Circular Dichroism Studies on Calcium Binding to Two Series of Ca^{2+} Binding Site Mutants of *Drosophila melanogaster* Calmodulin†

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ABSTRACT: The Ca^{2+}-induced structural changes in mutant calmodulins from *Drosophila melanogaster* have been studied by circular dichroism. The proteins comprise eight site-specific mutants, in which a bidentate glutamic acid (at position 12 in each Ca^{2+} binding loop) is replaced with either glutamine (BQ series) or lysine (BK series). Previous studies of these proteins indicate that Ca^{2+} binding at the mutated site is effectively eliminated by each of these substitutions, with additional effects at nonmutated sites. Circular dichroism has now been used to assess Ca^{2+}-induced changes in secondary and tertiary structure in these proteins. In the absence of Ca^{2+}, the helical content of these mutant calmodulins is close to that of the wild-type protein. In excess Ca^{2+}, calmodulins with a mutation in the N-terminal sites show Ca^{2+}-induced increases in helicity (CD at 222 nm) that are similar to those of the wild-type protein. In contrast, much less additional helix is induced by Ca^{2+} in calmodulins with mutations in the C-terminal sites, with the two mutations to site IV showing a particularly poor response. Ca^{2+}-induced changes to the environment of the single tyrosine of *Drosophila* calmodulin (Tyr-138 in site IV of the C-terminal domain) have been monitored via CD at 280 nm. The signal from this residue is significantly altered in the Ca^{2+}-free form of almost all these mutants, including those in the N-terminal domain. This indicates significant interaction between the N- and C-terminal domains of these mutants. For the N-terminal mutants, the amplitude of the Ca^{2+}-induced change in the CD at 280 nm is very similar to the wild-type response. It is significantly smaller in mutations to site III, and effectively abolished in mutations to site IV. For the *Drosophila* wild-type calmodulin and the N-terminal mutants, the change in the CD properties of Tyr-138 is >80% complete at two Ca^{2+} ions per calmodulin, consistent with the initial occupancy of sites III and IV, whereas the changes in peptide conformation occurs progressively during the binding of four and three Ca^{2+} ions, respectively, for wild-type and N-terminal mutants. By contrast, for the C-terminal mutants, the smaller changes in peptide backbone conformation and tyrosine conformation change continuously during Ca^{2+} titration. These calmodulin mutants also show significant interactions between the N-terminal and C-terminal domains. The results show the important structural role of the bidentate glutamate in the Ca^{2+}-induced conformational changes of calmodulin. Modifications of this single residue in the binding site can have far-reaching effects, substantially altering the secondary and tertiary structural changes generated in response to Ca^{2+} binding. These effects are greatest in mutants at sites III or IV, where near- and far-UV CD shows that mutation at either site appears to affect the conformation of the whole C-terminal domain. Particularly for site IV, the bidentate glutamate Glu-140 appears to contribute to the stabilization of the F-helix within the EF-hand structure.

Calmodulin is a highly conserved Ca^{2+} binding protein which is responsible for the Ca^{2+}-dependent activation of many target enzymes [for a review, see Klee (1988)]. Calmodulin contains four "EF-hand"-type calcium binding sites (numbered I–IV starting from the N-terminus), and X-ray crystallographic studies (Babu et al., 1985, 1988) on the Ca^{2+}-saturated form of the protein show that the amino (N)- and carboxy (C)-terminal pairs of sites form two discrete globular domains.

Many spectroscopic studies of Ca^{2+} binding to calmodulin have made use of the two tyrosine residues as probes; for example, UV absorption difference spectroscopy (Klee, 1977; Crouch & Klee, 1980), intrinsic fluorescence (Wang et al., 1982; Kilhoffer et al., 1981a,b), near-UV circular dichroism (Walsh et al., 1979; Kilhoffer et al., 1980, 1981a,b; Burger et al., 1984; Crouch & Klee, 1980), and NMR (Seamon, 1980; Ikura et al., 1983; Forsén et al., 1986). These generally show an apparent end point at a $[Ca^{2+}]/[CaM]$ ratio of approximately 2. On the other hand, it is found (Klee, 1977; Wang et al., 1982; Burger et al., 1984; Hennessey et al., 1987) that the Ca^{2+}-induced changes in the polypeptide backbone conformation, seen in the far-UV circular dichroism spectrum, are continuous until the $[Ca^{2+}]/[CaM]$ ratio reaches at least 4. These results have led to the conclusion that the two tyrosine-containing ion binding sites in the C-terminal domain (numbered III and IV) have a significantly higher affinity for Ca^{2+} than do the two sites in the N-terminal domain (numbered I and II) [for a review, see Forsén et al. (1986) and Linse et al. (1991)]. This interpretation has been supported by stopped-flow studies on calmodulin and its tryptic fragments [e.g., see Martin et al. (1985)]. Calcium dissociation from the N-terminal pair of sites is generally faster than dissociation from the C-terminal pair by a factor of 10 or more. A number of models for the Ca^{2+} binding properties of calmodulin have been presented [e.g., see Burger et al. (1984), Iida and Potter (1986), and Wang (1985)]. More recent work (Linse et al.,

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Table I: Amino Acid Sequences for the Four Loop Regions of *Drosophila melanogaster* Calmodulin

		sequence											
loop	position	1	2	3	4	5	6	7	8	9	10	11	12
I	20–31	D	k	D	g	D	g	T	i	T	t	k	E
II	56–67	D	a	D	g	N	g	T	i	D	f	p	E
III	93–104	D	k	D	g	N	g	F	i	S	a	a	E
IV	129–140	D	i	D	g	D	g	Q	v	N	y	e	E

[a] Upper case letters in the sequence show residues at Ca^{2+}-liganding positions. The residue modified in each of the mutants studied is the conserved glutamate at position 12.

Table II: Definition of Mutant Proteins

Q series mutants	residue changed	K series mutants	residue changed
B1Q	$E_{31} \rightarrow Q$	B1K	$E_{31} \rightarrow K$
B2Q	$E_{67} \rightarrow Q$	B2K	$E_{67} \rightarrow K$
B3Q	$E_{104} \rightarrow Q$	B3K	$E_{104} \rightarrow K$
B4Q	$E_{140} \rightarrow Q$	B4K	$E_{140} \rightarrow K$

1991) confirmed the higher affinity and greater degree of cooperativity of the pair of C-terminal binding sites (III and IV), compared with the N-terminal binding sites (I and II).

