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Articles

Recovery of Native Structure by Calcium Binding Site Mutants of Calmodulin upon Binding of *sk*-MLCK Target Peptides†

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ABSTRACT: The calcium-dependent binding of two synthetic 18-residue peptides derived from the calmodulin binding region of skeletal myosin light chain kinase to wild-type *Drosophila melanogaster* calmodulin and four calcium binding site calmodulin mutants has been investigated using optical spectroscopy. The WFF peptide (with W4 and F17) and the FFW peptide (with F4 and W17) both bind to wild-type calmodulin with 1:1 stoichiometry and K_d values of ≤ 0.2 and 1.6 nM, respectively. Near-UV CD spectra of the protein−peptide complexes suggest that both peptides bind in the same orientation, with the side chain of residue 4 interacting with the C-domain of calmodulin and that of residue 17 with the N-domain [as in the structure of the calmodulin−M13 peptide complex determined by Ikura et al. [Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., & Bax, A. (1992) *Science 256*, 632−638]]. Both peptides have lower affinities for all the mutant calmodulins than for the wild-type protein. Fluorescence measurements suggest that mutation of calcium binding site 2 in the N-domain does not affect the interaction of the W4 side chain of the WFF peptide with the C-domain of calmodulin. However, the E67Q (B2Q) but not the E67K (B2K) mutation (site 2, N-domain) alters the interaction of W17 of the FFW peptide with the protein. In contrast, the E140K (B4K) mutation has a much greater effect than the E140Q (B4Q) mutation (site 4, C-domain) on the interaction of calmodulin with both peptides. Far-UV CD spectra show that the four mutant calmodulins have less α-helical structure than the wild-type calmodulin, and near-UV CD spectra indicate that the tertiary structure is also markedly different, particularly for the site 4 mutants. Binding of the target peptides by the mutant proteins causes a significant recovery of both secondary and tertiary structure, substantially overcoming the structural deficiencies introduced by the mutation. This may explain the ability of the mutant calmodulins to activate intact *sk*-MLCK, in spite of their significantly reduced affinities for calcium and altered conformations.

Calmodulin (CaM)[1] is a ubiquitous eukaryotic calcium binding protein which regulates a number of different enzymes in a variety of metabolic pathways in a calcium-dependent manner. Calmodulin interacts with many of its

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[1] Abbreviations: CaM, calmodulin; Ca_4-CaM, calcium-saturated calmodulin; MLCK, myosin light chain kinase; *sk*-MLCK, skeletal muscle myosin light chain kinase; *sm*-MLCK, smooth muscle myosin light chain kinase; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; B2Q, E67Q calmodulin mutant; B2K, E67K calmodulin mutant; B4Q, E140Q calmodulin mutant; B4K, E140K calmodulin mutant; M13, *sk*-MLCK residues 577−602; M5, *sk*-MLCK residues 577−593.

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target proteins with very high affinity (K_d values in the nanomolar range) and has also been shown to bind target peptides whose sequences are derived from the calmodulin binding regions of many of these proteins. The 26-residue target peptide M13 derived from skeletal muscle myosin light chain kinase (*sk*-MLCK, residues 577−602) binds with high affinity ($K_d \approx 1$ nM) (Blumenthal et al., 1985). This peptide and related sequences adopt a predominantly α-helical conformation upon binding to the protein (Klevit et al., 1985; Garone & Steiner, 1990; Ikura & Bax, 1992). Similar conclusions were obtained from studies with the corresponding target peptide (RS20) from smooth muscle myosin light chain kinase (*sm*-MLCK) (Lukas et al., 1986; Roth et al., 1991). O'Neil and DeGrado (1990) have suggested that the basic amphipathic helix is a common structural motif for target sequences from a number of different calmodulin-regulated proteins.

The crystal structure of calmodulin in the calcium-bound form from mammalian or insect sources has been solved to high resolution (Babu et al., 1985; Kretsinger et al., 1986; Babu et al., 1988; Taylor et al., 1991; Chattopadhyaya et al., 1992). The structure shows two domains, each comprising two helix−loop−helix calcium binding motifs. Each calcium binding loop consists of twelve residues with the amino acids in positions 1, 3, 5, 7, 9, and 12 acting as ligands for the calcium [for a review of this and other "EF-hand" type calcium binding proteins, see Strynadka and James (1989)]. The two calcium binding loops in each domain are connected by a short antiparallel β-sheet involving two hydrogen bonds between the hydrophobic residues in position 8. Sites 1 and 2 in the N-terminal domain have slightly lower affinity for calcium than sites 3 and 4 in the C-domain, and there is evidence for cooperativity of calcium binding to the two sites in each domain (Linse et al., 1991). In the calcium-bound form of calmodulin (Ca_4-CaM), the helices in each EF-hand are almost at right angles to each other, exposing hydrophobic "pockets" in the middle of each domain.

Maune et al. (1992b) have studied two series of single-site mutants of *Drosophila melanogaster* calmodulin, each of which has the conserved glutamic acid residue at position 12 in one of the calcium binding loops mutated to either glutamine or lysine. In general, calcium binding to the mutated site appears to be effectively eliminated. Mutations to calcium binding site 4 (residue 140) seem to be more deleterious than those to site 3 (residue 104) in the C-domain, and those to site 2 (residue 67) seem more deleterious than those to site 1 (residue 31) in the N-domain, in terms of structural changes in the protein upon calcium binding (Maune et al., 1992a), alteration of calcium binding to the nonmutated sites (Maune et al., 1992b), and ability of calmodulin to activate several target enzymes (Gao et al., 1993).

