Fluorescence Energy Transfer Analysis of Calmodulin·Peptide Complexes†

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ABSTRACT: The interactions between calmodulin and the tryptophan residues of synthetic peptides corresponding to the calmodulin binding domains of skeletal muscle myosin light-chain kinase and the plasma membrane calcium pump were examined. The single tryptophan residue contained in each peptide became relatively immobilized and inaccessible to iodide ion upon binding to calmodulin, indicating that the indole side chain was inserted into a hydrophobic cleft in the surface of calmodulin. Fluorescence energy transfer from peptidyl tryptophan residues to an AEDANS moiety attached to cysteine-26 of spinach calmodulin was measured. Included in these analyses was a tryptophan-containing peptide analog of the calmodulin binding domain of neuromodulin. These data indicated that the indole ring of each peptide inserted 32–35 Å away from cysteine-26 and may therefore interact with the carboxyl-terminal lobe of CaM in its "bent" conformation [Persechini & Kretsinger (1988a) *J. Cardiovasc. Pharmacol. 12* (Suppl 5), S1–S12; Ikura et al. (1992) *Science 256*, 632–638; Vorherr et al. (1992) *Eur. J. Biochem. 204*, 931–937]. The interchange of tryptophan-3 and phenylalanine-21 of the calcium pump peptide increased the efficiency of energy transfer to the AEDANS-moiety approximately 12-fold, reducing the calculated distance to 20 Å. These data suggest that phenylalanine-21 of the calcium pump peptide interacts with the hydrophobic cleft in the amino-terminal lobe of CaM.

Calmodulin (CaM)[1] is expressed in all eukaryotic cells and serves as a major intracellular receptor for calcium. Although only 16 700 daltons, calcium·CaM is able to bind to and activate a wide range of target enzymes including adenylyl cyclases, phosphodiesterases, kinases, and ion pumps [reviewed in Klee and Vanaman (1982), Cohen and Klee (1988), and Means (1988)]. These numerous interactions provide an interesting example of protein·protein recognition and may provide a useful model system to study the interactions of proteins.

The primary structure of CaM is remarkably conserved throughout phylogeny (Toda et al., 1985). This conservation is reflected by the ability of plant CaM to activate mammalian enzymes including the plasma membrane calcium pump (Strasburg et al., 1988) and skeletal muscle myosin light-chain kinase (skMLCK) (Zot et al., 1990). According to X-ray crystallography studies, CaM is shaped like a dumbbell with an amino-terminal lobe containing two EF-hand motifs comprising calcium binding domains I and II and a carboxyl-terminal lobe containing two additional EF-hands comprising calcium binding domains III and IV (Babu et al., 1985, 1988). The dimensions of each lobe are approximately 25 × 20 × 20 Å. These domains are joined by a long apparently flexible α-helix (Persechini & Kretsinger, 1988a; 1989b; Heidorn et al., 1991; Kataoka et al., 1991; Ikura et al., 1991, 1992). Upon binding of calcium, hydrophobic surfaces are exposed on each lobe of CaM which are thought to constitute important determinants for the high-affinity interaction of CaM with other molecules (LaPorte et al., 1980; Krebs et al., 1984; Johnson et al., 1986; Babu et al., 1988).

Peptides which bind to CaM with high affinity are generally able to form amphiphilic α-helices with hydrophobic and positively charged residues on opposite faces [reviewed by O'Neil and DeGrado (1990)]. Fluorescence (O'Neil et al., 1987) and circular dichroism spectroscopy (Cox et al., 1985; Klevit et al., 1985; McDowell et al., 1985; Vorherr et al., 1990) studies of a variety of CaM binding peptides have been consistent with the formation of α-helices when complexed with CaM. Finally, NMR spectroscopy studies of melittin and the CaM binding domain of smooth muscle myosin light-chain kinase (smMLCK) have confirmed that these peptides form α-helices when bound to CaM (Seeholzer et al., 1986; Roth et al., 1991; Ikura et al., 1992). These data suggest that CaM binding domains form a characteristic hydrophobic surface which may complement the calcium induced hydrophobic surfaces on CaM.