The Ca^{2+} binding sites in calmodulin consist of the strongly conserved helix–loop–helix motif (or "EF-hand") of some 30 amino acids that was originally observed in parvalbumin by Kretsinger and Nockolds (1973). Calcium, bound in the 12-residue loop region of this motif, coordinates to residues at positions 1, 3, 5, 7, 9, and 12 of the loop. The glutamate at position 12, which provides two carboxylate oxygens as liganding groups, is considered to be important because it appears to enclose the Ca^{2+} within the binding site (Beckingham, 1991). The sequences of the four loop regions of *Drosophila melanogaster* calmodulin are shown in Table I. The amino acid sequence of the *Drosophila* calmodulin differs from the mammalian sequence at only three positions [99, 143, and 147; see Smith et al. (1987)]. The absence of Tyr-99 in the *Drosophila* protein has important consequences for the spectroscopic properties in the near-UV region.

Eight mutant calmodulins have been used in this work (see Table II for nomenclature). Each mutant has *either* a glutamate to glutamine substitution (Q series) *or* a glutamate to lysine substitution (K series) at position 12 in *one* of the four Ca^{2+} binding loops. The change from glutamate to glutamine is conservative in the sense that it should produce only a minimal perturbation of the protein structure. However, this substitution should, in fact, weaken Ca^{2+} binding by eliminating one of the seven liganding groups. The change from glutamate to lysine was chosen as a highly nonconservative modification, involving a charge reversal (Maune et al., 1992).

The effect of these modifications on the equilibrium and kinetic Ca^{2+} binding properties of *Drosophila* calmodulin has been described in detail by Beckingham and co-workers (Maune et al., 1992; Maune 1991; Martin et al., 1992). The binding of calcium to the wild-type protein in a buffer containing 100 mM KCl and 1 mM Mg^{2+} is described, as for bovine calmodulin, by a four-site model with a high-affinity pair of sites showing positive cooperativity and a low-affinity pair of sites showing little or no cooperativity. In the mutants, binding at the mutated site is effectively eliminated, and binding is generally described by a three-site model. For mutants with modifications in the N-terminal sites, the model involves a high-affinity pair (with positive cooperativity) and a single site with lower affinity. Although a similar model may be used to describe binding of Ca^{2+} to mutants with modifications in the C-terminal domain, the binding is described equally well by a model with three independent sites. Studies by Maune (1991) using a buffer containing 100 mM KCl but *no* Mg^{2+} indicate that in the absence of Mg^{2+}, the affinity for Ca^{2+} is increased somewhat at all sites on the protein. In the absence of KCl, all the stoichiometric constants increase uniformly (Haiech et al., 1981; Iida & Potter, 1986). These constants allow calculation of the relative concentrations of the stoichiometric species Ca–CaM, Ca_2–CaM, Ca_3–CaM, and Ca_4–CaM. However, knowledge of these concentrations does not imply a specification of the distribution of individual occupied sites with a given stoichiometric species (Haiech et al., 1981).

In this paper, we report the effect of Ca^{2+} binding on the near- and far-UV circular dichroism for members of these two series of mutant calmodulins. We address the question of the relative affinities for Ca^{2+} of the binding sites in the two sets of mutants and investigate the possible conformational effects leading to enhanced interaction of the N-terminal and C-terminal domains of calmodulin which have been implicated in recent stopped-flow studies of the kinetics of Ca^{2+} dissociation from these mutant calmodulins (Martin et al., 1992).

MATERIALS AND METHODS

Protein Preparation. The generation of an expression vector for wild-type *Drosophila* calmodulin and the production of site-directed mutants are described elsewhere (Maune et al., 1992). Protein concentrations were calculated using extinction coefficients for the calcium-saturated form of the proteins (in the range 1540–1600 $M^{-1}\cdot cm^{-1}$ at 278 nm for the mutant calmodulins used here) given by Maune et al. (1992). All other chemicals were of reagent grade and were obtained from local suppliers. All solutions were prepared in a buffer containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes)[1]/KOH, pH 7.6, and were stored in plastic containers.

Apocalmodulin samples were prepared for circular dichroism measurements as follows. Lyophilized (Ca^{2+}-containing) samples were dissolved in 20 mM Hepes/KOH buffer at a concentration of approximately 600 μM and desalted on a Pharmacia PD10 (G25) column equilibrated in water. The resulting solution was made 10 mM in HCl [in the absence of salt, the protein remains soluble, and Ca^{2+} affinity is very low (Haiech et al., 1981)] and passed through a further PD10 column that had been equilibrated with 10 mM HCl. Finally, the buffer composition and pH were adjusted (to 20 mM Hepes/KOH, pH 7.6) by addition of KOH and concentrated

[1] Abbreviations: CaM, calmodulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; TR1C and TR2C, tryptic fragments of calmodulin [residues 1–77 (TR1C) and 78–148 (TR2C)]; $\Delta\epsilon$, molar circular dichroic extinction coefficient ($M^{-1}\cdot cm^{-1}$) [note: this may be evaluated per mole of peptide residue ($\Delta\epsilon_{res}$) or (for near-UV spectra) per mole of tyrosine ($\Delta\epsilon_M$)]; $[\theta]_{mrw}$, molar residue ellipticity ($deg\cdot cm^2\cdot dmol^{-1}$), given by $3300\Delta\epsilon_{res}$; *r*, $[Ca^{2+}]/[CaM]$, molar ratio of total Ca^{2+} concentration to calmodulin concentration.

Table III: Far-UV Circular Dichroism Properties ($\Delta\epsilon_{222}$) of *Drosophila melanogaster* Calmodulin for the Apo and Holo Forms Recorded in 20 mM Hepes/KOH at pH 7.6[a]

	$\Delta\epsilon(-Ca^{2+})$	$\Delta\epsilon(+Ca^{2+})$	$\Delta\epsilon(+Ca^{2+})/\Delta\epsilon(-Ca^{2+})$
wild type	−4.29	−5.42	1.26
B1K	−4.05	−5.21	1.29
B1Q	−3.96	−5.17	1.31
B2K	−4.32	−5.83	1.34
B2Q	−4.17	−5.82	1.39
B3K	−4.09	−4.71	1.15
B3Q	−3.94	−4.79	1.22
B4K	−4.32	−4.71	1.09
B4Q	−3.96	−4.43	1.12
bovine CaM	−3.81[b]	−4.65[b]	1.22,[b] 1.28[c]
TR2C	−3.56[b]	−4.59[b]	1.29,[b] 1.28[c]
TR1C	−4.16[b]	−4.53[b]	1.09,[b] 1.16[c]

[a] The spectrum for the holo form was taken at 100 μM protein plus 1 mM Ca^{2+}. [b] Values from Martin and Bayley (1986). [c] Values from Drabikowski et al. (1982).