The solution structure of a complex of calmodulin with the M13 peptide determined from high-resolution NMR data shows that on complex formation the two domains of calmodulin effectively surround the peptide, which has adopted an α-helical conformation (Ikura et al., 1992). The peptide lies in a hydrophobic channel with its N-terminal portion interacting primarily with the C-domain of calmodulin and its C-terminal portion interacting primarily with the N-domain of the calmodulin. The hydrophobic side chains of Trp4 and Phe17 of the peptide are exclusively involved in contacts with the carboxy- and amino-terminal domains of CaM, respectively, and appear to anchor the peptide to the two domains by fitting into the hydrophobic pockets. Recent crystal structures of complexes of calmodulin with target peptides from *sm*-MLCK and CaM kinase II also have this mode of peptide binding (Meador et al., 1992, 1993). These structures show that the details of the peptide−protein interaction can vary, since the two domains of calmodulin are oriented differently and located closer together in the complex with the CaM kinase II peptide.

In the present work we have characterized the binding of two 18-residue synthetic peptide analogues of the M13 calmodulin binding region of skeletal muscle myosin light chain kinase (*sk*-MLCK) with *Drosophila* calmodulin, in order to investigate the role of aromatic residues 4 and 17 in the interaction. Since the calmodulin itself has no tryptophan residues, the tryptophan in position 4 or position 17 of the synthetic peptides allows the interaction of specific residues of target peptides with individual domains of calmodulin to be monitored by absorbance, CD, and fluorescence spectroscopy. We have studied binding of the two peptides to wild-type calmodulin and to the site 2 and site 4 calmodulin mutants in order to see how point mutations which effectively eliminate calcium binding to one of the EF hands affect the interaction of calmodulin with target sequences. These results also rationalize the ability of calmodulin mutants to activate *sk*-MLCK (and other calmodulin-dependent enzymes) to different degrees (Gao et al., 1993).

MATERIALS AND METHODS

Proteins and Peptides. Drosophila melanogaster calmodulin expressed in *Escherichia coli* was purified as described by Maune et al. (1992b), but with incorporation of ion-exchange chromatography on DEAE-Sephacel (Sigma) as a first step instead of the batchwise phenyl-Sepharose treatment described. *E. coli* cells expressing wild-type *Drosophila* calmodulin were grown and temperature induced essentially as described previously (Maune et al., 1992b). From a 40-L culture, cells were spun down, resuspended in 1.5 L of 25 mM Tris (pH 7.0) and 50 mM KCl, lysed, and centrifuged to remove cell debris, and the supernatant was frozen. An aliquot of 250 mL of supernatant was thawed, diluted 1:1 with 25 mM Tris (pH 7.0), 25 mM KCl, and 1 mM $CaCl_2$ (buffer A), and loaded on a 250-mL column of DEAE-Sephacel resin (Sigma) equilibrated with the same buffer. The column was washed with 500 mL of buffer A containing 100 mM KCl and then with 500 mL of buffer A containing 200 mM KCl. Calmodulin was eluted with buffer A containing 400 mM KCl, loaded on a 100-mL column of phenyl-Sepharose CL-4B resin (Sigma), and washed with 500 mL of buffer A containing 400 mM KCl and then with 500 mL of 25 mM Tris and 1 mM $CaCl_2$; calmodulin was then eluted with 25 mM Tris and 1 mM EDTA. The protein was concentrated about 10-fold using an Amicon ultrafiltration cell (Model 8050) with Diaflo Ultrafiltration membranes of 3K molecular weight cutoff (Amicon Inc.). Aliquots of 2.5 mL of concentrated calmodulin (≈20 mg/mL) were loaded on a G-50 gel-filtration column (2.5 × 50 cm) in 25 mM Tris (pH 7.5) containing 1 mM $CaCl_2$ to remove some minor high molecular weight contaminants. The purified calmodulin then yielded a single band on SDS−PAGE (15% gel−Laemmli system) and was stored at −20

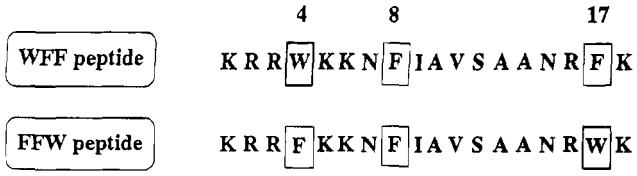

FIGURE 1: Sequences of the two synthetic *sk*-MLCK target peptides.

°C. Mutant calmodulins were purified as described by Maune et al. (1992b).

Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer and had the sequences KKRWKKNFIAVSAANRFK (WFF peptide) and KKRFKKNFIAVSAANRWK (FFW peptide) as shown in Figure 1. They were purified by HPLC on a C18 column (WFF peptide) or a C8 column (FFW peptide) and were provided with free carboxy and amino termini. All concentrations were determined spectrophotometrically after correcting the absorption spectra for light scattering, by plotting absorbance versus log(wavelength) from 400 to 350 nm and extrapolating to 280 or 259 nm as appropriate. An extinction coefficient of 5560 M^{-1} cm^{-1} at 280 nm was used for the peptides (calculated for 2 Phe and 1 Trp), and published extinction coefficients were used for *Drosophila* calmodulin and the four mutants in the presence of calcium: ϵ(259 nm) of 2120 (WT), 2109 (B2Q), 2088 (B2K), 2116 (B4Q), and 2180 M^{-1} cm^{-1} (B4K) (Maune et al., 1992b). Because of the low extinction coefficient for calmodulin, the absorption spectra are unusually sensitive to small amounts of contaminants or light-scattering material, and the uncertainties on absolute molar values of CD intensities ($\Delta\epsilon_M$ and $\Delta\Delta\epsilon_M$) are therefore estimated as ±5%.

