The specificity of interaction of the isolated N- and C-terminal domains of calmodulin with peptide WFFp (Ac-KRRKKKNFIAVSAANRFK-amide) and variants of the target sequence of skeletal muscle myosin light chain kinase was investigated using CD and fluorescence. Titrations show that two molecules of each domain bind to 18-residue target peptides. For WFFp, the C-domain binds with 4-fold higher affinity to the native compared with the non-native site; the N-domain shows similar affinity for either site. The selectivity of the C-domain suggests that it promotes occupancy of the correct binding site for intact calmodulin on the target sequence. Far UV CD spectra show the extra helicity induced in forming the 2:1 C-domain-peptide or the 1:1 C-domain-N-domain-peptide complex is similar to that induced by calmodulin itself; binding of the C-domain to the Trp-4 site is essential for developing the full helicity. Calmodulin-MLCK-peptide complexes show an approximate two-fold rotational relationship between the two highly homologous domains, and the 2:1 C (or N)-domain-peptide complexes evidently have a similar rotational symmetry. This implies that a given domain can bind sequences with opposite peptide polarities, significantly increasing the possible range of conformations of calmodulin in its complexes, and extending the versatility and diversity of calmodulin-target interactions.

Calmodulin is a regulatory protein involved in a variety of Ca\(^{2+}\)-dependent cellular signaling pathways. Its importance as a mediator of the second messenger Ca\(^{2+}\) is reflected in its high conservation throughout evolution. This apparently contrasts with its unique ability to interact strongly with and to regulate selectively a variety of proteins (at least 30) without any obvious sequence homology in their calmodulin binding region (see Refs. 1–5 for reviews). Recently, structures of calmodulin and calmodulin-peptide complexes at atomic resolution have been determined (reviewed in Refs. 2, 6, and 7), showing two similar domains to the target enzyme is not necessarily sufficient for activation of enzymes which are not activated by the Ca\(^{2+}\) saturated form of calmodulin to the channel composed by the two domains, with the predominant interactions being those between the N- and C-terminal domains and the C- and N-terminal portions of the target, respectively.

Calmodulin can be cleaved by trypsin to generate two half-molecules, i.e. the C-terminal and the N-terminal Ca\(^{2+}\) binding domain (20, 21). The equilibrium (+2–24) and kinetic properties (25, 26) of intact calmodulin in the Ca\(^{2+}\) binding and dissociation reactions, as well as the secondary structure (24, 27), are well represented by a summation of the properties of these fragments, suggesting that the two domains are effectively independent structures. The isolated domains are capable of activating target proteins, but the degree of activation varies with the target protein (26, 28–35). In particular, skeletal muscle myosin light chain kinase (sk-MLCK) is activated best by a 1:1 mixture of the domains (85% activation compared with calmodulin), but less by either the C-domain (65%) or the N-domain (20%) (31). This activation pattern is reproduced when calmodulin chimeras consisting of two linked N-domains or two C-domains are used (36). Although the C-domain activates target enzymes better than the N-domain in several cases (29–31, 35), this is not always so (31, 33). Therefore, differences in domain sequence and structure may contribute to the versatility of calmodulin's regulatory functions. Binding of the domains to the target enzyme is not necessarily sufficient for activation since isolated domains can inhibit calmodulin-induced activation of enzymes which are not activated by the domain itself (28, 29, 31, 35).

Although the overall structure of the two domains show marked similarities (37), differences between them in sequence and Ca\(^{2+}\) affinity have apparently been well conserved during evolution (38). This points toward possible functional differences of the two domains, which can be further amplified by variations in target sequences. Studies of enhancement of Ca\(^{2+}\) affinities of calmodulin by target sequences suggest that calmodulin triggers its activation. Despite the wide target range, target affinities are strong ($K_d \sim 1 \text{nM}$) (2–4). The interaction is apparently mediated by both hydrophobic and electrostatic forces (17). NMR (18) and x-ray structures (19) of two related Ca\(^{2+}\)-calmodulin-target-peptide complexes show that the $\alpha$-helical MLCK target peptide lies in a hydrophobic channel composed by the two domains, with the predominant interactions being those between the N- and C-terminal domains and the C- and N-terminal portions of the target, respectively.

1 The abbreviations used are: sk-MLCK, skeletal muscle myosin light chain kinase; FFFp, Ac-KRRKKKNFIAVSAANRFK-amide; FFp, Ac-KRRKKKNFIAVSAANRFK-NH$_2$; FFW, Ac-KRRKKKNFIAVSAANRFK-NH$_2$; FFWu, unprotected version of FFWp; FW10p, Ac-IAVSAANRFK-NH$_2$; FW10u, unprotected version of FW10p; sm-MLCK, smooth muscle myosin light chain kinase; WF10p, Ac-KRRKKKNFIAVSAANRFK-NH$_2$; WF10u, unprotected version of WF10p; WFFp, Ac-KRRKKKNFIAVSAANRFK-NH$_2$; WFFu, unprotected version of WFFp; X-WFFp-Y and related abbreviations, domain-WFFp-complex in which domain X interacts with the Trp-containing N-terminal portion of WFFp and domain Y with the C-terminal portion; M13 and RS20, target sequences of sk-MLCK and sm-MLCK, respectively; HPLC, high performance liquid chromatography.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by Wellcome Research Fellowship 044093/Z/95/Z/IGS/JS/CG.

§ To whom correspondence should be addressed. Tel.: 44-181-959-3666; Fax: 44-181-906-4419; E-mail: p-bayley@nirn.mrc.ac.uk.

(Received for publication, May 29, 1997, and in revised form, October 15, 1997)
calmodulin which is half-saturated with Ca$^{2+}$ could bind to the target protein by only one domain without activation, allowing a rapid response in enzyme activity to an increase in Ca$^{2+}$ concentration (39).