Hepes. Protein samples treated in this way contain Ca^{2+} at levels less than 0.03 mol/mol of calmodulin, as measured by using atomic absorption spectroscopy (Török et al., 1992).

Circular Dichroism Measurements. Circular dichroism spectra were recorded at 22 °C using a Jasco J-600 spectropolarimeter. The instrument time constant was generally 1 s, and at least three scans were averaged for each reported spectrum. Near-UV spectra (330–255 nm) were recorded at a protein concentration of approximately 100 μM (1.7 mg/mL) using fused silica cuvettes with a path length of 10 mm. Far-UV spectra (260–200 nm) were recorded at the same protein concentration using a demountable cuvette with a path length of 0.1 mm. The use of such high protein concentrations for the far-UV measurements greatly reduces any problems associated with interference from extraneous calcium but limits the measurements to wavelengths greater than 200 nm. The reported near- and far-UV spectra are presented in terms of the mean residue circular dichroism, $\Delta\epsilon$, on the basis of a mean residue weight of 112.7. Since *Drosophila* calmodulin contains only one tyrosine residue, the values of $\Delta\epsilon_{res}$ for the mean residue near-UV CD can be compared on a molar basis ($\Delta\epsilon_M$) with those for tyrosine model compounds using $\Delta\epsilon_M = \Delta\epsilon_{res} n_{res}$ where n_{res}, the number of amino acid residues per mole of calmodulin, is 148.

Calcium titrations were performed by direct addition of aliquots of concentrated $CaCl_2$ up to a $[Ca^{2+}]/[CaM]$ ratio $r = 10$. Two independent titrations were performed for each protein studied. Titrations were performed with [CaM] ≈100 μM.

RESULTS

Spectroscopic Properties of the Wild-Type Drosophila Calmodulin. The far-UV CD spectrum (260–200 nm) of the wild-type *Drosophila* calmodulin is very similar in band shape to that of the bovine calmodulin for both the Ca^{2+}-free (apo) and Ca^{2+}-saturated (holo) forms (data not shown). This indicates that the overall secondary structure content of the two calmodulins is very similar, as expected given the overall sequence similarity between the two proteins (Smith et al., 1987). Although the absolute intensities of the 222-nm signal for the apo and holo forms of the *Drosophila* calmodulin are somewhat greater than those previously reported for the bovine protein (Martin & Bayley, 1986; Table III), the Ca^{2+}-induced increase in intensity is comparable for the two proteins (see Table III). Thus, the Ca^{2+}-induced increase in helicity is quantitatively very similar for these two calmodulin species.

Figure 1 shows the near-UV (or aromatic, 310–250 nm)

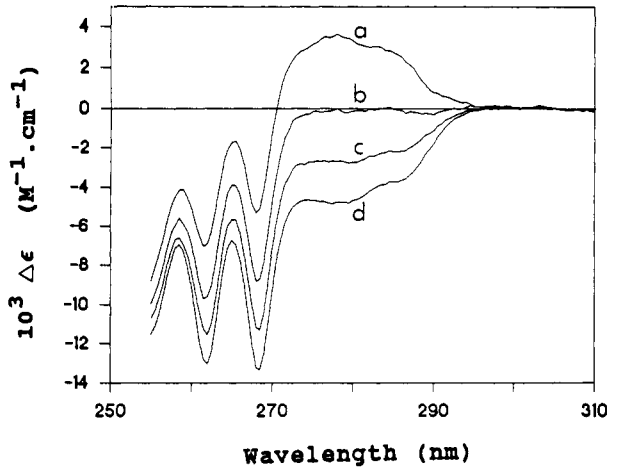

FIGURE 1: Near-UV CD spectra of wild-type *Drosophila* calmodulin in 20 mM Hepes/KOH, pH 7.6, 22 °C, at $[Ca^{2+}]/[CaM]$ ratios of 0 (a), 1 (b), 2 (c), and 10 (d). CD intensities are expressed in terms of mean residue weight.

CD spectra of the wild-type *Drosophila* calmodulin in the apo form and in the presence of added Ca^{2+} at r values of 1, 2, and 10. The latter spectrum (curve d) represents the holo form. The prominent bands at 262 and 268 nm are attributed to phenylalanine residues; the signal above 275 nm arises from the single tyrosine residue, located at position 138 in the C-terminal half of the molecule. The conformational change induced by the binding of Ca^{2+} obviously perturbs the environment of both phenylalanine and tyrosine residues [see Martin and Bayley (1986)]. The spectrum of the Ca^{2+}-saturated form is surprisingly similar in intensity to that of the protein from bovine brain (which contains a second tyrosine at position 99 in addition to Tyr-138); $\Delta\epsilon_{280} = -0.0048$ $M^{-1}\cdot cm^{-1}$ for the *Drosophila* calmodulin compared with $\Delta\epsilon_{280} = -0.0053$ $M^{-1}\cdot cm^{-1}$ for the bovine protein (Martin & Bayley, 1986). In contrast, the spectra of the apo forms are very different for the two proteins: $\Delta\epsilon_{280} = +0.0036$ $M^{-1}\cdot cm^{-1}$ for the *Drosophila* calmodulin compared with $\Delta\epsilon_{280} = -0.0028$ $M^{-1}\cdot cm^{-1}$ for the bovine protein (Martin & Bayley, 1986).