Fluorescence and Affinity Measurements. For the mutant calmodulins, peptide at a concentration of 1 or 2 μM in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5 and 30 °C was titrated with aliquots of a stock solution of 20 μM calmodulin. UV-transmitting plastic cuvettes were used since at low concentrations significant amounts of the peptides were observed to bind to quartz surfaces. Fluorescence titration spectra were recorded using a SPEX FluoroMax fluorimeter with excitation at 280 nm (bandwidth, 1.7 nm), and emission was scanned from 310 to 390 nm (bandwidth, 5 nm). For each titration, the value of the fluorescence intensity at 330 nm was plotted as a function of calmodulin concentration and fitted using the Marquardt method (Bevington, 1969) to obtain the dissociation constant (K_d) and the fluorescence enhancement factor (F/F_o). At least three independent determinations were performed, and the average value was reported with its standard deviation. For wild-type calmodulin, peptide at a concentration of 50–200 nM was titrated with calmodulin, and spectra were recorded. Although these concentrations are at least 100-fold higher than the K_d for the WFF peptide, fitted values between 0.2 and 0.6 nM were consistently obtained in independent titrations. The reported average value of (2.0 ± 0.5) × 10^{-10} M was obtained for titrations at 50 nM peptide (the lower limit for detection by this method) and hence represents an upper limit for the K_d value.

Circular Dichroism Spectra. CD spectra were recorded on a Jasco J-600 spectropolarimeter at room temperature. Far-UV CD spectra (190–260 nm) of 7.5 μM peptide, calmodulin, or a peptide/calmodulin (1.1:1) mixture in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ were measured in a 0.1-cm path length cuvette. No further change was observed upon increasing the peptide:protein ratio to 1.5:1. Near-UV CD spectra (250–340 nm) of 20 μM calmodulin or a peptide/calmodulin (1.1:1) mixture in the same buffer were measured in a 1-cm path length cuvette. Multiple scans were averaged (16 for near-UV and 8 for far-UV), baselines were subtracted, and a small degree of numerical smoothing was applied. CD spectra are presented as the molar CD absorption coefficient $\Delta\epsilon_M$ (= $\epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar absorption coefficients for left- and right-handed circularly polarized light). This value is calculated on the basis of the molar concentration of peptide or protein rather than on a per residue basis to facilitate direct comparison of free protein, peptide, and protein–peptide complex. For far-UV CD, the value of $\Delta\epsilon_{MRW}$ is obtained by dividing $\Delta\epsilon_M$ by the appropriate number of peptide residues. Alternative units of CD are mean residue weight ellipticity, e.g., $[\theta]_{MRW}$ (deg cm^2 $dmol^{-1}$) = 3300 $\Delta\epsilon_{MRW}$ (M^{-1} cm^{-1}). For a completely helical polypeptide of 18 residues we have calculated values of $[\theta]_{MRW} = -34\,100$ deg cm^2 $dmol^{-1}$ and $\Delta\epsilon_{MRW} = -10.3$ M^{-1} cm^{-1} at 222 nm (Scholtz et al., 1991, 1993). This corresponds to a molar CD extinction coefficient $\Delta\epsilon_M = -10.3 \times 17 = -176$ M^{-1} cm^{-1} for an α-helical 18-residue peptide.

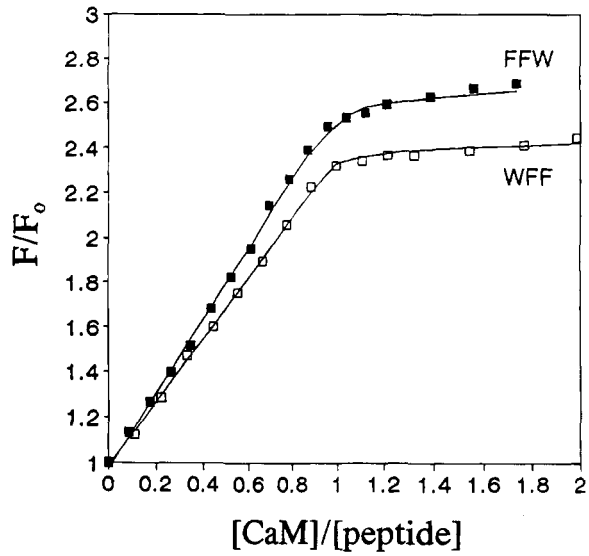

FIGURE 2: Titration curves for 200 nM peptides with wild-type calmodulin in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5 and 30 °C. The lines show the best fits to the data, obtained with $K_d = 2.2 \times 10^{-10}$ M for the WFF peptide and $K_d = 1.3 \times 10^{-9}$ M for the FFW peptide.