In the present work, we address on a molecular level the specificity of the interaction of the individual calmodulin domains with target peptides derived from the target sequence of sk-MLCK. Previously, spectroscopic studies have been reported on the interaction of the domains with the short peptides maltosanin (40–42). Here, the binding affinities, the molecular interactions in the complex, and the effects of domain binding on the conformation of the target peptides are investigated using a variety of spectroscopic techniques. The striking finding is that two molecules of either domain bind with good affinity to the 18-residue target peptides. The symmetry of the resulting complexes is considered by comparison with the calmodulin-MLCK peptide structure (2), and is discussed in relation to the known versatility of calmodulin in its specific interactions with a range of target proteins. The feasibility of calmodulin domains to bind with reversed polarity and alternative positions on a target sequence greatly extends the potential range of conformations of calmodulin in its bound form, and provides added diversity to the calcium sensitivity of calmodulin-dependent activation processes.

**MATERIALS AND METHODS**

**Proteins and Peptides—**Drosophila melanogaster calmodulin was purified as described previously (43). The purified protein ran as a single band on an SDS-polyacrylamide gel electrophoresis (15% gel; Laemmli system). The tryptic fragments of calmodulin were prepared as described in Ref. 31 with an additional gel filtration step (G75 column) included after the cleavage and before the purification. The purified protein ran as a single band on an SDS-polyacrylamide gel electrophoresis. Mass spectrometry, reverse-phase HPLC, and ion exchange chromatography. The purified protein was used for the C-domain of calmodulin assuming that the absorption of the domain is revealed only indirectly via the competition of the two molecules of domain per molecule of peptide. The simplest model for binding assumes that the optical signal monitors binding of a domain to the Trp-chromophore of the peptide. Titration curves of WF10p and WF101p peptide with either domain were fitted with a stoichiometry of 1:1, using standard fitting procedures (46). In the case of the long peptides WFFp and WFFwp, the titration curves clearly indicated that saturation of the optical signal was achieved via the complex formation of two molecules of domain per molecule of peptide. The simplest model for binding assumes that the optical signal monitors binding of a domain to the Trp-containing portion of the peptide; binding of a second molecule of the domain is revealed only indirectly via the competition of the domain between the two sites. The analysis is based on the known structure of the complex of calmodulin with the sk-MLCK target M13 peptide, of which the C-domain of calmodulin, interacting exclusively with the Trp residue of the peptide, binds predominantly with the C-terminal portion of the peptide. The two sites on the peptide are therefore designated according to their position in the peptide sequence as site N and site C; in the case of binding to peptide WFFp, binding at site N with K$^{\text{N}}$ produces an optical signal, and binding at site C with K$^{\text{C}}$ is optically "silent," whereas for peptide WFFwp, the optical properties of sites N and C are reversed. The mechanism for the binding of two molecules of a given domain (D) per molecule of peptide with sites N and C (understood to be oriented N-peptide-C) is shown in Scheme 1.

According to the scheme, in the course of a titration of a fixed amount of peptide (P) with a single domain, D, the optical signal at any point is determined by 2X$^*$S$^*$, where X$^*$ is the mole fraction of various peptide-containing species (and $X^* = 1$), together with S$^*$, their intrinsic spectroscopic properties. On the above model, for WFFp peptide, and indices 1–4 referring to free peptide, D-peptide-, peptide-D, and D-peptide-D, respectively, then S$^* = S_1$ (known from free peptide) and S$^*$ = S$^*_2$ (known from, or fitted to, the plateau titration curve). To evaluate the term 2X$^*$S$^*$ for a given complex of peptide (P) and variable concentration of added domain (D), the analysis requires the values of K$^{\text{N}}$, K$^{\text{C}}$, and f are then refined by least squares methods in fitting to the experimental titration data. Conversely, simulations were made, using chosen values of these parameters to examine their individual effects on model titration curves.

Fluorescence Measurements—Uncorrected fluorescence emission spectra were recorded in UV-transmitting plastic cuvettes or in quartz cuvettes using a SPEX Fluoromax fluorimeter. Excitation was at 300 nm for measurements at >50 µM peptide concentration, otherwise at 290 or 295 nm for the C-domain and at 280 or 300 nm for the N-domain (bandwidth 0.9 nm). The emission was scanned from 300 or 310 nm to 400 nm (bandwidth 4.3 nm). The temperature was 20 °C and the buffer 25 mM Tris/HCl, 100 mM KCl, 1 mM CaCl$_2$ at pH 8.0. Data for the fluorescence titrations were obtained either by integrating the spectra in the region of the largest fluorescence change (300–350 nm) or by measuring the change in the emission at a wavelength between 322–334 nm with bandwidth 17 nm. Unless otherwise stated, the dissociation constants are derived from at least three independent titrations.

Circular DICHROISM (CD) Measurements—CD spectra were recorded in fused silica cuvettes using a Jasco J-600 spectropolarimeter. The measurements were made at 20 °C in 25 mM Tris/HCl, 100 mM KCl, 1 mM CaCl$_2$ at pH 8.0. For UV-CD measurements (200–280 nm) with calmodulin and all peptides as well as with WFFp and isolated domains were made using 1-mm cuvettes with peptide and protein concentrations in the range 7–25 μM. With isolated domains and FFFp or WFFwp the measurements were made in a 0.1-mm demountable cuvette at 10-fold higher concentrations. Spectra are presented as the molar CD absorption coefficient (Δε) at a fixed wavelength of 222 nm; the molar residue weight of the protein rather than the mean residue weight for the normalization. In the case of the domain mixture complexes, the concentration of the complex was used for the normalization to facilitate the comparison with the spectra of calmodulin (46). The difference between the CD absorption coefficient at 222 nm in the presence and the absence of the peptide was used to establish the number of adopting a helical structure upon complex formation. The results of three experiments were averaged. The difference was expressed as molar in peptide (not protein) concentration and compared with the Δε$_{\text{calmod}}$ value of fully helical peptides of different lengths, which were calculated according to Ref. 45.