For *Drosophila* calmodulin, the $\Delta\epsilon_{280}$ values quoted above (which are calculated on a per residue basis) correspond to $\Delta\epsilon_M = +0.53$ $M^{-1}\cdot cm^{-1}$ *per tyrosine* for the apo form and $\Delta\epsilon_M = -0.71$ $M^{-1}\cdot cm^{-1}$ *per tyrosine* for the holo form. These values are large, but within the range of values reported for tyrosine model compounds (Strickland, 1974) for which such intensities indicate restricted freedom of rotation and, possibly, individual rotational isomers. The corresponding values for the bovine protein (representing a mean intensity for the two residues, Tyr-99 and Tyr-138) are −0.21 $M^{-1}\cdot cm^{-1}$ *per tyrosine* for the apo form and −0.39 $M^{-1}\cdot cm^{-1}$ *per tyrosine* for the holo form (Martin & Bayley, 1986). Assuming additivity of the two tyrosine residues in the bovine protein and that Tyr-138 has the same $\Delta\epsilon_M$ as in the *Drosophila* calmodulin, then the $\Delta\epsilon_{280}$ values for Tyr-99 in bovine calmodulin are −0.95 $M^{-1}\cdot cm^{-1}$ [(−0.21 × 2) − 0.53] for the apo form and −0.07 $M^{-1}\cdot cm^{-1}$ [(−0.39 × 2) + 0.71] for the holo form.

Spectroscopic Properties of the Mutant Calmodulins. The far-UV CD spectra of all eight mutant proteins were very similar to the wild-type spectrum and gave Ca^{2+}-free $\Delta\epsilon_{222}$ values close to the wild type in all cases. The range was −3.94 $M^{-1}\cdot cm^{-1}$ (B3Q) to −4.32 $M^{-1}\cdot cm^{-1}$ (B2K and B4K) (see Table III). The accuracy of individual $\Delta\epsilon_{222}$ values is approximately ±0.2 $M^{-1}\cdot cm^{-1}$, mainly determined by protein concentration estimation (i.e., <5%). Thus, the apo forms of all of the mutant calmodulins have a rather similar secondary structure to the

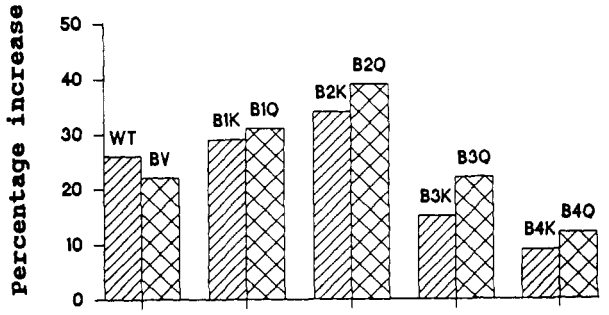

FIGURE 2: Percentage increase in $\Delta\epsilon_{222}$ on binding Ca^{2+} for wild-type *Drosophila* calmodulin (WT), bovine calmodulin (BV), and the eight *Drosophila* calmodulin mutants. The $[Ca^{2+}]/[CaM]$ ratio was 10 for the plus Ca^{2+} (holo) spectra.

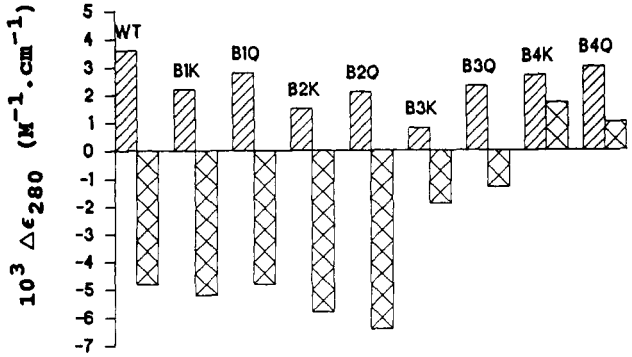

FIGURE 3: Values of $\Delta\epsilon_{280}$ for the apo (hatched bars) and holo forms (cross-hatched bars) of wild-type *Drosophila* calmodulin (WT) and the eight *Drosophila* calmodulin mutants. The $[Ca^{2+}]/[CaM]$ ratio was 10 for the plus Ca^{2+} (holo) spectra.

wild-type protein, and the net effect of each mutation on the overall backbone structure of the apoprotein is rather small. In contrast, the $\Delta\epsilon_{222}$ values for the holo forms cover a much greater range, between $-4.43\ M^{-1}\cdot cm^{-1}$ (B4Q) and $-5.83\ M^{-1}\cdot cm^{-1}$ (B2Q). Circular dichroism measurements monitor *relative* changes with considerable accuracy, and these differences are therefore significant. The increase in $\Delta\epsilon_{222}$ (expressed as a percentage signal increase on going from the apo to the holo form) is shown in Figure 2. For the mutants with substitution in the N-terminal half of the molecule, the percentage increase in $\Delta\epsilon_{222}$ is similar to (site I mutants) or slightly greater than (site II mutants) that for the wild-type calmodulin. For mutants with substitutions in the C-terminal half of the molecule, the percentage increase in $\Delta\epsilon_{222}$ is significantly less than for the wild-type protein; this is especially noticeable for the site IV mutants. The smaller increase observed for mutants with substitutions in the C-terminal sites is consistent with the observation that TR2C (C-terminal tryptic fragment of calmodulin, residues 78–148) shows a significantly greater intensity increase than does TR1C (N-terminal tryptic fragment, residues 1–77) upon binding Ca^{2+} (Martin & Bayley, 1986; Drabikowski et al., 1982; see Table III). Thus, elimination of Ca^{2+} binding at one or more of the C-terminal sites has a pronounced influence on calmodulin conformation. In general, $\Delta\epsilon_{222}$ is closely similar for a given pair of K and Q mutants, in either the absence or the presence of Ca^{2+}, the greatest differences being found for B4K and B4Q. However, both show a similar percentage change in the presence of Ca^{2+}.

Although the apo forms of the eight mutant calmodulins studied have a secondary structure which is clearly very similar to that of the wild-type protein, the environment of Tyr-138 as assessed by near-UV circular dichroism measurements is clearly different in individual mutant proteins. All the apoproteins show positive CD at 280 nm, but the intensities vary considerably (see Figure 3). Duplicate measurements established that these differences were reproducible to ±5%. A low-pH procedure (see Materials and Methods) was used to prepare the apo forms of the proteins for these measurements in order to ensure that they were indeed Ca^{2+}-free. As a control to establish that this procedure did not itself produce artifactual conformational changes, CD spectra for apo and holo forms generated by an alternative route were recorded. Apo forms were prepared by use of 2.5 mM Tris/EGTA and holo forms by addition of Ca^{2+} to a level of $r = 10$, in excess of the Tris/EGTA [note: Tris/EGTA is used since Na/EGTA can affect the near-UV CD of apocalmodulin (Török et al., 1992)]. In all cases, the near-UV CD spectra for these samples were very similar to those recorded for the corresponding samples prepared by low-pH treatment.