RESULTS

Interaction of Peptides WFF and FFW with Wild-Type Calmodulin. We have investigated the binding to calmodulin of two synthetic 18-residue peptides derived from the calmodulin binding region of skeletal muscle myosin light chain kinase (*sk*-MLCK). As shown in Figure 1 the WFF peptide corresponds to residues 577–594 of rabbit *sk*-MLCK (and residues 1–18 of the M13 peptide). In the FFW peptide the tryptophan residue in position 4 is interchanged with the phenylalanine residue in position 17. Both target peptides bind to wild-type calmodulin in the presence of calcium with a 1:1 stoichiometry and very high affinity as shown in Figure 2. The K_d for the complex of the FFW peptide with calmodulin is 1.6 nM, and that for the WFF peptide is

Table 1: Dissociation Constants, Fluorescence Enhancements, and Fluorescence Emission Maxima for the Interaction of Wild-Type and Four Mutant *Drosophila* Calmodulins with WFF and FFW Peptides

	K_d (M)	F/F_o	λ_{max} (nm)
WFF peptide			
wt CaM	$(\leq 2.0 \pm 0.5) \times 10^{-10}$	2.4	333
B2Q	$(2.1 \pm 0.8) \times 10^{-8}$	2.8	333
B2K	$(5.2 \pm 1.7) \times 10^{-9}$	2.7	334
B4Q	$(2.1 \pm 0.9) \times 10^{-8}$	2.5	335
B4K	$(4.8 \pm 1.7) \times 10^{-8}$	2.2	336
FFW peptide			
wt CaM	$(1.6 \pm 0.4) \times 10^{-9}$	3.0	334
B2Q	$(3.2 \pm 2.1) \times 10^{-7}$	2.0	341
B2K	$(1.9 \pm 0.7) \times 10^{-8}$	3.1	335
B4Q	$(3.8 \pm 1.3) \times 10^{-8}$	2.9	335
B4K	$(3.4 \pm 0.3) \times 10^{-7}$	2.4	336

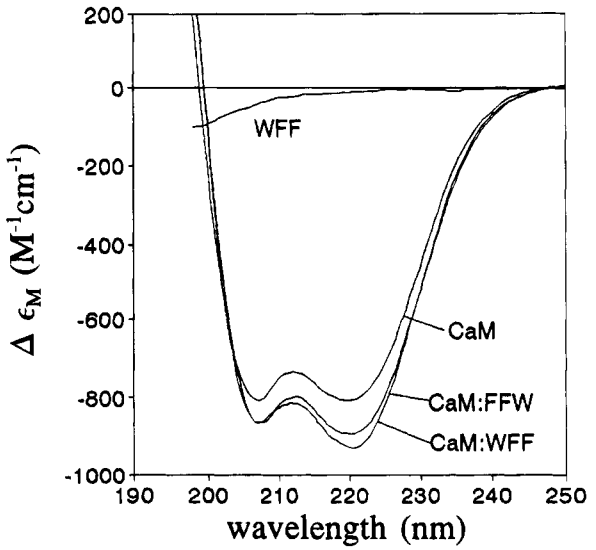

FIGURE 3: Far-UV CD spectra of free peptide, calmodulin, and complexes (1:1 protein:peptide) of calmodulin with the WFF and FFW peptides. [CaM] = 7.5 μM, and [peptide] = 7.8 μM, in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5.

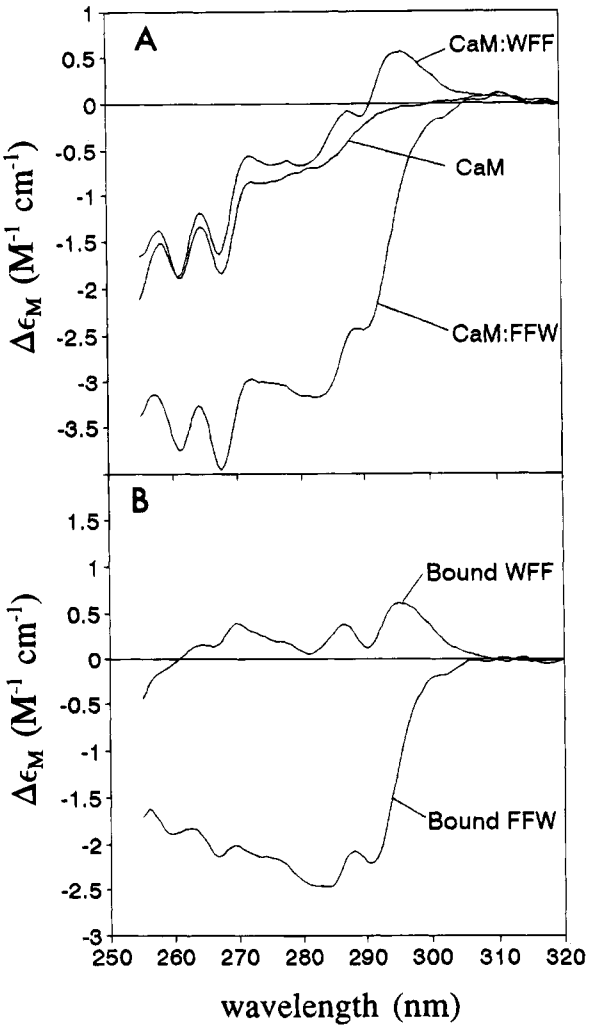

FIGURE 4: (A) Near-UV CD spectra of 20 μM wild-type calmodulin and complexes (1:1 protein:peptide) of calmodulin with the WFF and FFW peptides in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5. (B) Near-UV CD difference spectra for WFF and FFW peptides bound to wild-type calmodulin calculated as CaM−peptide complex minus free CaM.

estimated at ≤ 0.2 nM. Upon binding to calmodulin, the wavelength of maximum tryptophan fluorescence for each peptide is shifted from 356 to 336 nm, with an enhancement in fluorescence intensity at 330 nm of about 2.4-fold for the WFF peptide and 3-fold for the FFW peptide (Table 1). These results indicate that the tryptophan residue is in a hydrophobic environment when either peptide binds to the protein and are consistent with the solution structure of the complex of calmodulin with a 26-residue *sk*-MLCK peptide (known as M13) determined by Ikura et al. (1992), which showed that the Trp4 and Phe17 side chains of the M13 peptide are buried in the hydrophobic regions of the C- and N- domains of calmodulin, respectively.