The CaM binding domains of many proteins including myosin light-chain kinase, the plasma membrane calcium pump, the α-subunit of phosphorylase kinase, and the *Bordetella pertussis* adenylyl cyclase contain a tryptophan residue near their amino termini [reviewed by O'Neil and DeGrado (1990) and James et al. (1991)]. Several studies have shown that replacement of this residue can dramatically reduce the affinity of synthetic peptides and target proteins for CaM, indicating that this residue is an important determinant for binding (O'Neil et al., 1987; Glaser et al.,

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[1] Abbreviations: 1,5-IAEDANS,5-[[[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; DTT, dithiothreitol; CaM, calmodulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NMR, nuclear magnetic resonance; Phe, phenylalanine; skMLCK, skeletal muscle myosin light-chain kinase; smMLCK, smooth muscle myosin light-chain kinase; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; Trp, tryptophan. Peptides: FP57-Trp, CaM binding domain peptide from bovine neuromodulin modified to contain a tryptophan residue in place of the native phenylalanine; M5, the calmodulin binding domain from rabbit skeletal muscle myosin light-chain kinase; C24W, the calmodulin binding domain from the human erythrocyte plasma membrane calcium pump; C24F, same as C24W except with residues Trp-4 and Phe-21 interchanged.

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Table I

skeletal muscle myosin light-chain kinase	M5[a]	KRRWKKNFIAVSAANRFG
plasma membrane calcium pump	C24W[b]	QILWFRGLNRIQTQIRVVNAFRSS
	C24F	QILFFRGLNRIQTQIRVVNAWRSS
neuromodulin	FP57-Trp[c]	QASWRGHITRKKLKGEK

[a] Kennelly et al. (1987). [b] Kataoka et al. (1991). [c] Alexander et al. (1988).

1989; Vorherr et al., 1990).

In this study we have characterized the interaction of the Trp residue of synthetic peptides corresponding to the CaM binding domains of the plasma membrane calcium pump and skMLCK. Because little is known about the structural determinants of CaM which interact with target peptides, the major goal of this study was to define the region of CaM which interacts with these Trp residues. For comparison, we also mapped the insertion site of the Trp of an analog of the CaM binding domain of the neural-specific growth cone protein neuromodulin [also designated GAP-43, B-50, F1; reviewed by Skene (1989)]. Unlike most classical CaM binding proteins, neuromodulin binds to CaM with equal or greater affinity in the absence of free calcium (Andreasen et al., 1983; Alexander et al., 1987). Therefore, its CaM binding domain may interact with CaM in a distinct manner.

EXPERIMENTAL PROCEDURES

Materials. AEDANS was purchased from Molecular Probes (Eugene, OR).

Calmodulin and Peptides. Spinach CaM was purchased from Sigma Chemical Co. (St. Louis, MO), and bovine brain CaM was purified by the method of Masure et al. (1986). Preparation and purification of synthetic peptides corresponding to the CaM binding domains of neuromodulin (designated FP57-Trp), skMLCK (designated M5), and the erythrocyte plasma membrane calcium pump (designated C24W) have been described previously (Alexander et al., 1988; Kennelly et al., 1987; Kataoka et al., 1991). The C24W analog, C24F, was synthesized according to Kataoka et al. (1991) and purified by reverse-phase HPLC. Peptide concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 5600 M^{-1} cm^{-1}. A summary of these peptides is given in Table I.

We found that C24W, and to a greater extent C24F, had a tendency to precipitate in the buffer of 10 mM MOPS, pH 7.4, 1 mM $CaCl_2$, and 150 or 500 mM KCl typically used in our experiments. Therefore, all experiments with these peptides were also carried out in deionized distilled water plus 1 mM $CaCl_2$. In general, the only parameter which appeared to be significantly affected was the fluorescence anisotropy of the peptides free in solution. In addition, because the interaction of FP57-Trp with CaM is highly sensitive to ionic strength (Chapman et al., 1991), the energy transfer measurements with this peptide were made in the absence of KCl to ensure stoichiometric complex formation.

Fluorescent Labeling of Calmodulin. Spinach CaM (19 nmol) was dissolved in 250 μL of 6 N guanidine, 200 mM Tris, pH 8.0, and 10 mM EDTA and reduced with 2.5 μmol of DTT for 2 h at 37 °C. The sample was cooled to room temperature and 10 μmol of 1,5-IAEDANS was added. The sample was incubated at room temperature for 1.5 h, and then the labeling reaction was terminated by the addition of 100 μL of β-mercaptoethanol. Free label was removed by gel filtration on a 5-mL Pierce excellulose GF-5 size-exclusion column equilibrated with 6 N guanidine and 1 mM EGTA. The AEDANS–CaM eluate was dialyzed exhaustively against 5 mM MOPS, pH 7.4. The protein concentration was determined using the Pierce BCA (bicinchoninic acid) protein assay reagents with bovine serum albumin as a standard. The concentration of the fluorescent label was determined by measuring the absorbance of the AEDANS at 337 nm using an extinction coefficient of 6300 M^{-1} cm^{-1} and an equal concentration of unlabeled CaM as a blank. Labeling stoichiometries were 0.85–0.95 mol of AEDANS/mol of CaM.