Figure 3 shows that the mutant apocalmodulins with substitutions in the N-terminal sites (I and especially II) have near-UV CD spectra with significantly different contributions from Tyr-138. Since this chromophore is located in site IV in the C-terminal domain, this must mean that the conformation of the apo C-terminal domain is affected by mutation of the N-terminal domain binding sites. This strongly suggests a direct interaction between parts of the N- and C-terminal domains in the apo forms of these mutant calmodulins.

For all the mutant calmodulins studied, there is a Ca^{2+}-induced change in the near-UV CD; the magnitude of this change varies significantly for the different mutants, as shown in Figure 3. In the presence of Ca^{2+}, mutant calmodulins with modifications in the N-terminal sites develop the strong negative CD associated with the wild-type holocalmodulin spectrum. The changes in intensity are evidently much smaller for mutant calmodulins with substitutions in the C-terminal sites and are smallest when Ca^{2+} binding to site IV (which is closest to Tyr-138) is effectively eliminated by the mutation (B4K and B4Q). In this case, even in the presence of Ca^{2+}, the near-UV CD retains much of the character of the spectrum of apocalmodulin.

Calcium Binding to the Wild-Type Drosophila Calmodulin. The macroscopic binding constants for Ca^{2+} binding to wild-type *Drosophila* calmodulin (Maune, 1991; Maune et al., 1992) show that it contains two pairs of binding sites with different affinity, with positive cooperativity between the higher affinity pair, sites III and IV in the C-terminal domain. The low-affinity pair are sites I and II in the N-terminal domain [cf. Forsén et al. (1986) and Linse et al. (1991) and references cited therein].

Figure 4 shows the data obtained for the titration of the wild-type protein with Ca^{2+}; the data are presented as a percentage of the total signal change as a function of added Ca^{2+} with the 0 and 100% values taken as the signal observed at $r = 0$ and $r = 10$, respectively. Absolute values of $\Delta\epsilon$ at 280 nm (near-UV) and 222 nm (far-UV) for both the apo and holo forms are given in Table III and Figure 3. The results shown in Figure 4 are similar to those previously obtained for the bovine protein. Most of the change in $\Delta\epsilon_{280}$ (some 75–80%) occurs for $r < 2$ [see Kilhoffer et al. (1980, 1981a,b), Burger et al. (1984), and Crouch and Klee (1980)]. These results are also consistent with data for Ca^{2+} titrations of *Drosophila* calmodulin (Maune et al., 1992) in which UV difference spectra were used to monitor binding. Here it was found that changes were approximately 80–85% complete at $r = 2$. Both results indicate that 75–80% of the first two calcium ions added to the apoprotein are bound to sites III and IV in the C-terminal (tyrosine-containing) domain. Thus,

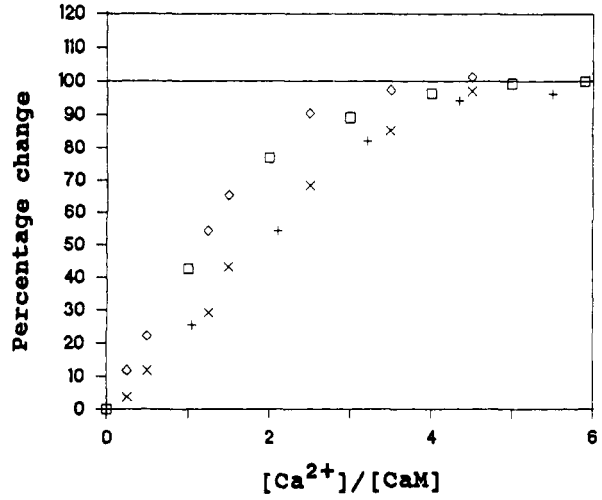

FIGURE 4: Near-UV (280 nm, □ and ◇) and far-UV (222 nm, + and ×) circular dichroism as a function of $[Ca^{2+}]/[CaM]$ ratio for wild-type *Drosophila* calmodulin in 20 mM Hepes/KOH at pH 7.6. The 100% signal was taken as the signal at a $[Ca^{2+}]/[CaM]$ ratio of 10. The different symbols at each wavelength show results from separate, independent experiments.

the intrinsic site binding constants (β_i) can be ordered as (β_{III} and β_{IV}) > (β_I and β_{II}). This is consistent with the stoichiometric binding constants of Maune et al. (1992).

Interestingly, the change in the phenylalanine signal (at both 262 and 268 nm) also appears to be largely saturated (75–80%) at $r = 2$ (data not shown, but see Figure 1), although five of the nine phenylalanine residues in *Drosophila* calmodulin are located in the N-terminal half of the molecule (Smith et al., 1987). However, it is consistent with the above interpretation of the first two calcium ions binding at sites III and IV, since the effect of Ca^{2+} binding on the phenylalanine CD signal of bovine calmodulin fragment TR1C (residues 1–77) is very much smaller than for TR2C (residues 78–148) (Martin & Bayley, 1986).

In contrast to the results obtained on monitoring the environment of the aromatic residues, the value of $\Delta\epsilon_{222}$ (which monitors changes in the conformation of the peptide backbone) varies approximately linearly for $r = 0$–4 (Klee, 1977; Wang et al., 1982; Burger et al., 1984; Hennessey et al., 1987). This result is also consistent with the fact that both tryptic fragments of bovine calmodulin (TR1C, residues 1–77, sites I and II; and TR2C, residues 78–148, sites III and IV) show changes in $\Delta\epsilon_{222}$ on binding Ca^{2+} [see Table III and Drabikowski et al. (1982) and Martin and Bayley (1986)].