Near UV-CD spectra (255–340 nm) were measured using 15–30 repetitive scans for peptide and protein concentrations in the range 80–380 μM with 10 nm cuvettes. The spectral range of 310–340 nm was used to zero the curves. A base-line spectrum was recorded, smoothed over 10 nm, and subtracted, and the resulting spectra were slightly smoothed. The spectra are the average of at least two independent measurements. For near UV CD titrations, spectra of 10–20 scans were recorded between 280 and 320 nm, and were zeroed using the data between 250 and 320 nm. The baseline spectrum was further 10 nm, subtracted, and the CD signal of the resulting spectrum integrated between 280 and 293 nm for titrations with the N-domain and between 292 and 299 nm for titrations with the C-domain. At least four independent titrations were evaluated. Under the conditions of the CD experiments with the N-domain and the short peptides WF10p and WF101p, incomplete complex formation is expected owing to the low affinity of the interaction. Using the dissociation constants of 80 and 75 μM (see below), it is calculated that 53% (N-WF10p) and 48% (WF101p-N) of the peptides are bound under the conditions of the experiment, and these values were used to subtract the free peptide contribution to the spectra and to normalize them to 1 M complex concentration.

Data Analysis and Determination of Dissociation Constants—Titrations were performed by addition of the domain solution to the peptide solution, and recording changes in fluorescence or CD signals deriving from the Trp chromophore of the peptide. Titration curves of WF10p and WF101p peptide with either domain were fitted with a stoichiometry of 1:1, using standard fitting procedures (46). In the case of the long peptides WFFp and WFFwp, the titration curves clearly indicated that saturation of the optical signal was achieved via the complex formation of two molecules of domain per molecule of peptide. The simplest model for binding assumes that the optical signal monitors binding of a domain to the Trp-containing portion of the peptide; binding of a second molecule of the domain is revealed only indirectly via the competition of the domain between the two sites. The analysis is based on the known structure of the complex of calmodulin with the sk-MLCK target M13 peptide, of which the C-domain of calmodulin, interacting exclusively with the Trp residue of the peptide, binds predominantly with the Trp-containing N-terminal portion of the peptide, and the N-domain binds predominantly with the C-terminal portion of the peptide. The two sites on the peptide are therefore designated according to their position in the peptide sequence as site N and site C; in the case of binding to peptide WFFp, binding at site N with K$^{\text{N}}$ produces an optical signal, and binding at site C with K$^{\text{C}}$ is optically "silent," whereas for peptide WFFwp, the optical properties of sites N and C are reversed.
Calmodulin Domain Interaction with sk-MLCK Target Sequences

To simplify the analysis, the determination of the two binding constants \( K_d^N \) and \( K_d^C \) was done in two steps. First, in titration of the fluorescence and the CD signals at high peptide concentration, the ratio \( K_d^N/K_d^C \) was determined. This value was then used as a fixed parameter in the fits to the fluorescence titrations at low concentration, which finally resulted in the individual \( K_d \) values. The rationale behind this approach, demonstrated by simulation, is that titration curves at high concentration (i.e. \( > K_d^C \) and \( K_d^N \)) depend strongly on the ratio \( K_d^N/K_d^C \) and less on their absolute values, whereas the opposite is true at lower concentrations.

The simplest analysis is where the two sites are non-interacting, i.e. there is no thermodynamic co-operativity in the binding of the second copy of the domain. For this case, the co-operativity factor in the scheme is given by \( f = 1 \). Interaction can be included with \( f > 1 \) (negative co-operativity) or \( f < 1 \) (positive co-operativity). In titrations of a full-length peptide with a single domain, it was found that the inclusion of an additional co-operativity parameter \( f \) gave no significant improvement to the numerical fitting, and thus fits were generally done with the simplest model with \( f = 1 \). In calculations based on studies of the 1:1 complex of C-domain plus N-domain plus WFFp peptide, the inclusion of an \( f \sim 0.5 \) was deduced, suggesting a small degree of positive co-operativity; in one out of four cases, evidence was found for a spectroscopic interaction. These analyses are discussed under “Results.”

RESULTS

Fluorescence Spectra—Solutions of the free Trp-containing peptides have a fluorescence emission maximum at 358 nm. The maximum of the enhanced fluorescence emission of all the peptide-domain complexes (Table I) lies between 335 and 338 nm, except for the N-WF10p complex (348 nm), suggesting that the N-domain is less effective than the C-domain in burying the Trp residue of this short peptide in a hydrophobic environment. As discussed in more detail below, two molecules of either domain were found to bind to one molecule of full-length peptide (WFFp or FFWp). Complexes are represented as e.g. X-WFFp-Y, indicating that domain X binds to the N-terminal portion of the peptide and domain Y to the C-terminal portion.

Near UV CD Spectra—Near UV CD spectra of the domains in the absence and the presence of a target peptide, as well as spectra of the free peptides are shown in Fig. 1. The free peptides WFFp (Fig. 1A), FFWp (Fig. 1B), and WF10p (data not shown) have similar near UV CD spectra; below 290 nm, the signal increases steadily and without fine structure to a \( \Delta_{EM} = 0.4 \text{ m}^{-1} \text{ cm}^{-1} \) at 255 nm. Only the WF10p peptide (Fig. 1C) shows a signal above 290 nm.

The spectrum of the N-domain is composed of the Phe signals below 270 nm (Fig. 1B, bold line). The spectrum of the C-domain (bold line in Fig. 1C) shows the additional contribution of the single Tyr residue above 270 nm. Spectra of the isolated domains of bovine testis calmodulin (24) and of the C-domain of Drosophila calmodulin (39) have been reported elsewhere. The spectrum of a 1:1 domain mixture (Fig. 1A) is closely similar to that of intact calmodulin (39, 46) confirming earlier reports that the near UV CD spectrum of calmodulin can be represented by a summation of the CD spectra of the individual domains (24).