Fluorescence Spectroscopy Instrumentation. All fluorescence anisotropy measurements as well as spectra for the overlap integrals were made using an SLM-4800S spectrofluorometer assembled in the T-format and interfaced with a Hewlett-Packard 9815A microprocessor. Routine fluorescence spectra were obtained using a Shimadzu RF5000 spectrofluorometer. Spectra for quantum yield determinations were obtained using an SLM-8000 spectrofluorometer. Sample temperatures were maintained at 25 °C.

KCl Dissociation of Peptide·CaM Complexes. The fluorescence anisotropy of 5 μM solutions of CaM binding domain peptides in 10 mM MOPS, pH 7.4, 150 mM KCl, and 1 mM $CaCl_2$, or deionized water plus 1 mM $CaCl_2$, in the presence and absence of 7 μM bovine brain CaM was measured as a function of increasing KCl. Samples were excited at 295 nm, incident light was resolved through a 2-nm slit, and emitted light was filtered through WG 335 filters. The fraction of peptide bound was determined as described previously (Alexander et al., 1988).

Iodide Quenching. The emission spectra of 5 μM solutions of CaM binding domain peptides in 10 mM MOPS, pH 7.4, 500 mM KCl, 1 mM $Na_2S_2O_3$, and 1 mM $CaCl_2$, or deionized water plus 1 mM $Na_2S_2O_3$ and 1 mM $CaCl_2$, in the presence and absence of 7 μM bovine brain CaM were obtained as a function of increased KI. Samples were excited at 295 nm with 1.5-nm resolution, and the emission spectra were corrected for blank and dilution and integrated from 310 to 460 nm, yielding the fluorescence intensity (F). These values were divided into the initial fluorescence intensity value obtained in the absence of added KI (F_0) and plotted as a function of the final iodide ion concentration.

Distance Measurements Using Fluorescence Energy Transfer. Förster energy transfer has been shown to be useful to measure distances in the 10–60-Å range and has been thoroughly reviewed by Fairclough and Cantor (1978), Stryer (1978), and Lakowicz (1983). The efficiency, E, of energy transfer from donor to acceptor is defined by

$$E = 1 - F_{DA}/F_D \quad (1)$$

where F_{DA} and F_D are the fluorescence intensity of the donor in the presence and absence of the acceptor, respectively. From E, the distance, r, between the donor and acceptor can be calculated:

$$E = R_0^6/(r^6 + R_0^6) \quad (2)$$

R_0 is the distance at which $E = 0.5$ and is defined, in

centimeters, by

$$R_0^6 = (8.79 \times 10^{-25}) n^{-4} K^2 \phi_D J \quad (3)$$

where n is the refractive index of the medium, taken to be 1.4, K^2 is the orientation factor and will be described in more detail in Results, J is the spectral overlap integral (in M^{-1} cm^3) between the emission spectra of the donor, and $F_D(\lambda)$, and the absorption spectra of the acceptor, $\epsilon_A(\lambda)$, and is defined by

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda / \int F_D(\lambda) \, d\lambda \quad (4)$$

J was determined by the numeric integration of the emission spectra of the M5·CaM complex and the absorption spectra of AEDANS–CaM using Excel on a Macintosh computer. ϕ_D is the quantum yield of the donor in the absence of the acceptor and was determined using (Epps et al., 1987)

$$\phi_D = \phi_{RF}(I_D/I_{RF})(A_{RF}/A_D) \quad (5)$$

where I_D and I_{RF} are the respective fluorescence intensities of the donor and a reference compound and A_{RF} and A_D are the respective absorbances of the reference compound and donor. ϕ_{RF} is the quantum yield of the reference compound L-tryptophan and was taken to be 0.14 (Chabbert et al., 1991).