Calcium Binding to Mutant Calmodulins with Modifications in the N-Terminal Domain. Macroscopic binding constants for the binding of Ca^{2+} to the mutants with modifications in the N-terminal domain show that one binding site (presumably the mutated one) is greatly reduced in affinity by the mutation and a three-site model is generally sufficient to describe the binding process (Maune et al., 1992). Modeling of the binding data shows that there are two high-affinity sites (with reduced cooperativity compared with the corresponding pair of the wild-type protein) and a third independent site with reduced affinity compared with *either* of the low-affinity sites in the wild-type protein. The interpretation advanced by Maune et al. (1992) is that the mutation of one of either of the N-terminal pair of sites modifies the binding properties of the C-terminal pair and diminishes the affinity of the nonmutated N-terminal partner. UV-difference absorption studies (Maune et al., 1992) clearly indicate that the two C-terminal sites are still the high-affinity pair in these mutants.

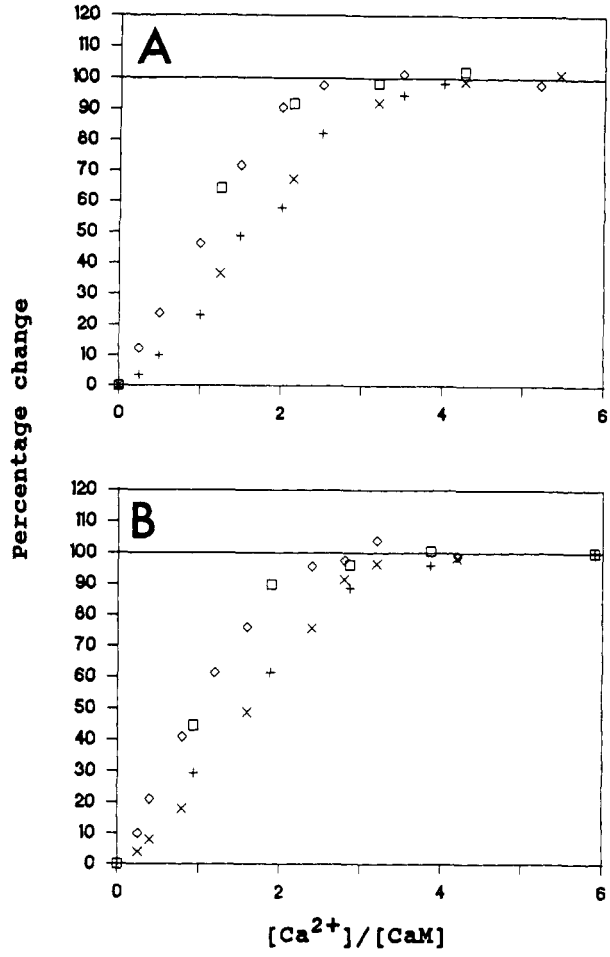

FIGURE 5: Near-UV (280 nm, □ and ◇) and far-UV (222 nm, + and ×) circular dichroism as a function of $[Ca^{2+}]/[CaM]$ ratio for *Drosophila* calmodulin mutants B1K (A) and B2Q (B) in 20 mM Hepes/KOH at pH 7.6. The 100% signal was taken as the signal at a $[Ca^{2+}]/[CaM]$ ratio of 10.

Figure 5A shows the data obtained for the titration of mutant B1K with Ca^{2+}; absolute values of $\Delta\epsilon$ at 280 nm (near-UV) and 222 nm (far-UV) for both the apo and holo forms of mutant B1K are given in Table III. The bulk of the change in $\Delta\epsilon_{280}$ (some 90–94%) occurs for $r < 2$. UV absorption difference studies (Maune et al., 1992) also show changes which are essentially complete at $r = 2$. These results show that >90% of the first two calcium ions added to the apo mutant B1K are bound to sites III and IV. The change in $\Delta\epsilon_{222}$ again varies approximately linearly with r (see above) and is more than 90% complete at $r = 3$. This is consistent with the idea that binding at the mutated site is effectively eliminated for the Ca^{2+} concentration range employed here. Very similar results are obtained for the near- and far-UV CD titrations of mutant B2Q (see Figure 5B) and for mutants B1Q and B2K (data not shown).

Calcium Binding to Mutant Calmodulins with Modifications in the C-Terminal Domain. Macroscopic binding constants measured for Ca^{2+} binding to mutants with modifications in the C-terminal domain again show that the affinity of one binding site is strongly reduced (Maune et al., 1992). Although the binding data are satisfactorily modeled by three independent binding sites, they are also fitted by a model indentical to that found for the N-terminal domain mutants with one interacting pair and a third weaker site (Maune et al., 1992). The latter possibility is physically less attractive in the case of these C-terminal mutants since it appears to imply a degree of cooperativity either between the

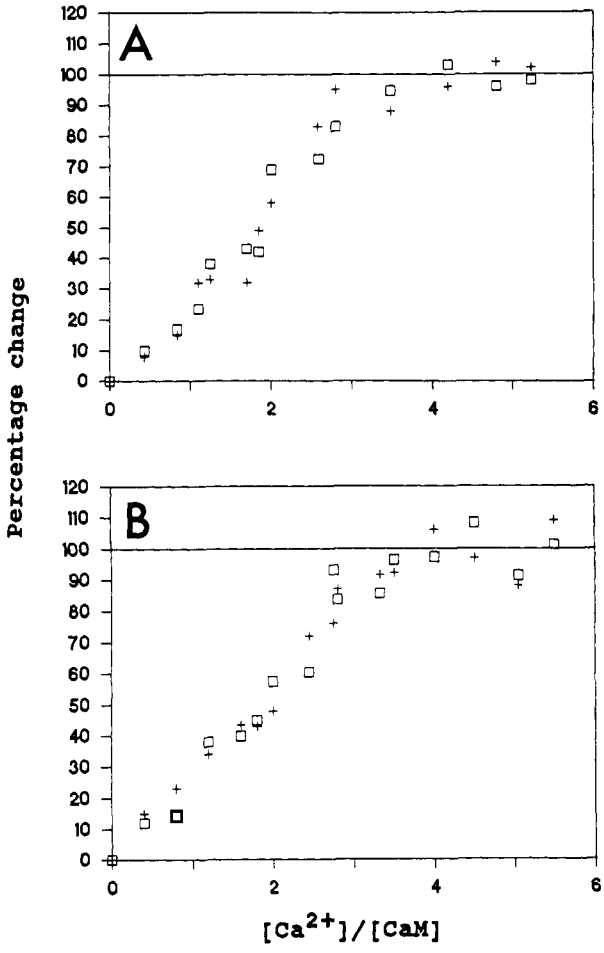

FIGURE 6: Near-UV (280 nm, □) and far-UV (222 nm, +) circular dichroism as a function of $[Ca^{2+}]/[CaM]$ ratio for *Drosophila* calmodulin mutants B3K (A) and B4Q (B) in 20 mM Hepes/KOH at pH 7.6. The 100% signal was taken as the signal at a $[Ca^{2+}]/[CaM]$ ratio of 10.