Fig. 1 (D–F) shows the spectral change induced by binding of a given target peptide to the domains, i.e. the difference spectrum: domain-peptide complex minus domain normalized for bound peptide concentration. These spectra are dominated by the contribution of the \( ^1L_a \) and \( ^1L_b \) transitions of the bound Trp residue. (An empirical method for separation of these contributions is currently in preparation.) Contributions from Tyr-138 of the C-domain and (\( \approx 275 \) nm) from the Phe residues of the domains and the bound peptide were found to be only minor, \( \Delta_{EM} < 0.4 \text{ m}^{-1} \text{ cm}^{-1} \) at \( \lambda > 265 \) nm) using the peptide FFFp as control.

Near UV CD Spectra of Complexes with Peptide WFFp—The near UV CD difference spectrum of the bound Trp in the C-WFFp-C complex (Fig. 1D) is stronger than that with the shorter WF10p peptide (Fig. 1D). The Trp signal is also affected by the end protection of the peptides, the unprotected versions (suffix “u”) showing the weaker signal (39). At 295 nm, the signal decreases in the order: C-WFFp-C \( \geq \) calmodulin-WFFp \( \geq \) C-WFFp-N \( > \) calmodulin-WFFu = C-WFFu > C-WF10p > calmodulin-WF10u = C-WF10u. These variations could be due to relatively localized conformational differences of the Trp residue relative to the binding pocket and the peptide backbone, and the effects of different degrees of mobility of the target peptide sequence.

| Complex       | Stoichiometry | \( K_d^N \) | \( K_d^C \) | \( K_d^N/K_d^C \) | \( \Delta G/kJ \cdot mol^{-1} \) |
|---------------|---------------|-------------|-------------|------------------|-----------------------------|
| C-WFFp-C     | 2:1           | 335         | 18 (± 8) \( \text{nM} \) | 45 (± 25) \( \text{nM} \) | 2.5 (± 0.5)                 | -43.4, -41.2               |
| C-WF10p      | 1:1           | 335         | 10 (± 4) \( \text{nM} \) | 80 (± 35) \( \text{nM} \) | 8.0 (± 0.5)                 | -44.9, -39.8               |
| N-WFFp-N     | 2:1           | 335         | 560 (± 30) \( \text{nM} \) | 143 (± 30) \( \text{nM} \) | 18.0 (± 45) \( \text{nM} \) | 1.26 (± 0.05)              | -38.4, -37.8               |
| N-WF10p      | 1:1           | 348         | 60 (± 14) \( \mu \text{M} \) | 2.0 (± 1.1) \( \mu \text{M} \) | 370 (± 150) \( \text{nM} \) | 0.19 (± 0.05)              | -32.0, -36.1               |
| C-FFWp-C     | 2:1           | 337         | 560 (± 30) \( \text{nM} \) | 2.0 (± 1.1) \( \mu \text{M} \) | 6.4 (± 3.5) \( \mu \text{M} \) | 0.38 (± 0.09)              | -29.5, -31.8               |
| FW10p-C      | 1:1           | 338         | 60 (± 14) \( \mu \text{M} \) | 2.0 (± 1.1) \( \mu \text{M} \) | 75 (± 18) \( \mu \text{M} \) | 0.38 (± 0.09)              | -29.5, -31.8               |
| N-FFWp-N     | 2:1           | 335         | 5.5 (± 2.6) \( \mu \text{M} \) | 2.1 (± 0.5) \( \mu \text{M} \) |              |                           |                            |
| N-FW10p-N    | 1:1           | 337         | 5.5 (± 2.6) \( \mu \text{M} \) | 2.1 (± 0.5) \( \mu \text{M} \) |              |                           |                            |

- Calculated using the \( K_d \) ratio determined from CD experiments.
- Calculated using the \( K_d \) ratio determined from fluorescence experiments.
Trp and surrounding residues. We conclude that there is some variation in the binding mode of the C-domain to the Trp residue in the different peptides.

The Trp near UV CD difference spectrum of N-WFFp-N is characterized by a negative signal (Fig. 1D). The spectrum of the complex N-WF10p has the same shape, but a reduced intensity (not shown). Although binding to a different binding site cannot be ruled out, the preserved shape may suggest that the Trp is still bound to the same binding site. However, the fluorescence spectra indicate that the environment of the WF10p Trp residue is significantly less hydrophobic than that of the WFFp Trp residue. It is possible that the hydrophobic environment is only maintained as long as the Trp residue is restricted in mobility, but with increased mobility Trp gains access to less hydrophobic regions, which are probably close to the water surface.

Near UV CD Spectra of Complexes with Peptide FFWp—The spectrum of N-FFWp-N has the same form as that of the complex of calmodulin with FFWu (46) (and FFWp; data not shown), but with intensity reduced to 50%, suggesting that the Trp residue is bound similarly in both complexes but with increased mobility within the N-FFWp-N complex. For F10p-N,
a significantly weaker signal than for N-FFWp-N is observed, which is not the case when the calmodulin complexes with FFWu and FW10u are compared (39).

C-FFWp-C shows the weakest CD spectrum of the complexes with the long peptides, and this is the only case where the spectrum with the corresponding short peptide FW10p does not show weaker intensity and is significantly different in shape. Therefore, the Trp binding modes are different for the long and the short peptide and are possibly a superposition of spectra of distinct complex conformations.

Near UV CD Spectra of Complexes with a 1:1 Domain Mixture—To find out whether the 1:1 mixture of C- and N-domain binds in a manner similar to that of intact calmodulin, near UV CD spectra were recorded of the mixture with and without peptide WFFp or FFWp. The Trp spectrum of the 1:1:1 complex with WFFp is very similar in shape to that of C-WFFp-C and of the calmodulin-WFFp complex (Fig. 1D). It is estimated that the Trp spectrum of N-WFFp-N contributes less than 15% to the 1:1:1 complex spectrum. Thus, in the 1:1:1 complex, the Trp residue of the WFFp peptide effectively binds only to the C-domain, resulting in the C-WFFp-N complex, whose spectrum closely resembles that of intact calmodulin-WFFp.