RESULTS

Insertion of CaM Binding Domain Peptide Trp Residues into CaM. The sequences of the peptides utilized in this study are given in Table I. The peptides C24W and M5 correspond to the CaM binding domains of the plasma membrane calcium pump and skMLCK, respectively. FP57-Trp is an analog of the calmodulin binding domain of neuromodulin (also designated GAP-43, B50, or F1) in which the native phenylalanine has been replaced with a Trp residue. The emission spectra of the Trp residues of M5, C24W, and FP57-Trp exhibited pronounced blue shifts as well as increases in their fluorescence intensity upon binding to calcium·CaM, consistent with their transfer to an apolar environment (Alexander et al., 1988; Garone & Steiner, 1990; data not shown). We also found that the indole side chains of M5 and C24W became inaccessible to iodide ion (Figure 1) and yielded marked increases in fluorescence anisotropy when saturated with either bovine or spinach CaM (Table II). Iodide quenching and fluorescence anisotropy measurements of FP57-Trp·CaM yielded similar results (Chapman et al., 1991; Table II). These data strongly indicate that the indole ring of each of these peptides becomes inserted into a hydrophobic cleft in CaM. We also found, in experiments carried out to control for the effects of ionic strength in the iodide quenching studies, that C24W and M5·CaM complexes remained stable at KCl concentrations up to 2 M (data not shown). These findings are consistent with the proposal that hydrophobic interactions constitute the major component of the total CaM·peptide binding energy. In contrast, the CaM binding domain analog of neuromodulin, FP57-Trp, was readily dissociated from CaM by submolar concentrations of KCl (Chapman et al., 1991). This finding, and the paucity of hydrophobic residues within this domain, indicate that the interaction of FP57-Trp with CaM has a higher degree of ionic character.

Interestingly, the interchange of Trp-4 and Phe-21 of C24W, resulting in C24F (Table I), did not dramatically change the iodide quenching properties (Figure 1) or quantum yields of the tryptophanyl fluorescence in the peptide·CaM complex (Table II). In addition, the tryptophanyl emission spectra and steady-state fluorescence anisotropy of C24W and C24F

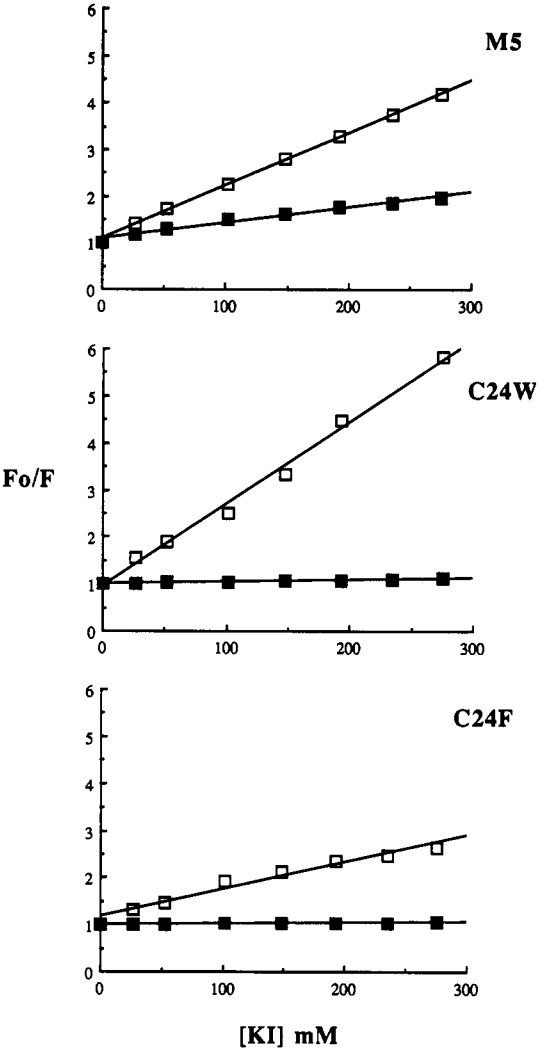

FIGURE 1: Iodide quenching of the tryptophanyl fluorescence of calmodulin binding domain peptides in the presence and absence of calmodulin. The emission spectra of peptides (5 μM) in the presence (closed squares) or absence (open squares) of CaM (7.5 μM), in 10 mM MOPS, 500 mM KCl, and 1 mM $CaCl_2$ (for M5) or unbuffered 1 mM $CaCl_2$ (for C24W and C24F) was measured 10 min after each addition of 500 mM KI, 1 mM $CaCl_2$, and 1 mM $Na_2S_2O_3$. Samples were excited at 295 nm with 1.5-nm resolution. The emission spectra were corrected for blank and dilution and integrated from 310 to 460 nm. The initial fluorescence intensity (F_0), divided by the fluorescence intensity after each addition of KI (F), is plotted as a function of the final KI concentration.

bound to CaM were almost identical (data not shown, Table II). These data suggest that the native Phe-21 of C24W may also interact with a hydrophobic cleft in CaM.