N-terminal pair (and little such cooperativity is detected for the wild-type protein, see above) or between a pair of sites in separate domains.

Titration data for mutants B3K and B4Q are shown in Figure 6A,B. A precise interpretation of these data is difficult because the signal changes are small compared with those seen for the wild-type protein and for the N-terminal mutants (see Figures 2 and 3 and Table III). In general, it appears that changes in $\Delta\epsilon_{280}$ and $\Delta\epsilon_{222}$ occur effectively in parallel and saturate at an r value of approximately 3. The CD data appear to be most readily interpreted with a binding model of three approximately equivalent sites. If a cooperative model is involved (see above), the cooperativity would have to be significantly less than is observed in the case of the site III/site IV interactions in wild-type or N-terminal mutants. The CD saturation curves (Figure 6) do not allow us to specify the relative affinity of individual sites in the case of the calmodulin mutants with substitutions in the C-terminal domain.

DISCUSSION

Roles of the Individual Binding Sites in Ca^{2+} Binding and Conformational Changes. These studies address two aspects of the Ca^{2+}-induced conformational change at the level of secondary and tertiary structure, and consider the implications for the interactions of the N- and C-terminal domains. For wild-type *Drosophila* calmodulin, far-UV CD spectra establish that a substantial increase in helical content, comparable to that observed for bovine calmodulin (Martin & Bayley, 1986), is induced upon Ca^{2+} binding. From Ca^{2+} titration of the far-UV CD changes, all four Ca^{2+} binding events appear to contribute to this increase in helicity. Near-UV CD establishes that the single tyrosine of the protein is in an asymmetric environment prior to Ca^{2+} binding and that upon Ca^{2+} binding the rotational freedom of this residue remains restricted. This is consistent with the buried conformation of this residue in both bovine and *Drosophila* calmodulins (Babu et al., 1985, 1988; Taylor et al., 1991). In contrast to the Ca^{2+}-induced increase in helicity, the changes to the properties of Tyr-138 are largely effected by the first two Ca^{2+} binding events.

In the interpretation of the far-UV CD changes of the mutants, it is important to note that there is a 2.5-fold greater change in the CD of the (wild-type) C-terminal domain on binding calcium, compared to the (wild-type) N-terminal domain (see Table III, data for TR1C and TR2C). Thus, on the assumption that the individual mutation acts to produce an apo-type conformation at that site, the conformational effects would be expected to be intrinsically larger for mutations in the C-terminal domain than in the N-terminal domain. For the N-terminal mutants, the far-UV CD spectra indicate Ca^{2+}-induced increases in helicity that are comparable to or slightly greater than those seen for the wild-type protein. Data from ^1H NMR (Starovasnik et al., 1992) and from studies with a hydrophobic reporter molecule (Beckingham, 1991) indicate that the Ca^{2+}-bound form of the N-terminal domain is somewhat different from wild type in both the B1Q and B2Q mutants. The ^1H NMR studies suggest an altered conformation of the peptide backbone within the β-sheet region that lies between binding sites I and II. Such changes could modify the relative orientation of sites I and II with little overall change in α-helical content.

The changes in the far-UV CD spectra of the N-terminal mutants are largely complete upon addition of 3 equiv of Ca^{2+}, with little further change observed on addition of up to 10 equiv of Ca^{2+}. The ^1H NMR studies of B1Q and B2Q indicate weak Ca^{2+} binding at the mutated site in each of these proteins, although for the B2Q mutant this binding does not elicit the normal resonance change associated with binding at site II. These CD studies suggest that any weak binding at the mutated sites elicits no further change in the overall helical content.

The changes to the environment of Tyr-138 in these N-terminal mutants are closely similar to those of the wild-type protein, with the full change (>85%) occurring with 50% site occupancy as in the nonmutated protein. This suggests that the Ca^{2+}-bound conformation of the C-terminal domain in mutants with modifications in the N-terminus is not significantly altered in secondary or tertiary structure from that of the wild-type holoprotein. This finding correlates well with results from UV difference spectroscopy (Maune et al., 1992), ^1H NMR (Starovasnik et al., 1992), and binding of a hydrophobic reporter molecule (Beckingham, 1991).

Studies of the activation of target enzymes (calcineurin,[2] calmodulin kinase II,[3] and skeletal muscle myosin light chain kinase[4] have established that, in contrast to other pairs of mutants at sites I, II, and III, the B1Q and B1K mutations differ in their capacity to activate these targets, with the B1K mutant showing an activity much more like that of the wild-type protein (Maune et al., 1992). Modeling studies using the crystal structure of *Drosophila* calmodulin (Taylor et al.,

[2] J. F. Maune and C. B. Klee, unpublished observations.
[3] S. George and K. Beckingham, unpublished observations.
[4] Z. H. Gao, M. F. Van Berkum, A. R. Means, K. Beckingham, and J. T. Stull, manuscript in preparation.

1991) suggested that this difference could result from the capacity of the positively charged lysine substitution in B1K to mimic the effect of a Ca^{2+} ion in site I and thus to induce a conformation at site I close to the wild-type Ca^{2+}-bound form. However, the CD spectra presented here provide little evidence for conformational differences between B1Q and B1K in either to apo or the holo forms. The effects of the lysine residue in B1K might therefore involve an interaction within the B1K–target enzyme complex.

For the C-terminal mutants, the far- and near-UV CD spectra indicate that both the Ca^{2+}-induced increase in helicity and the environmental change to Tyr-138 are substantially reduced compared to the wild-type protein. This emphasizes the strong level of coupling between conformational change and cooperative Ca^{2+} binding at the two C-terminal sites. Mutation of either site III or site IV destroys the cooperativity between these two otherwise high-affinity sites (Maune et al., 1992), and the C-terminal domain is unable to adopt either the secondary or the tertiary structure of the wild-type protein when the residual nonmutated site binds Ca^{2+}.