In contrast, the spectrum of the 1:1:1 complex of C- and N-domain with FFWp is significantly different from those of N-FFWp-N (Fig. 1E) or the complexes of calmodulin with FFWu (46) and FFWp, but is very similar to that of FW10p-N and, despite a deviation at 295 nm, to the C-FFWp-C spectrum (Fig. 1F). This may suggest that the isolated C-domain can bind (in part) to the unusual Trp containing C-terminal site on the peptide.

Binding Stoichiometry and Affinities of Individual Calmodulin Domains for the Target Peptide—The fluorescence and CD spectra (Fig. 1, D–F) show that each domain binds to the single Trp in the N-terminal portion of WFFp and the C-terminal portion of FFWp and to the short peptides WF10p and FW10p. The fluorescence and CD signals from this Trp residue were used to determine the affinities of the domains for the peptides and the stoichiometry of the complexes.

For the short WF10p and the FW10p peptides, the fluorescence titrations with either domain were fitted well with 1:1 stoichiometry and the $K_d$ values obtained are listed in Table I. For the full-length peptides, titrations of the CD and fluorescence signals with a given domain show that the stoichiometry is clearly greater than 1 (see Fig. 2). These data were analyzed with the two binding site model described under “Materials and Methods.” Fig. 2 shows typical examples of two CD titrations (C-domain and WFFp, Fig. 2A; N-domain and WFFp, Fig. 2B) and of two fluorescence titrations at low peptide concentration (C-domain and WFFp, Fig. 2C; N-domain and WFFp, Fig. 2D). The dissociation constants obtained are illustrated in Fig. 3 and listed in Table I together with the corresponding Gibbs free energies, calculated as $RT \log K_d$.

The $K_d^{C}/K_d^{N}$ ratios obtained from the CD and the fluorescence titrations at high peptide concentrations were generally in good agreement and only the value derived from the less noisy fluorescence experiments was used. The exception was in titrations of WFFp with the C-domain; in this case $K_d^{C}/K_d^{N} = 2.5 \pm 0.5$ was obtained from the CD experiments (at [WFFp] =

![Fig. 2](image-url)
80–350 μM, Fig. 2A), whereas $K_{d}^{C}/K_{d}^{N} = 8.0 \pm 0.5$ was obtained from the fluorescence experiments (at [WFFp] = 50 μM data not shown). Using these ratios as fixed parameters in the fits to the fluorescence titrations at low peptide concentration (0.2 and 1 μM, four titrations each, see Fig. 2B) gave dissociation constants of $K_{d}^{N} = 18 \pm 8$ nM and $K_{d}^{C} = 45 \pm 25$ nM using the ratio from the CD data, and $K_{d}^{N} = 10 \pm 4$ nM and $K_{d}^{C} = 80 \pm 35$ nM using the ratio from the fluorescence data. These values may be averaged to give $K_{d}^{N} = 14$ nM with confidence limits 6 and 26 nM, and $K_{d}^{C} = 63$ nM with confidence limits 20 and 115 nM.

The independent site model used for these fits assumes that only two distinct spectroscopic species have to be accounted for. However, it seems possible from the differences in the $K_{d}^{C}/K_{d}^{N}$ ratios that the spectroscopic properties of the Trp site may be affected to a small degree by binding of a domain to the silent binding site, i.e. $S_{2} \neq S_{4}$ (Scheme 1). Simulations show the following results. (i) For the CD data, $S_{4} = 1.3 \times S_{2}$, was needed to give $K_{d}^{C}/K_{d}^{N} = 8.0 \pm 0.5$, giving slightly better fits compared with the assumed $S_{2} = S_{4}$. With these parameters, $K_{d}^{N} = 10 \pm 4$ nM and $K_{d}^{C} = 80 \pm 5$ nM were obtained with the low concentration fluorescence titrations. (ii) Alternatively, for the high concentration fluorescence data, $S_{4} = 0.8 \times S_{2}$ was needed to give $K_{d}^{C}/K_{d}^{N} = 2.5 \pm 0.5$, giving a fit only slightly worse than that with $S_{2} = S_{4}$. With these parameters, $K_{d}^{C} = 25 \pm 11$ nM and $K_{d}^{C} = 63 \pm 3$ nM were obtained with the low concentration fluorescence data. There is therefore only marginal evidence of spectroscopic interaction between the two domains, and this makes relatively small differences in the derived $K_{d}^{C}$ and $K_{d}^{N}$ values.

Far UV CD Spectra of Complexes of Calmodulin or Calmodulin Domains with Target Peptides—Secondary structure changes upon WFFp, FFFp, or FFWp binding to the domains have been monitored using far UV CD spectroscopy. The domain and the complex spectra were normalized using the protein concentration, except in the case of the domain mixture, where the concentration of one domain was used to facilitate the comparison with the spectra of calmodulin (46). The spectra of the free domains are dominated by the contributions from $\alpha$-helical structures with the characteristic minima near 207 and 222 nm. The spectrum of the 1:1 domain mixture agreed within experimental error with the corresponding spectrum of calmodulin confirming earlier results for bovine testis and brain calmodulin (24, 27) that the domain structure is preserved in the tryptic fragments. Upon addition of the (unstructured) peptide to the domains, there is an increase in $\alpha$-helicity consistent with the peptide adopting an $\alpha$-helical structure in the complex. The conformation of the calmodulin domains is largely unaffected by binding of the target sequence (18), although slight structure perturbations have been reported (47). Far UV CD was measured at different domain:peptide ratios and the change in the CD spectrum was normalized to peptide concentration. The corresponding $\Delta \delta$ values at 222 nm are collected in Table II together with the change in the number of helical residues. Calmodulin, the C-domain, and the domain mixture induce the same high degree of helicity in each peptide, whereas the N-domain is as effective only with FFFp. The induced helicity tends to be highest for peptide WFFp, decreasing with the number of replacements for FFFp and FFWp. The exception again is the N-FFFp-N complex, which shows the same induced helicity as N-WFFp-N. Table II shows that the increased helicity saturates at a 2:1 domain:peptide ratio for peptides WFFp, FFFp, or FFWp. A 1:1 domain:peptide ratio induces more than 50% of the maximum helicity, suggesting
that binding of the first molecule of either domain to the long peptides induces more helicity than binding of the second.