Structural changes upon formation of the two peptide−protein complexes have been monitored using far-UV CD spectroscopy. Figure 3 shows the far-UV CD spectra of wild-type *Drosophila* calmodulin in the presence of calcium and with the WFF and FFW peptides. The spectrum of free Ca_4-CaM is dominated by the contributions from α-helical structure, with the characteristic negative bands centered at 207 and 222 nm. The value of $\Delta\epsilon_{222nm}$ (on a per residue basis) is -5.5 M^{-1} cm^{-1} [similar to the value reported in Maune et al. (1992a)]. Under the same conditions, the free peptides have spectra with an intense negative band at ≤ 198 nm, characteristic of a largely random or unordered structure.

The spectra of the binary complexes of wild-type Ca_4-CaM with either WFF or FFW peptide are more intense than the sum of the spectra of free calmodulin and free peptide. This shows that formation of the calmodulin−peptide complex results in a conformational change in one or both components and is consistent with the peptide adopting a predominantly α-helical conformation when bound to calmodulin (see below).

Figure 4A shows the near-UV CD spectra of wild-type *Drosophila* calmodulin in the presence of calcium and in complexes with the WFF and FFW peptides. Free calmodulin shows prominent bands at 262 and 268 nm, which derive from the nine Phe residues. The signal at longer wavelengths ($\lambda > 275$ nm) derives from the single Tyr located at position 138 in the C-terminal domain. The $\Delta\epsilon_M$ for this Tyr at 280 nm is 0.58 M^{-1} cm^{-1} [cf. Maune et al. (1992a)]. The free peptides show negligible circular dichroism in this wavelength range, implying considerable conformational freedom of the Trp and Phe residues. The spectra of the binary complexes of Ca_4-CaM with the WFF or FFW peptide show clear evidence of a major contribution from the Trp residue in the peptide. Tryptophan model compounds (Strickland, 1974) generally show CD spectra

corresponding to the L_b transition, with one band at 289–294 nm and a second approximately 7 nm to shorter wavelength, and the L_a transition (265–275 nm), which shows little fine structure. The $\Delta\epsilon_M$ values for these bands are expected to lie in the range ± 3 M^{-1} cm^{-1} (Strickland, 1974). If it is assumed that the CD spectrum of calmodulin is not changed in the complex, then the appropriate difference spectra (Figure 4B) should correspond to the spectra of the bound peptides. Although these spectra conform to the general pattern of indole derivatives as described for Trp CD, the two spectra differ significantly in both magnitude and sign. The high negative value for the Trp residue in the CaM-FFW peptide complex is diagnostic of a high degree of immobilization.

These differences in the near-UV CD spectra indicate that the environments of the Trp residues in the two peptide complexes are significantly different. This would be consistent with interaction of the tryptophans with different domains, as would be expected if both peptides bind to calmodulin in the same orientation as the M13 peptide from *sk*-MLCK (Ikura et al., 1992), i.e., with residue 4 interacting with the C-domain of calmodulin and residue 17 interacting with the N-domain.

Interaction of WFF and FFW Peptides with Calmodulin Mutants. We have measured the affinities of the two peptides for four calcium binding site mutants of calmodulin: the B2K and B2Q CaMs have Glu67 (in binding site 2) mutated to Lys and Gln, respectively, and the B4K and B4Q CaMs have Glu140 (in binding site 4) mutated to Lys and Gln, respectively (Maune et al., 1992b). The residue mutated is in each case the conserved Glu in position 12 of the indicated calcium binding loop, and it has been shown that each of the mutations effectively eliminates calcium binding to the altered site (Maune et al., 1992b).

As shown in Table 1, the affinities of either peptide for any of the mutant proteins are at least 10-fold lower than those for wild-type calmodulin. The B2K mutant has the highest affinity for both peptides, suggesting that it is the least altered in function. The B4K mutant has the lowest affinity for both peptides—more than 200-fold lower than that of wild-type calmodulin. The B2Q and B4Q mutants both have about 100-fold lower affinity for the WFF peptide than wild-type CaM, but there is a 10-fold difference in their affinities for the FFW peptide. Relative to wild-type CaM, the affinity of B4Q for the FFW peptide is decreased by a factor of 24, but that of B2Q is decreased by a factor of about 200. The fluorescence enhancement upon binding of FFW peptide to the B2Q mutant is also much lower than that for any of the other proteins, and the λ_{max} of fluorescence emission is much higher. This suggests that this mutation in site 2 has significantly altered the interaction of the N-domain of calmodulin with the side chain of residue 17 of the peptide. The fluorescence enhancement upon binding of either peptide to the B4K mutant is somewhat lower than that of the wild-type calmodulin and, in combination with the decrease in affinity for either peptide, suggests a change in the overall protein–peptide interaction.

