, we assume that it would have a higher affinity for calcium than site IV in the F92W/D133E mutant. Therefore, K_1 is tentatively assigned to site III and K_2 to site IV in the F92W/D133E mutant (Table 2). The calcium dissociation constant of site IV in the F92W/D133E mutant

is 2760-fold greater than the same site in F92W CaM. The dissociation constant of site III in the F92W/D133E mutant is 24-fold greater than that in F92W CaM. These results demonstrate that substitution of Glu for Asp at the $+Z$ position in the loop region, a very conservative single-residue change, induces a drastic reduction in calcium affinity of the corresponding site and, at the same time, significantly decreases the calcium binding affinity of the paired, unmutated site in the same domain.

Falke and colleagues summarized the sequences of 567 hlh motifs using PROSITE sequence analysis software (Falke et al., 1994). Among the 567 hlh motif sequences, there are 295 Asp, 131 Ser, 123 Asn, 9 Thr, 8 Gly, and only 1 Glu at the $+Z$ position. This comparison suggests that the $+Z$ position in the loop of an hlh motif is relatively variable among Asp, Ser, and Asn but prefers Asp over Glu. Even though there is no charge change by substitution of Glu for Asp at the $+Z$ position in F92W/D133E CaM, the larger Glu residue may cause unfavorable local interactions such as with Asp in the $+Y$ position, or with Glu in the $-Z$ position. Since Glu is longer than Asp by a methylene group, charge repulsion with Asp in the $+Y$ position or with Glu in the $-Z$ position may become significant enough to distort the calcium binding loop around the $+Z$ position resulting in reduced calcium affinity. Alternatively, substitution of a larger Glu for Asp in the $+Z$ position may simply cause a smaller binding cavity at the site resulting in a reduced calcium affinity. The reduction in calcium binding affinity in site IV may cause a loss of the positive cooperativity between site III and site IV in the C-terminal domain leading to a reduction in calcium affinity in site III although no mutation occurs in site III. Falke also found results supporting electrostatic repulsion between coordinating oxygens as a possible explanation for changes in ion selecting in the *E. coli* galactose binding protein engineered with different residues in the $-X$ position (Drake & Falke, 1996; Drake et al., 1996).

Previous studies have shown that point mutations of Glu to Ala at the highly conserved $-Z$ position in site II (E67A) and site IV (E140A) of VU-1 CaM reduce the calcium binding affinity of the N- and C-terminal domains, respectively (Haiech et al., 1991). The mutation of the invariable Glu in the $-Z$ position to Ala reduces the calcium affinity 100−300-fold, whereas our relatively conservative D/E mutation of the variable $+Z$ position drops the calcium affinity for the site nearly 3000-fold. Beckingham et al. have reported the effect of a point mutation of Glu to either Gln or Lys at the $-Z$ position in each of the loops in *Drosophila melanogaster* CaM on the calcium binding affinity (Maune et al., 1992). Calcium binding at the mutated site was undetectable in most of the CaM mutants, E31Q, E67Q, E67K, E104Q, E104K, E140Q, and E140K, in the presence of 1 mM magnesium. However, in the absence of magnesium, E104Q (mutation in site III) and E140Q (mutation in site IV) gave calcium dissociation constants of 1250 and 200 μM for the mutated sites, respectively. And at the same time, the unmutated partner sites, site IV in E104Q and site III in E140Q, have values of 100 and 16.7 μM, respectively. Again the values reflect higher affinities for the invariable $-Z$ position mutation when compared to our less drastic mutation of a variable position in the loop region.

The CaM binding peptide, W4I-M13, increases the macroscopic calcium affinity of all three recombinant calmodulins in the present study (Table 3). These results are consistent with a study in which the CaM binding peptide, mastoparan, or the CaM binding fragment of caldesmon increases the calcium affinity of scallop testis CaM (Yazawa et al., 1987). Another study also shows that a CaM binding peptide, RS20, increases the calcium affinity of VU-1 CaM, E67A CaM, and E140A CaM in the presence of 5 mM $MgCl_2$ (Haiech et al., 1991). Formation of a CaM-peptide complex possibly stabilizes the Ca^{2+}-bound form of CaM thereby kinetically decreasing the dissociation rate constant, and, at the same time, producing a positive cooperativity between the N- and C-terminal domains by bringing the two domains closer together (Yazawa et al., 1987). As a result, the calcium binding affinity of CaM increases in the presence of the CaM binding peptide. Unlike K_1 and K_2 (Table 2), K_1' and K_2' (Table 3) may not necessarily reflect the calcium affinities of the two sites in the C-terminal domain because of the aforementioned positive cooperativity between the N- and C-terminal domains. Alternatively, they might reflect the macroscopic calcium affinities of the two domains in the protein.

F92W/D133E CaM has a reduced PDE regulatory activity when compared with F92W CaM in the presence of 50 μM calcium (Figure 6A and Table 4). However, the two calmodulins exhibit similar PDE regulatory activity when calcium concentration is increased to 15 mM (Figure 6B and Table 4). Since the calcium dissociation constants of sites III and IV of F92W/D133E CaM are 335 μM and 2.76 mM, respectively, the C-terminal domain of this protein is not saturated when the calcium concentration is 50 μM but is saturated when the calcium concentration is 15 mM. These results suggest not only that the calcium-bound form of CaM is essential for PDE regulation but that the D/E mutation alters calcium regulation of CaM mediated PDE activity without affecting CaM interaction with the enzyme. It is also obvious that the affinity of F92W for PDE is reduced 6-fold in the presence of 15 mM calcium which may indicate a detrimental ionic effect of the high calcium concentration on CaM/PDE interactions.

In summary, a Trp residue has been successfully introduced into VU-1 CaM as a spectral probe for monitoring the calcium-induced conformational transition in the C-terminal domain. A novel CaM mutant, D133E, has been prepared, in which the calcium binding affinity of the mutated site IV is decreased 2760-fold and that of the unmutated paired site III is reduced 24-fold. This D/E mutation causes greater changes in calcium binding than the mutations which alter the highly conserved $-Z$ position to uncharged Ala or Gln residues. The D/E CaM mutant also has a 3-fold lower PDE activation activity with a 25-fold lower PDE binding affinity than F92W CaM. These results suggest that a very conservative D/E single-residue change in CaM induces a decrease in calcium affinity followed by a significant reduction in PDE regulatory activity under the near-physiological conditions.

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