**DISCUSSION**

**The Number of Domain Binding Sites on the 18-residue Peptides WFFp and FFWp**—In the complex of calmodulin with the target sequence of sk-MLCK, the C-domain of calmodulin binds mainly to residues in the N-terminal portion of the target peptide and the N-domain mainly to those in the C-terminal portion (18). This work clearly shows that two molecules of either the C-domain or the N-domain bind to the 18-residue sk-MLCK target peptide WFFp and to the variants FFFp and FFWp. In agreement with this, either domain will bind with 1:1 stoichiometry to the short peptides WF10p or FW10p, which represent the N- and the C-terminal portion of the WFF and FFW target sequences, respectively.

**The Hydrophobic Pockets of the Domains**—The maximum of the enhanced fluorescence at 335–338 nm (Table I) shows that either domain binds the bulky Trp residue of the peptides, which becomes buried in a hydrophobic pocket. Generally, the enhanced fluorescence at 335–338 nm shows that binding of the first molecule of either domain to the long peptide promotes the efficient formation of the appropriate intermediate complex, en route to the correct binding site on the peptide and its target sequence. This indicates that it is an intrinsic property of the C-domain and N-domain when present together to form a complex of almost identical structure as with calmodulin.

**The Role of the Individual Domains in the Target Recognition Process**—Both C-domain and N-domain form complexes with either WFFp or FFWp with a stoichiometry of 2 mol of peptide. The N- and the C-domain bind to both sites on the peptide with surprisingly similar affinities (see Table I), with the highest affinity interactions being those between the C-domain and the Trp-containing portion of the WFFp or FFWp target sequences. Similar conclusions were reached from studies of sk-MLCK activation (31); two binding sites for calmodulin domains were found on the enzyme with dissociation constants for the C-domain of $K_D^C = 300 \text{ nM}$ and $K_D^N = 20 \mu \text{M}$ and for the N-domain of $K_D^N = 12 \mu \text{M}$ and $K_D^C = 3–5 \mu \text{M}$ (the values are presented according to the nomenclature in this paper from the tentative assignment in the original paper). The $K_D$ values obtained here with a target peptide tend to be 1–2 orders of magnitude smaller than those derived from the enzyme activation studies. This may suggest that binding of the domains to the whole enzyme may require energetically costly disruption of interactions between the calmodulin binding site and other parts of the enzyme (1, 2).

**The subtle affinity differences of the two domains for the binding sites on the peptide support the view that the key recognition process between calmodulin and the native target peptide is due to the interaction of the C-domain with the N-terminal portion of the target sequence (31, 39, 48): its preference for the “correct” binding site on the peptide and its higher affinity for this site compared with the N-domain ensure the correct orientation of the complex. Consistent with this, a 1:1 mixture of the separated N- and C-domains appears to bind to the native target sequence WFFp in the same orientation as intact calmodulin (see “Results”). The resulting C-WFFp-N complex is virtually indistinguishable spectroscopically from the calmodulin-WFFp complex, in terms of the interactions leading to the exact mode of Trp binding and helicity induced in the peptide. This indicates that it is an intrinsic property of the C-domain and N-domain when present together to form a complex of almost identical structure as with calmodulin.

Evidence has recently been presented for a potential functional difference between the two domains. At intermediate Ca$^{2+}$ concentrations ($\sim 1 \mu \text{M}$) in the presence of the target peptide, the C-domain binds Ca$^{2+}$ preferentially and is therefore able to bind to the peptide, while the N-domain remains in the apo state (39). The intrinsic preference of the C-domain for the correct binding site on the peptide promotes the efficient formation of the appropriate intermediate complex, en route to the full complex required for activating the enzyme in response to an elevated Ca$^{2+}$ signal. The mechanism outlined above, which resembles that proposed for the Ca$^{2+}$-dependent activation of troponin C with tropomyosin (49), suggests that the conservation of sequence differences in the domains of the calmodulin-WFFp-N complex is virtually indistinguishable spectroscopically from the calmodulin-WFFp complex, in terms of the interactions leading to the exact mode of Trp binding and helicity induced in the peptide. This indicates that it is an intrinsic property of the C-domain and N-domain when present together to form a complex of almost identical structure as with calmodulin.
calmodulin molecule is important in its function.  

**The Role of the Trp Residue in the Target Sequence on the Recognition Process**—The experiments with the FFWp and FFFp variant peptides show the importance of the interaction between Trp-4 of the peptide and the C-domain. Replacing Trp-4 by Phe appears to decrease the helicity in the N-terminal part of the bound peptide, and diminishes the selectivity of that peptide portion for the C-domain. With FFWp, the C-terminal binding site shows the higher affinity for both C- and N-domain. Binding of the C-domain with the C-terminal site of FFm implies a type of interaction that is not observed in the calmodulin-FFm complex, which apparently has the same peptide orientation as WFFp (48). This suggests a possible functional role of linking the two domains together in one molecule, which may ensure a defined orientation of the interaction.

**Cooperativity and the Energetics of the Domain-Peptide Interactions**—The formation of the 1:1:1 mixed complex C-WFFp-N raises the question of co-operativity in the interactions of calmodulin domains with the target sequence. The sum of the total Gibbs free energy change ($\Delta G_{\text{binding}}$) for formation of C-WFFp and WFFp-N as calculated from the values in Table I is $-82$ kJ/mol and is larger than the corresponding value, $-78.9$ kJ/mol, obtained for formation of WFFp-C and N-WFFp. This shows that the C-WFFp-N complex is energetically preferred, as deduced from the near UV CD spectra (see “Results”). Simulations using the $K_d$ values of Table I show that, under the conditions of the CD experiment with the 1:1 domain mixture, up to 34% of the complexes would involve “non-native” binding of the peptide Trp to the N-domain, exceeding the upper limit ($<15\%$) estimated from the near UV CD data. This suggests a somewhat higher degree of specificity of the individual domains in the 1:1 C:N-domain mixture for their “native” binding sites on WFFp than is predicted from the interaction of two identical domains with the peptide. Introducing in the simulation a positive cooperativity factor of $\beta = 0.5$ in forming the C-WFFp-N complex decreases the proportion of non-native complex to 11%, *i.e.* consistent with the near UV CD data. Thus, this factor of 0.5, corresponding to a change of less than 5% of the free energy change in the interaction, is sufficient to reconcile the homodomain titrations with the mixed domain near UV CD results. Thus, although the independent binding site model is generally adequate for the homodomain case, there is some evidence for a small degree of cooperativity between the complex of WFFp with the mixed N- and C-domains, and it is likely that this would be further enhanced in the interaction of calmodulin itself with the target sequence.