Table 2 shows the intensity of the far-UV CD signal at 222 nm for calmodulin and the mutants alone and upon formation of the complexes with either the WFF or FFW peptide. The values for the mutant calmodulins alone appear somewhat lower that those previously reported (Maune et al., 1992a) probably due to uncertainties on the spectroscopic determination of protein concentrations (see Materials and Methods). In the presence of either the WFF or the FFW peptide, the intensity of the CD signal at 222 nm increases for all the complexes. For the wild-type Ca_4-CaM this increase in intensity is consistent with the peptide adopting a predominantly α-helical conformation. For the B2K mutant with either peptide and for the B2Q mutant with the WFF peptide the increase in molar ellipticity on forming complex is similar to that of wild-type calmodulin. However for the B2Q mutant with the FFW peptide, the increase is much smaller, suggesting that either less structure is induced in the peptide or the protein structure is altered on binding this peptide. Larger increases in intensity ($\Delta\Delta\epsilon_M$) are observed for the two site 4 mutants upon binding either peptide.

Table 2: Far-UV Circular Dichroism Extinction Coefficients (M^{-1} cm^{-1}) of Wild-Type and Four Mutant Calmodulins Alone and in Complex with WFF or FFW Peptide[a]

		$\Delta\epsilon_M$ (222 nm)		$\Delta\Delta\epsilon_M$ (222 nm)	
calmodulin	no peptide	+WFF	+FFW	+WFF	+FFW
wild type	−811	−942	−926	−131	−115
B2Q	−738	−863	−813	−125	−75
B2K	−712	−878	−855	−166	−143
B4Q	−673	−857	−849	−184	−176
B4K	−578	−804	−783	−226	−205

[a] The value of the mean residue CD $\Delta\epsilon_{MRW}$ is related to the molar CD by the equation $\Delta\epsilon_{MRW} = \Delta\epsilon_M/n$, where n is the number of peptide bonds in either calmodulin (147) or the complex of calmodulin plus peptide (147 + 17 = 164). The use of $\Delta\epsilon_M$ facilitates comparison of the far-UV CD of the complexes in terms of $\Delta\Delta\epsilon_M = \Delta\epsilon_M$(complex) − $\Delta\epsilon_M$(calmodulin).

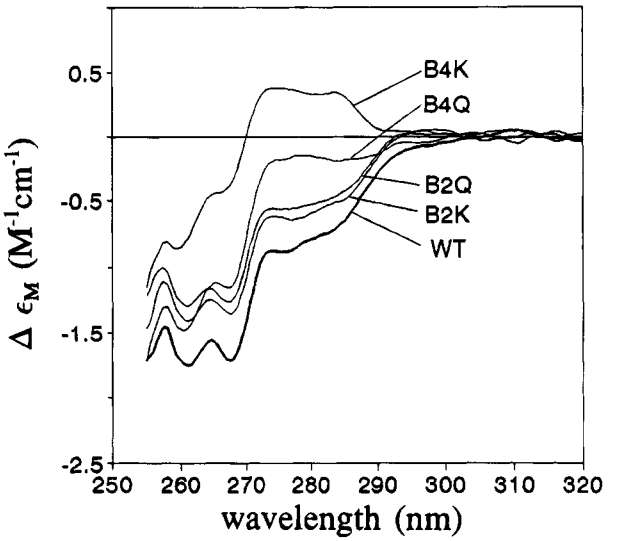

FIGURE 5: Near-UV CD spectra of wild-type and four mutant calmodulins. [CaM] = 20 μM in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5.

The near-UV CD spectra of the four mutant calmodulins are quite different from that of the wild-type protein as shown in Figure 5. In contrast, the near-UV CD spectra of the five complexes with the WFF peptide are very similar as shown in Figure 6A. Closer examination of the region of the spectrum between 290 and 300 nm which is due primarily to the bound tryptophan (see above) indicates that the spectra of the B2K and B2Q complexes are effectively identical to that of the wild-type complex, whereas those of the two site 4 mutants have somewhat lower intensity in this region. The near-UV CD spectra of the complexes with the FFW peptide

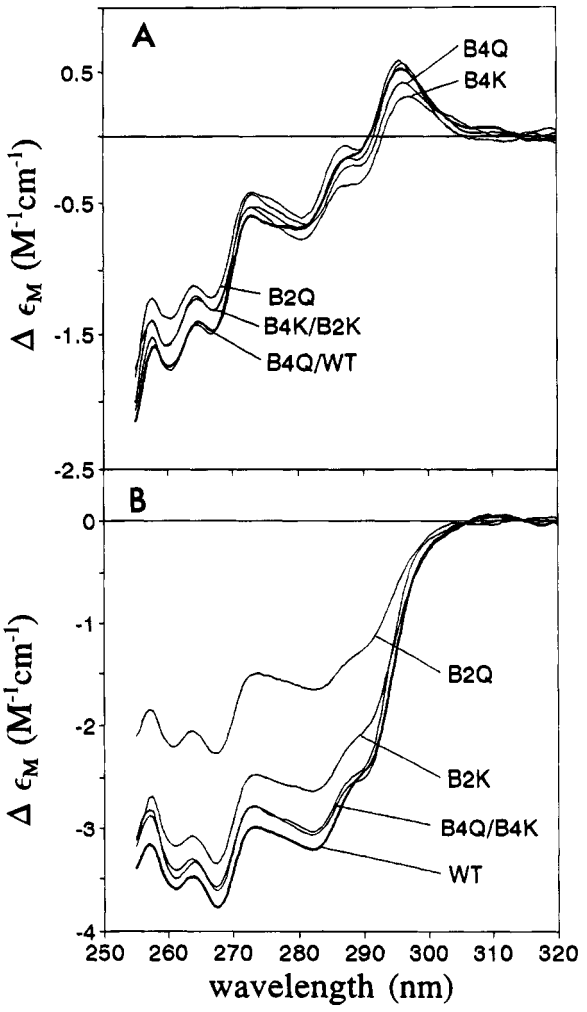

FIGURE 6: Near-UV CD spectra of complexes (1:1 protein:peptide) of wild-type and four mutant calmodulins with (A) WFF peptide and (B) FFW peptide. [CaM] = 20 μM in 25 mM Tris, 100 mM KCl, and 1 mM $CaCl_2$ at pH 7.5.