Localization of the Trp Insertion Sites Using Förster Energy Transfer. To localize the region on CaM in which the peptide indole side chains are inserted, we measured fluorescence energy transfer from the Trp residues of each peptide to an acceptor species, AEDANS, covalently attached to CaM. These experiments were facilitated by the use of spinach CaM which contains a single cysteine residue that can be readily covalently labeled with reporter molecules (Toda et al., 1985; Mills et al., 1988). Strasburg et al. (1988) have demonstrated that this cysteine residue can be specifically labeled with 1,5-IAEDANS in the presence of 6 N guanidine. Furthermore, the position of this cysteine, residue 26 of spinach CaM (Lukas et al., 1984; Toda et al., 1988), is conveniently located near the extreme amino-terminal end of the three-dimensional structure of the molecule (Babu et al., 1985, 1988; Figure 3).

Table II [a]

fluorophore	A	A_l	E(%)	ϕ_D	R_0 (Å)	r (Å)
M5	0.030	0.065				
+ CaM	0.14 (0.13)	0.19	16	0.21	24	32
FP57-Trp	0.035					
+ CaM	0.11 (0.12)	0.13	6.5	0.11	22	34
C24W	0.032					
+ CaM	0.15 (0.15)	0.17	4.6	0.074	21	35
C24F	0.040					
+ CaM	0.15 (0.15)	0.16	54	0.090	21	20
AEDANS-CaM	0.035	0.14				

[a] A and A_l represent anisotropy and limiting anisotropy values obtained at 25 °C for an excitation wavelength of 295 nm; emitted light was filtered with Schott WG335 filters. E is the efficiency of energy transfer from the peptide Trp to the AEDANS moiety attached to Cys-26 of CaM. ϕ_D is the quantum yield of the donor, corresponding to the Trp of the peptide·CaM complex. R_0 is the Förster critical distance at which $E = 0.5$; see text. $J = 6.7 \times 10^{-15}$ cm^3 M^{-1}, $n = 1.4$, $K^2 = {}^2/_3$. r is the calculated distance between the donor and acceptor. Parentheses denote results from peptides complexed with bovine brain calmodulin; all other measurements were made using spinach calmodulin.

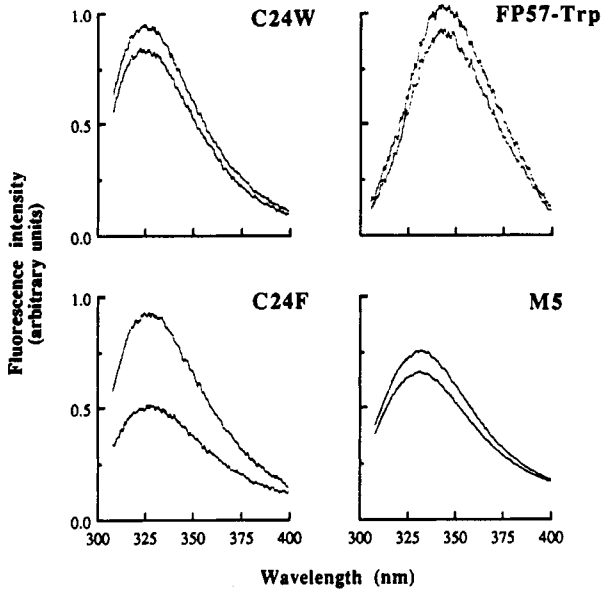

FIGURE 2: Fluorescence energy transfer from the tryptophan residues of calmodulin binding domain peptides to AEDANS-calmodulin. Peptides (1 μM) in 10 mM MOPS, 150 mM KCl, and 1 mM $CaCl_2$ (for M5) or unbuffered 1 mM $CaCl_2$ (for FP57-Trp, C24W, and C24F), in the presence of 1.2 μM CaM or AEDANS-CaM, were excited at 295 nm with 1.5-nm resolution. The total volume of each sample was 375 μL. From the emission spectra, appropriate blanks were subtracted. Shown are representative examples of each spectrum. In each plot, the upper and lower traces correspond to the emission spectra in the absence and presence, respectively, of the AEDANS acceptor.