These CD studies demonstrate quantitative differences between mutations in sites III and IV of Tyr-138. Previous conformational studies have revealed qualitative differences in the Ca^{2+}-induced conformational changes shown by these two sets of mutants. Thus, when the environment of Tyr-138 is examined by UV difference spectroscopy (which monitors changes in the hydrophobicity of the environment) (Maune et al., 1992), differences are found for mutants at site III and site IV. In site III mutants (plus Ca^{2+}), the environment of Tyr-138 appears similar to the wild type, but in the B4Q mutant it is substantially altered. ^1H NMR analysis (Starovasnik et al., 1992) of resonances from His-107 (site III) and Tyr-138 (site IV) in both B3Q and B4Q establishes that either mutation affects both residues but each mutation produces strikingly different perturbations of the two resonances. Combined, these data indicate that mutating either site III or site IV causes conformational effects in both EF-hand regions of the C-terminal domain but these changes are different, depending on whether the mutation is in site III or site IV.

The near- and far-UV CD changes detected here in the site III and site IV mutants have very low magnitude and are apparently complete upon addition of 3 equiv of Ca^{2+}, showing no further effects even when Ca^{2+} is added in 10-fold molar excess. However, the conformational changes detected by UV difference spectroscopy and ^1H NMR continue beyond addition of 3 equiv of Ca^{2+}. These results indicate that even at sufficiently high [Ca^{2+}], when there is apparently some binding at the mutated site, the native conformation of wild-type Ca_4–calmodulin cannot be induced in these site III and site IV mutants by Ca^{2+} alone.

The dramatic reduction in the magnitude of the conformational change shown by the glutamine mutants B3Q and B4Q results from a minimal change to a single glutamate residue of the protein. This emphasizes the critical importance of this highly conserved glutamate at position 12 of sites III and IV in stabilizing the EF-hand structure of each of these sites and, by a cooperative effect, in promoting the structure of the partner EF-hand. Clearly, the single-site mutation has an influence well beyond the immediate site of replacement, producing surprisingly far-reaching effects on structure and presumably function. The conformational changes shown by *both* of the site IV mutants (B4Q and B4K) are strikingly reduced, which correlates well with the previous finding that these mutants are particularly affected in terms of Ca^{2+} binding (Maune et al., 1992). It appears that both Ca^{2+} binding and related conformational changes in the protein are especially sensitive to mutation of the position 12 glutamic acid in binding site IV. Since this residue (Glu-140) is within the F-helix of site IV (residues 139–148), it is reasonable to hypothesize that the bidentate chelation function of Glu140 makes an important contribution to the stabilization of this C-terminal helix. Mutation of Glu-104 (in mutants B3K and B3Q) also produces a lower helical content (reduced $\Delta\epsilon$; see Table III). By analogy with site IV, this could indicate loss of helix, or alternatively, since sites III and IV interact, this could be a wider effect involving parts of both EF-hands. At the same time, it is interesting to note that deletion of the bidentate function of Glu-31 (site I) or Glu-67 (site II) has much less effect on the helical conformation.

Implications for Interdomain Interactions in the Protein. We have previously measured the Ca^{2+} dissociation rates for *Drosophila* calmodulins, both wild type and these two series of mutants. The N-terminal mutants were found to produce a small but significant increase in the rate of dissociation from the C-terminal sites whereas the C-terminal mutants were found to reduce considerably the rate of Ca^{2+} dissociation from the N-terminal sites (Martin et al., 1992). These effects were interpreted as involving enhanced interaction between the N- and C-terminal domains of these mutants as compared to the wild-type protein. In principle, such interactions could be present in either the apo or the Ca^{2+}-loaded form of the mutant proteins. A consideration of the absolute CD intensities measured here for these mutants allows some further deductions to be made. Thus, *for the N-terminal mutants*, the near-UV CD indicates that the Ca^{2+}-loaded form of the C-terminal domain is in a conformation very similar to that of the Ca^{2+}-loaded form of the C-terminal domain in the wild-type whereas the apo form of the C-terminal domain clearly has a significantly different conformation from that of the apo form of the C-terminal domain in the wild type (see Figure 3). This suggests that the interactions in these mutants which affect dissociation from the C-terminal domain may involve the Ca^{2+}-free form of this domain of the protein. *For the C-terminal mutants*, both the apo and holo forms of the C-terminal domain are different from the corresponding apo and holo forms of the wild-type C-terminal domain in near-UV CD (see Figure 3). However, effects are observed on the kinetics of Ca^{2+} dissociation from the N-terminal sites under conditions where the sites in the mutated C-terminal domain are effectively unoccupied. This indicates the interaction of the N-terminal domain with at least some component of the Ca^{2+}-free form of the mutated C-terminal domain.

A number of recent studies suggest that the central helix identified in the crystal structure of calmodulin (Babu et al., 1985, 1988) is discontinuous in solution and that close proximity of the two globular domains may occur under these conditions (Heidorn & Trewhella, 1988; Persechini & Kretsinger, 1988a,b; Ikura et al., 1990, 1991). Furthermore, recent spectroscopic studies provide evidence that apocalmodulin (wild type) has a more compact form in solution than the holo form (Bayley et al., 1988; Small & Anderson, 1988; Török et al., 1992). We conclude that the two halves of the calmodulin molecule, identified as the N-terminal and C-terminal domains of the X-ray crystallographic structure, do indeed interact with one another in solution and can influence one another's properties. These interactions appear to be most pronounced when one or the other or both domains are in the apo state, either in the absence of calcium or when the Ca^{2+}

affinity has been significantly reduced by mutation of a critical Ca^{2+}-liganding residue in one of the Ca^{2+} sites. The effect of such mutations is greatest in the binding sites of the C-terminal domain (sites III or IV) where, as a result, the Ca^{2+} affinity of the whole domain is severely impaired. In considering the extensive effects of the single amino acid replacements studied here, we propose the following general mechanism by which long-range intramolecular reactions may occur. Elimination of Ca^{2+} binding at a given site by mutation of the position 12 ligand may release structural elements within the domain in question and thus permit interaction with the nonmutated domain. Such interactions could affect residues within the nonmutated domain which are either structurally or dynamically involved in Ca^{2+} binding.

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Registry No. Ca^{2+}, 7440-70-2; glutamic acid, 56-86-0; glutamine, 56-85-9; lysine, 56-87-1.