show more variation as seen in Figure 6B, although they all show strong negative CD intensity at 280 nm. The complexes with B4K and B4Q mutants appear very similar to the wild-type complex. The near-UV CD spectrum of B2K–FFW peptide complex is somewhat less intense, and that of the B2Q–FFW peptide complex is much less intense, than the spectrum of the complex with the wild-type protein. These results confirm that mutation of calcium binding site 4 in the C-domain alters the interaction of calmodulin with the side chain of residue 4 of the target peptide, whereas mutation in site 2 in the N-domain alters the interaction with the side chain of residue 17 of the target peptide, consistent with the postulated orientation of binding of the peptides to wild-type calmodulin.

DISCUSSION

Both the WFF and FFW peptides bind to calmodulin with very high affinity and 1:1 stoichiometry in the presence of calcium. By use of the intrinsic fluorescence of the tryptophan residue of the peptide, the dissociation constant of calmodulin and WFF peptide is estimated to be $\leq$0.2 nM, which is similar to the value for the M13 peptide deduced from enzyme inhibition studies (Blumenthal et al., 1985). It is significantly lower than the value of 500 nM previously reported for the analogous 17-residue peptide M5 (Garone & Steiner, 1990). The affinity of calmodulin for the FFW peptide is at least 8-fold lower than for the WFF peptide, reflecting the effect of interchanging the Trp-4 and Phe-17 residues in the target peptide. Vorherr et al. (1990) observed a decrease in affinity of >20-fold for calmodulin upon replacing the corresponding Trp of the target peptide from the erythrocyte calcium pump by either a Tyr or an Ala residue. In the case of both *sk*-MLCK and calcium pump targets, additional evidence indicates that alteration of the peptide does not change the orientation of binding but that replacing the Trp-4 residue leads to a loss of affinity in peptide interactions with the C-domain. Preliminary measurements of the binding to calmodulin of the peptide FFF, with Trp4 replaced by Phe, show that it binds about 10-fold weaker than WFF. The Trp residue in position 4 therefore increases the free energy of peptide binding, despite the fact that a Phe side chain has a higher hydrophobicity index (Eisenberg et al., 1984). Blumenthal and Krebs (1987) reported that truncated peptide analogues of M13 lacking Phe17 have a markedly lower affinity for calmodulin, further emphasising the role of aromatic residues in this interaction. The solution structure of the calmodulin–M13 peptide complex (Ikura et al., 1992) shows that peptide residues Trp4 and Phe17 have extensive interactions exclusively with the hydrophobic residues of the calmodulin C-domain and N-domain, respectively. The larger Trp residue is apparently involved in a greater number of protein–peptide contacts than the Phe, which may account for the decrease in affinity when the tryptophan is replaced.

The wild-type CaM–WFF and CaM–FFW complexes show an increase in the far-UV CD intensity over that of calmodulin alone, consistent with the peptide adopting an α-helical conformation when bound to calmodulin, as had been previously shown for the calmodulin–M13 peptide complex (Ikura & Bax, 1992). As described in Materials and Methods, an α-helical 18-residue peptide would have $\Delta\epsilon_M$(222 nm)= -176 M^{-1} cm^{-1}. The values for the WFF and FFW complexes of $\Delta\Delta\epsilon_M$(222 nm) of -131 and -115 M^{-1} cm^{-1} correspond to approximately 12 or 13 residues of each target peptide adopting α-helical conformation upon binding to wild-type Ca_4-CaM. Ikura and Bax (1992) showed that residues 3–21 of the M13 peptide adopt an α-helical conformation in the CaM–M13 peptide complex. Our calculated values may be a slight underestimate of the structural change in the peptide since Ikura et al. (1991) also showed that three residues in the linker region between the two domains of calmodulin underwent a small change in the opposite sense (from α-helical to extended conformation) upon binding of the M13 peptide.

The near-UV CD spectra indicate that the chiral environments of the tryptophan side chain of the WFF and FFW peptides are quite different in sign and magnitude when these peptides are bound to wild-type calmodulin. Model compound studies and theoretical calculations (Goux et al., 1976; Woody, 1994) show that the sign and magnitude of the near-UV CD spectra of tryptophan in proteins derive from electronic interactions with neighboring polarizable groups, as well as interactions with the peptide backbone. These marked differences between bound WFF and bound FFW would be consistent with W4 of the WFF peptide and W17 of the FFW peptide binding to different domains of calmodulin. This would imply that, although the presence of the Trp residue at position 4 is energetically important, it is not

the unique determining factor, and the relocation of the Trp side chain of the peptide from position 4 to position 17 does not reverse the orientation of binding of the helical peptide. Using fluorescence energy transfer, Chapman et al. (1992) obtained a similar result for calmodulin binding to the target sequence of the plasma membrane calcium pump: the same peptide orientation was found for two related peptide sequences in which residues W3 and F21 had been interchanged.