To ensure that the peptides (1 μM) were saturated with AEDANS-calmodulin (1.2 μM) in the energy transfer experiments, we assayed for free peptide by monitoring the iodide accessibility and fluorescence anisotropy of each peptide's Trp residue and did not detect free peptide (data not shown). Representative spectra from the energy transfer experiments are given in Figure 2. From these spectra, the efficiencies of transfer were determined and are listed, in addition to all of the other distance measurement parameters, in Table II. Table II also includes the limiting anisotropy of each donor as well as the AEDANS acceptor. These data were used to assign an error to K^2 as discussed in more detail below. For the present analysis K^2 was taken as ${}^2/_3$, the assumed value for a donor/acceptor pair which occupy all orientations during the lifetime of the donor. The overlap integral, 6.7×10^{-15} M^{-1} cm^3, was determined for the M5·CaM complex, and due to the similarity of the emission spectra of each of the peptide·CaM complexes, this value was used to calculate all distances. The approximate Förster critical distance (R_0) for a Trp/AEDANS donor/acceptor pair has been reported to be 22 Å (Fairclough & Cantor, 1978), in reasonable agreement with our values, which ranged from 21 to 24 Å (Table II). The calculated distances between the Trp and the AEDANS were 32, 34, and 35 Å for M5, FP57-Trp, and C24W, respectively. The interchange of Trp-4 and Phe-21 in C24F reduced the distance between the Trp and the AEDANS group to 20 Å (Table II).

According to the crystal structure of CaM, the overall length of the molecule is 65 Å (Babu et al., 1986, 1988). However, small-angle X-ray scattering studies have indicated that the maximum dimensions of the M5·CaM and the C24W·CaM complexes, in solution, are 49 and 52.5 Å, respectively (Heidorn et al., 1991; Kataoka et al., 1991). On the basis of modeling studies with the calmodulin binding domain of skMLCK, Persechini and Kretsinger (1988a) have proposed that there is a bend in the central helix of CaM which would reorient the two hydrophobic patches in each lobe for interactions with the hydrophobic face of target peptides. This notion is supported by dynamic simulations of calmodulin in a bent conformation modeled on the basis of the troponin 3-D template (Vorherr et al., 1992). Such a bent conformation would explain the disparity between the crystal and solution structure of CaM. Multidimensional NMR spectroscopy studies have, in fact, recently demonstrated that marked conformational changes occur in the central helix of chicken and *Drosophila* CaM when complexed with peptides corresponding to the CaM binding domains of smMLCK and skMLCK, respectively (Roth et al., 1992; Ikura et al., 1991, 1992).

Considering the overall length of the CaM·peptide complex determined by the small-angle X-ray scattering studies, our distance measurements suggest that the indole rings of M5 and C24W are inserted into the carboxyl-terminal lobe of CaM in its proposed bent conformation (Figure 3; Vorherr et al., 1992). The overall length of the FP57-Trp·CaM complex has not been determined. However, the 34-Å separation between its Trp residue and the AEDANS moiety strongly argues against the association of the indole ring within the amino-terminal lobe of CaM. Finally, our results indicate that the indole side chain of C24F becomes inserted within the amino-terminal lobe of CaM (Figure 3). Therefore, Trp-4 and the native Phe-21 of the calmodulin binding domain of the calcium pump may simultaneously interact with the hydrophobic clefts in the carboxyl- and amino-terminal domains of CaM, respectively. Utilizing amino- and carboxyl-terminal tryptic fragments of CaM, Yazawa et al. (1991) also reported that the calmodulin binding domain of the calcium pump can "bridge" the two lobes of CaM.

Orientation Factor Error Analysis. A major source of error in fluorescence energy transfer distance determinations is due to the inability to directly measure K^2. However, from the relative mobility of the donor and the acceptor, the magnitude of the error introduced by the orientation factor can be assessed. To address this issue we carried out Perrin analysis as described by LaPorte et al. (1981) (data not shown) and determined the limiting anisotropy of the acceptor and each of the donors (Table II). From the limiting anisotropy measurements, the corresponding polarizations were calculated (Lakowicz, 1983) and ranged from 0.18 to 0.26. According to Haas et al. (1978),