The estimated Gibbs free energy changes for formation of C-WFFp-N ($-82$ kJ/mol), C-WFFp-C ($-84.6$ kJ/mol), and N-WFFp-N ($-76.2$ kJ/mol) are significantly larger than that for WFFp binding to intact calmodulin ($-66.5$ kJ/mol).3 These values may be compared with data of Persichini et al. (31) for the activation of the sk-MLCK enzyme by calmodulin fragments. They derived a value of the ratio of $K_C/K_D/K_{\text{Cam}} = 0.9$ nM and equate this with an effective N-domain concentration for binding in the calmodulin-enzyme complex where the C-domain is already bound. ($K_C$ and $K_D$ are the dissociation constants of the isolated C-domain and N-domain, respectively, from their native sites on the target sequence; $K_{\text{Cam}}$ is the dissociation constant for calmodulin.) The values obtained from the present studies of domain binding to the sk-MLCK target peptide correspond to values of 0.9–1.8 nM, the higher value allowing for the inclusion of a small degree of cooperativity in domain binding. Given the completely different types of exper-

---

3 S. R. Martin, unpublished data.
Calmodulin Domain Interaction with sk-MLCK Target Sequences

Fig. 4. Structure of the calmodulin MLCK-peptide complex and schematic models of possible peptide complexes with isolated domains. A, structural representation of the calmodulin MLCK-peptide complexes (18) (data are taken from Protein Data Bank file 1CDL.pdb using Rasmol). Red, target peptide, with polarity indicated by position of Trp-4 and the N and C termini. I, II, III, and IV refer to Ca\(^{2+}\) ions (green) in the four EF-hands (turquoise). Helices (eight) are shown in purple, and odd-numbered ones H1, H3, H5, H7 are identified for clarity; loops joining two EF-hands within a domain are yellow, and the interdomain loop is orange. The position of the approximately two-fold rotational symmetry axis perpendicular to the paper and relating corresponding structural features of the N-domain (top right) and C-domain (bottom left) is indicated with +, B and C, illustrative schematic models for the 2:1 C-domain-peptide complex. The domains are shown with two representative binding pockets. The domains binding to the native and non-native sites on the peptide are shown as red and green, respectively. B, orientation of the domains with rotational symmetry, as observed (approximately) for the C- and the N-domain in the complexes with intact calmodulin (see A). C, the red domain binding to its native site on the peptide does so in the same orientation as in the complex with calmodulin, whereas the green domain at the non-native site is reversed relative to B, and the relative orientation of the domains shows translation + rotation (i.e. helical) symmetry.

(2), although binding of melittin to calmodulin is known to occur with reversed polarity.\(^4\) An analogous rotational relationship occurs in the complex of the regulatory light chain with myosin, where the EF-hands occur in the sequence III, IV, I, II (52, 53). The principle of binding of peptides with either polarity is also consistent with the lesser importance of interactions with the peptide backbone as compared with those with the peptide side chains (17), the binding of calmodulin to a peptide composed of D-amino acids (51), and the calmodulin-mediated activation of sm-MLCK containing a reversed calmodulin binding sequence (50).

Fig. 4 (B and C) shows that these considerations lead to some predictions about the relationship between target peptide sequence and the symmetry of the interactions. If a target sequence contains a palindromic feature (e.g. X-Y-Z ... Z'-Y'-X'), where the prime indicates at least a conservative homology between X and X' etc., if not a direct identity X = X'), then the calmodulin domains would be most likely to adopt a two-fold rotational relationship. In fact, there is something of a palindromic relationship between residues 2 and 8 of M13 (R-R-W-K-K-N-F) and residues 13–19 (A-A-N-R-F-K-K), where bold type indicates the two key hydrophobic residues Trp-4 and Phe-17. By contrast, if a target sequence shows a repeating feature within a linear structure (e.g. X-Y-Z ... X'-Y'-Z'), then the domains would be most likely to adopt a helical (translational + rotational) relationship. This does not appear to have been seen for calmodulin; however, it is intriguing that there appears to be a double (or triple) repeated feature in the sequence 96–148 of skeletal and cardiac TnI apparently respon-

\(^4\) K. Johnson, K. Beckingham, and F. Quiocho, personal communication.
Acknowledgments—We thank Kate Beckingham for advice on expression systems, S. Howell for the mass spectra, P. Fletcher for synthesis of some peptides, and P. Browne for valuable discussion. We thank Dr Tony Wilkinson (University of York) for demonstration of the symmetry of the calmodulin-MLCK peptide complexes.