All of the mutant calmodulins have significantly lower affinity than the wild-type calmodulin in interaction with either peptide. This implies that the integrity of both domains is necessary for retention of the high-affinity binding of MLCK target sequences. The K_d values are still less than 1 μM, indicating that very stable protein−peptide complexes are formed.

The binding of either peptide to the B2K, B4Q, or B4K calmodulins causes a greater increase in total helical content of the complex than for the wild-type calmodulin. The difference in $\Delta\epsilon_M$ for the site 4 mutants is greater than would result from adoption of 100% α-helical conformation by the bound peptide (i.e. $\Delta\Delta\epsilon_M$ exceeds -176 M^{-1} cm^{-1}). This strongly suggests that complex formation with the mutant calmodulin can induce additional secondary structure in the protein, as well as α-helical structure in the peptide. The largest increase in helicity occurs upon binding of either peptide to the B4K mutant which has the most altered secondary structure (i.e., the lowest α-helical content) of all the mutant calmodulins in the absence of peptide.

Near-UV CD results confirm that all of the mutations have altered the tertiary structure of calmodulin in the region of Tyr138 in the C-domain to different degrees [cf. Maune et al. (1992a)]. Mutation of site 2 (B2Q, B2K) in the N-domain has relatively little effect on the magnitude of the conformational change induced in the C-domain on binding calcium, as monitored by $\Delta\epsilon_{280}$. However, mutations of site 4 (B4Q, B4K) in the C-domain effectively prevent the calcium-induced conformational change in this domain which produces the characteristic intense negative CD of Tyr138 found for the wild type calmodulin. By contrast the complexes of mutant calmodulins with either peptide demonstrate near UV CD properties almost identical with those of the wild type calmodulin−peptide complex as seen in Figure 6A for the WFF peptide and Figure 6B for the FFW peptide. This is a strong indication that the binding of either peptide induces the C-domain of the mutant protein to adopt a conformation more like that of wild-type calmodulin even when mutation in calcium binding site 4 has caused major structural change in the mutant protein. The only exception is the B2Q−FFW complex, which shows a much lower near-UV CD intensity than the other CaM−FFW complexes. The fluorescence enhancement upon peptide binding is also much lower for this complex, suggesting that the B2Q mutation has altered the interaction of W17 of the FFW peptide with the mutated N-domain.

The similarities in both near- and far-UV CD spectra for the peptide complexes with the four calcium binding site mutants examined here and those with wild-type calmodulin suggest that marked conformational deficiencies at the level of both secondary and tertiary structure in the mutants have been compensated upon binding of an MLCK target peptide. Apparently some of the free energy of binding the target sequence is being used to generate structure in the mutant calmodulin. This is somewhat analogous to the free energy coupling between target protein binding and calcium binding by calmodulin leading to an increase in calcium affinity as described by Olwin and Storm (1985). Similar results have been obtained with mutants of calmodulin and a *sm*-MLCK target peptide by Haiech et al. (1991) who reported that the presence of the peptide enhances the binding of calcium by a site 2 mutant (E67A) and a site 4 mutant (E140A) analogous to the mutant calmodulins studied here. These mutant proteins were also able to activate intact *sm*-MLCK. Mukherjea and Beckingham (1992) found that the complexes formed by either of the model peptides mastoporan or mellitin with any of the mutant calmodulins studied here were conformationally distinct from the corresponding complexes formed with wild-type calmodulin.

It is also of interest to compare our results on target peptide interaction with previous work by Gao et al. (1993) on the ability of these mutants of calmodulin to activate calmodulin-dependent enzymes. They reported that at elevated concentrations these mutant calmodulins are able to activate skeletal MLCK to different degrees and that all four of the mutants used here had ≈20-fold lower affinity for the enzyme than the wild-type calmodulin. Affinities of the calmodulin mutants for the WFF peptide range from at least 20- to 200-fold lower than with wild type calmodulin. The extent of MLCK enzyme activation varies with the different mutants. Both site 2 mutants yield only 60% of the maximal activation obtained with wild type calmodulin. The B4Q mutant gave 90% activation, but with the B4K mutant only 30% activation was obtained. These results show that all the mutants activated *sk*-MLCK, with the weakest effect shown by the B4K mutant, which we find to have the weakest affinity for binding to WFF peptide as well as the most altered structure. It therefore appears that our results for affinity and conformational effects of interaction of the mutant calmodulins with the target peptides correlate with those for the affinity and activation of the intact *sk*-MLCK enzyme by the mutant calmodulins.

The approach adopted in the work described here of examining the conformational relationship of the components of the complex between the mutant calmodulin and a target peptide sequence allows a better understanding of possible effects of site-directed mutagenesis in calmodulin. In this case, mutations which cause observable effects on the structure and calcium binding properties of calmodulin still allow activation of the target enzyme. This may be explained by the observation of the reversal of the conformational change in the protein induced by the mutation, at the level of both secondary and tertiary structure, upon binding a target peptide. The deleterious effect of mutation on calmodulin structure has been effectively compensated for by the strength of binding to the target sequence, such that the mutant calmodulin has been restored toward the native or wild-type conformation, with its associated ability to activate the specific enzymic function. This concept indicates the need for molecular, mechanistic studies of the mutant protein−target sequence interaction, to complement the observation of more general enzymic effects of site-directed mutagenesis.

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