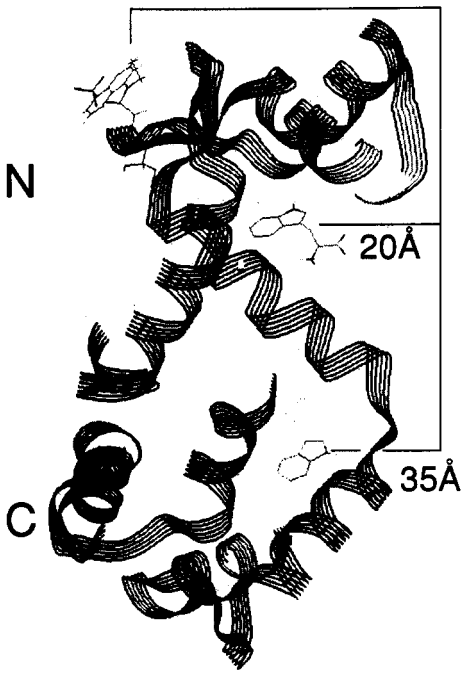

FIGURE 3: Application of distance measurements to the three-dimensional structure of CaM. CaM is modeled in its proposed bent conformation (Vorherr et al., 1992). Added is the AEDANS acceptor, attached to cysteine-26. Also included are Trp residues 20 and 35 Å from the AEDANS. These distances were determined from energy transfer measurements using C24F and C24W, respectively, as described in Experimental Procedures.

if the limiting polarization of the donor and acceptor are <0.3, the error in the distance calculated with $K^2 = {}^2/_3$ is unlikely to exceed ±10%. Using the contour plots generated by Dale et al. (1979), the limiting anisotropy values were also used to estimate the extreme maximum and minimum values of K^2 for each peptide·CaM complex. From this analysis the resulting ranges in the separations between the Trp residues and the AEDANS moiety were 28–42, 28–40, and 27–38 Å for C24W, M5, and FP57-Trp, respectively. The range of distances for C24F, 18–25 Å, do not overlap with the ranges for the other peptides, strongly suggesting that distinct nonoverlapping regions of CaM interact with the Trp residues of C24W and C24F.

DISCUSSION

CaM has the interesting property of being able to bind with high affinity, in a calcium-dependent manner, to a large number of nonhomologous target proteins. Recently, the CaM binding domains of a number of proteins have been identified and a general motif for the binding interaction has been proposed. These CaM binding domains are generally short (approximately 20 residues) linear sequences with the common ability to form amphiphilic basic helices [reviewed by O'Neil and DeGrado (1990)]. In general, the binding energy is thought to be largely derived from hydrophobic interactions (LaPorte et al., 1980; O'Neil & DeGrado, 1990). A Trp or Phe residue, conserved in the CaM binding domains in a number of CaM binding proteins, has been implicated as an important determinant for the binding interaction. We therefore studied the interaction of CaM with the Trp residue in the synthetic peptides M5 and C24W/C24F, corresponding to the CaM binding domains from skMLCK and the plasma membrane calcium pump (Table I). When bound to CaM, the indole side chains of each of these peptides became inaccessible to iodide ion, yielded marked increases in fluorescence anisotropy and displayed blue shifts as well as increases in their emission spectra. These data indicate that the indole side chain of each peptide is inserted into a hydrophobic cleft in CaM.

In order to determine and compare the region(s) of CaM which interacts with the indole side chains of these peptides, we applied fluorescence energy transfer measurements to localize the Trp insertion site(s). Included in these analyses was a Trp-containing analog of the CaM binding domain of neuromodulin (designated FP57-Trp; Table I). These measurements indicated the indole rings of M5, FP57-Trp, and C24W were inserted 32, 34, and 35 Å, respectively, from an AEDANS attached to cysteine-26 of spinach CaM, which lies near the amino-terminal end of the three-dimensional structure of the molecule. Small-angle X-ray scattering studies have indicated that the maximum dimensions of the M5·CaM and the C24W·CaM complexes are 49 and 52.5 Å, respectively (Heidorn et al., 1991; Kataoka et al., 1991). These data, coupled with modeling and NMR spectroscopy studies of CaM (Vorherr et al., 1992; Ikura et al., 1992) described in more detail below and in Figure 3, suggest the indole side chains insert into a common hydrophobic pocket within the carboxyl-terminal lobe of CaM.