REFERENCES

1. Vogel, H. J. (1994) Biochem. Cell Biol. 72, 357–376
2. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Struct. 24, 85–116
3. Klee, C. B. (1985) in Calmodulin (Cohen, P., and Klee, C. B., eds) pp. 35–56, Elsevier, Amsterdam
4. O’Neill, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 647–650
5. Persechini, A., Kretsinger, R. H., and Davis, T. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1084–1088
6. Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O., and Kretsinger, R. H. (1989) J. Biol. Chem. 264, 8052–8058
7. Persechini, A., Kretsinger, R. H. (1988) J. Cardiovasc. Pharmacol. 12, 61–66
8. Bayley, P., Martin, S. R., and Bayley, P. M. (1992) Biochemistry 31, 3452–3462
9. Bayley, P. M., and Martin, S. R. (1992) Biochim. Biophys. Acta 1160, 16–21
10. Barbatto, G., Ikura, M., Kay, L. E., Pastor, R. W., and Bax, A. (1992) Biochemistry 31, 5269–5278
11. Heidorn, D. B., and Trewella, J. (1988) Biochemistry 27, 909–915
12. Persechini, A., and Kretsinger, R. H. (1988) J. Cardiovasc. Pharmacol. 12, Suppl. 5, S1–S12
13. Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O., and Kretsinger, R. H. (1988) J. Biol. Chem. 264, 8052–8058
14. Persechini, A., Kretsinger, R. H. (1988) J. Cardiovasc. Pharmacol. 12, 61–66
15. Persechini, A., Kretsinger, R. H. (1988) J. Biol. Chem. 264, 8052–8058
16. Houdusse, A., and Cohen, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10644–10647
17. Afshar, M., Caves, L. S. D., Guimard, L., Hubbard, R. E., Calas, B., Grassly, G., and Haiech, J. (1994) J. Mol. Biol. 244, 554–571
18. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632–638
19. Meador, W. E., Meador, W. E., and Quiocho, F. A. (1991) Science 257, 1251–1255
20. Drabikowski, W., Kuznicki, J., and Grabarek, Z. (1977) Biochim. Biophys. Acta 485, 124–133
21. Walsh, M., Stevens, F. C., Kuznicki, J., and Drabikowski, W. (1977) J. Biol. Chem. 252, 7440–7443
22. Mizowiak, O., and Yagi, K. (1984) J. Biochem. (Tokyo) 96, 1175–1182
23. Linse, S., Helmersson, A., and Forsen, S. (1991) J. Biol. Chem. 266, 8050–8054
24. Martin, S. R., and Bayley, P. M. (1988) Biochem. J. 238, 485–490
25. Martin, S. R., Anderson Telemann, A., Bayley, P. M., Drakenberg, T., and Forsen, S. (1985) Eur. J. Biochem. 151, 543–550
26. Sato, J., Furukawa, Y., and Ogasawara, S. (1985) J. Biochem. 97, 224–228
27. Drabikowski, W., Brzeska, H., and Venyaminov, N. Yu. (1982) J. Biol. Chem. 257, 11584–11590
28. Newton, D., Klee, C., Woodgett, J., and Cohen, P. (1985) Biochim. Biophys. Acta 845, 533–539
29. Wolff, J., Newton, D. L., and Klee, C. B. (1986) Biochemistry 25, 7950–7955
30. Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., and Cohen, P. (1986) FEBS Lett. 130, 141–145
31. Persechini, A., McMillan, K., and Leakey, P. (1994) J. Biol. Chem. 269, 16148–16154
32. Guerini, D., Krebs, J., and Carafoli, E. (1984) J. Biol. Chem. 259, 15172–15177
33. Klee, C. B., and Haiech, J. (1994) Biochemistry 33, 182–191
34. Medvedeva, M. V., Kolobova, E. A., Wang, P., and Gusev, N. B. (1996) Biochem. J. 315, 1021–1026
35. Newton, D. L., Oldewurtel, M. D., Krints, M. H., Shiloach, J., and Klee, C. B. (1984) J. Biol. Chem. 259, 4419–4426
36. Persechini, A., Ganz, K. J., and Paresi, R. J. (1996) Biochemistry 35, 224–228
37. Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C. B., and Bax, A. (1995) Nat. Struct. Biol. 2, 768–776
38. Klee, C. B., and Vanaman, T. C. (1982) J. Biol. Chem. 257, 1215–1228
39. Bayley, P. M., Findlay, W. A., and Martin, S. R. (1996) Protein Sci. 5, 2125–2128
40. Steiner, R. F., Marshall, L., and Needleman, D. (1986) Arch. Biochem. Biophys. 246, 286–300
41. Sanyal, G., Richard, L. M., Carraway, K. L., and Puett, D. (1988) Biochemistry 27, 6229–6236
42. Ikura, M., and Ito, T. (1992) J. Biochem. (Tokyo) 112, 183–191
43. Maune, J. F., Klee, C. B., and Beckingham, K. (1992) J. Biol. Chem. 267, 5286–5290
44. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
45. Scholtz, J. M., Qian, H., York, E. J., Stewart, J. M., and Baldwin, R. L. (1991) Biopolymers 31, 1443–1470
46. Findlay, W. A., Martin, S. R., Beckingham, K., and Bayley, P. M. (1995) Biochemistry 34, 2087–2094
47. Zhang, M., Fabian, H., Mantese, H. H., and Vogel, H. J. (1994) Biochemistry 33, 10838–10888
48. Findlay, W. A., Gradwell, M. J., and Bayley, P. M. (1995) Protein Sci. 4, 2375–2382
49. Potter, J. D., and Johnson, J. D. (1981) in Calcium and Cell Function (Cheung, W. Y., ed) Vol. 2, pp. 145–173, Academic Press, New York
50. Shoemaker, M., Lau, W., Shattuck, R., Kwiatkowski, A., Matrisian, P., Guerra-Santos, L., Wilson, E., Lukas, T., Eldik, L. V., and Watterson, D. (1990) J. Cell Biol. 111, 1107–1125
51. Fisher, P. J., Prendergast, F. G., Ehnhardt, M. R., Urbauer, J. L., Wand, A. J., Sedarous, S. S., McCormick, D. J., and Buckley, P. J. (1994) Nature 368, 653–657
52. Afshar, M. (1995) Interactions of Calmodulin with Targets. Ph.D. dissertation, Université de Montpellier I, Faculté de Médecine, Montpellier, France
53. Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalakokis, V. N., Szent-Györgyi, A. G., and Cohen, C. (1994) Nature 368, 306–312
54. Pearlstone J. R., Sykes, B. D., and Smillie, L. B. (1997) Biochemistry 36, 7601–7606