We studied the orientation of peptide binding using an analog of C24W, termed C24F, in which Trp-4 and Phe-21 were interchanged. The fluorescence properties of the Trp residues of C24W and C24F, when bound to CaM, were remarkably similar in terms of their emission spectra and quantum yields. As described above, the indole side chains of both peptides appeared to be buried into CaM as evidenced by the Stern–Volmer plots and the marked increases in fluorescence anisotropy of the peptides' Trp residues when complexed with CaM. However, the efficiency of energy transfer from the tryptophan residue of C24F to the AEDANS moiety attached to cysteine-26 of spinach CaM was approximately 12-fold greater as compared to C24W. This increase in efficiency decreased the calculated separation between the Trp residue and the AEDANS-moiety to 20 Å. These measurements indicate that while Trp-4 of C24W is inserted into a hydrophobic pocket within the carboxyl-terminal lobe of CaM, the Phe residue near the carboxyl terminus of C24W, at position 21, is simultaneously inserted within the amino-terminal lobe of CaM. These results are modeled in Figure 3 where Trp residues are shown 20 and 35 Å from the AEDANS bound to cysteine-26 of CaM depicted in the bent conformation proposed by Vorherr et al. (1992).

It is notable that, in their original study, Blumenthal et al. (1985) isolated a 27-residue peptide denoted M13 from skMLCK which bound with high affinity to CaM. Subsequent analysis demonstrated that only the first 17 residues of M13 were required for high-affinity binding (Blumenthal & Krebs, 1987). This 17-residue peptide corresponds to M5, the peptide utilized for our studies (Table I; Kennelly et al., 1987). Interestingly, removal of Phe-17 resulted in a 50-fold loss in CaM affinity, indicating that Phe-17 may interact with hydrophobic determinants on CaM (Blumenthal & Krebs, 1987). Because our data suggest that the Trp residue of M5 may insert into the carboxyl-terminal lobe of CaM, Phe-17 may simultaneously insert into the hydrophobic cleft in the amino-terminal lobe of CaM analogous to Phe-21 of C24W. Consistent with this possibility, NMR spectroscopy studies have demonstrated that M13 and a 19-residue peptide corresponding to the CaM binding domain from smooth muscle MLCK (smMLCK) interact with both lobes of CaM (Klevit et al., 1985; Ikura et al., 1991, 1992; Roth et al., 1992).

Furthermore, the smMLCK peptide bound to CaM in the same orientation that we have proposed for the calcium pump peptide, with the amino- and carboxyl-terminal regions of the peptides interacting with the carboxyl- and amino-terminal lobes of CaM, respectively (Roth et al., 1992). This orientation of peptide binding to CaM is the opposite of that proposed by Persechini and Kretsinger (1988a) on the basis of modeling studies of M13·CaM but is consistent with cross-linking studies using model peptides and the CaM binding domain from smMLCK (O'Neil et al., 1989; O'Neil & DeGrado, 1989). Finally, during the preparation of this manuscript, Ikura et al. (1992) determined the solution structure of the *Drosophila* CaM·M13 complex using multidimensional NMR spectroscopy. When complexed with M13, the central helix of CaM was disrupted into two helices joined by a long flexible loop which allowed the two lobes of CaM to form a hydrophobic channel complementary in shape to the helical peptide. Specifically, Trp-4 and Phe-17 of M13 were found to anchor the amino- and carboxyl-terminal ends of the peptide to the methionine-rich hydrophobic domains in the carboxyl- and amino-terminal lobes of CaM, respectively. These findings are also consistent with the fluorescence data presented in this study, which also indicated that Trp-4 of M5 is inserted in the carboxyl-terminal lobe of CaM. From a comparison of the sequences of a number of CaM binding peptides, it was suggested that two hydrophobic residues separated by 12 residues may serve to anchor peptides to the hydrophobic determinants on CaM. The CaM binding domain of neuromodulin may prove to be an exception to this generalization, as this domain does not conform to the basic amphiphilic helix motif and possesses only a single hydrophobic residue nine residues carboxyl-terminal of the Trp residue (Chapman et al., 1991; Table I). In addition, FP57-Trp was readily dissociated from CaM by KCl (Chapman et al., 1991). In contrast, C24W and C24F could not be appreciably dissociated from CaM by 2 M KCl (data not shown). Furthermore, the iodide quenching and energy transfer data presented in this study are consistent with a role for Trp-4 and Phe-21 of C24W (as aligned in Table I) as hydrophobic anchors separated by 16 residues, which become inserted into the carboxyl- and amino-terminal lobes of CaM, respectively. It is notable that this is the same orientation of binding as reported for M13 (Ikura et al., 1992). Thus it appears that the simultaneous interaction of hydrophobic determinants of CaM binding domains with both lobes of CaM may represent a general binding mechanism for a class of CaM binding proteins.

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