Calcium-Induced Exposure of a Hydrophobic Surface on Calmodulin†

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ABSTRACT: Interactions between calmodulin (CaM) and several hydrophobic fluorescent probes were characterized in order to determine if CaM expresses hydrophobic binding sites in the presence of Ca^{2+}. Several classes of fluorescent probes capable of sensing exposure of hydrophobic binding sites on proteins were found to bind to CaM, and these interactions were greatly enhanced by Ca^{2+}. In the presence of Ca^{2+}, the fluorescence intensity of 9-anthroylcholine (9AC) was increased 24-fold by CaM, with a shift in the fluorescence emission maximum from 514 to 486 nm. The fluorescence intensity of 8-anilino-1-naphthalenesulfonate (Ans) was enhanced 27-fold with an emission maximum shift from 540 to 488 nm in the presence of CaM and Ca^{2+}. Similar results were obtained with the uncharged fluorescent ligand, *N*-phenyl-1-naphthylamine. With all three fluorescent dyes, the fluorescence changes caused by CaM in the absence of Ca^{2+} were minor compared to those observed with CaM and Ca^{2+}. Direct binding studies using equilibrium dialysis demonstrated that CaM can bind four to six molecules of 9AC or two to three molecules of Ans in a calcium-dependent manner. The effects of various amphiphilic compounds on the Ca^{2+}-dependent complex formation between CaM and the Ca^{2+}-sensitive phosphodiesterase or troponin I were investigated. Trifluoperazine (TFP) and 9AC inhibited CaM stimulation of the Ca^{2+}-sensitive phosphodiesterase. The Ca^{2+}-dependent binding of the phosphodiesterase to CaM–Sepharose was also inhibited by TFP, 9AC, and Ans. Furthermore, binding of CaM to troponin I–Sepharose was inhibited by these ligands. Consistent with these data was the observation that troponin I antagonized binding of 9AC to CaM. These data indicate that binding of Ca^{2+} to CaM results in exposure of a domain with considerable hydrophobic character, and binding of hydrophobic ligands to this domain antagonizes CaM–protein interactions. It is proposed that this hydrophobic domain may serve as the interface for the Ca^{2+}-dependent binding of CaM to the phosphodiesterase or troponin I.

Calmodulin (CaM)[1] was discovered as a regulatory protein that stimulated the Ca^{2+}-sensitive isozyme of phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970). It was subsequently determined that CaM binds Ca^{2+} (Teo & Wang, 1973) and that both CaM and Ca^{2+} are required for stimulation of the phosphodiesterase (Teo & Wang, 1973; Wolff & Brostrom, 1974; Lin et al., 1974; Teo et al., 1973). CaM forms calcium-dependent complexes with the phosphodiesterase and several other proteins, which are stable during Sephadex G-200 chromatography or electrophoresis on nondenaturing gels (Teshima & Kakiuchi, 1974; Lin et al., 1975; Amphlett et al., 1976; LaPorte & Storm, 1978). The following general model has been proposed for interactions between the phosphodiesterase (PDE) and CaM (Kakiuchi et al., 1973):

$$n\mathrm{Ca}^{2+} + \mathrm{CaM} \rightleftarrows \mathrm{CaM}\cdot\mathrm{Ca}^{2+}n$$

$$\mathrm{CaM}\cdot\mathrm{Ca}^{2+}n + \mathrm{PDE} \rightleftarrows \mathrm{CaM}\cdot\mathrm{Ca}^{2+}n\cdot\mathrm{PDE}$$

A variety of techniques have been used to demonstrate Ca^{2+}-induced conformational changes in CaM. These include optical rotatory dispersion (Liu & Cheung, 1976), circular dichroism (Wolff et al., 1977; Klee, 1977; Dedman et al., 1977a), differential sensitivity to proteolysis (Ho et al., 1975), differential sensitivity to chemical modification (Liu & Cheung, 1976; Walsh & Stevens, 1977; Richman & Klee, 1978), absorption spectroscopy (Klee, 1977; Richman & Klee, 1979), and enhancement of tyrosine fluorescence (Wang et al., 1975; Dedman et al., 1977a). The functional significance of these conformational changes is not defined, although they presumably result in formation of an interface site for CaM interactions with the phosphodiesterase and other protein systems.

It has been proposed that the major stabilizing forces for protein subunit associations are hydrophobic interactions (Chothia & Janin, 1975; Richards, 1977). The data reported in this study indicate that binding of Ca^{2+} to CaM results in formation of a domain with considerable hydrophobic character. It is proposed that this domain may function as the protein–protein interface between CaM and proteins which bind to CaM in the presence of Ca^{2+}.

Materials and Methods

Materials. Ans, 9AC, and *N*-phenyl-1-naphthylamine were purchased from Molecular Probes, Inc. TFP was a generous gift of Smith, Kline and French Labs. The dialysis membrane was a product of Spectrum Medical Industries, Inc. All other chemicals were of reagent grade or better.

Protein Preparations. CaM was prepared as previously described (LaPorte et al., 1979). Bovine heart CaM-sensitive phosphodiesterase was prepared as described previously (LaPorte et al., 1979). Troponin I was prepared by the method of Wilkinson (1974).

Fluorescence Measurements. All fluorescence measurements were made with an SLM 4800 spectrofluorometer thermostated at 30 ± 1 °C. Fluorescence spectra were acquired, corrected for background signal and instrument response, and integrated as previously described (LaPorte et al., 1980).

Fluorometric titration of CaM with 9AC was performed by preparation of samples containing 3.1 nmol of CaM and addition of the indicated amounts of 9AC and troponin I in 2 mL of 10 mM morpholinopropanesulfonic acid, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, 2 mM dithiothreitol (buffer A), and either 0.1 mM $CaCl_2$ or 0.25 mM EGTA. Samples were

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[1] Abbreviations used: CaM, calmodulin; 9AC, 9-anthroylcholine; Ans, 8-anilino-1-naphthalenesulfonate; TFP, trifluoperazine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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incubated at 30 °C for 3 h, to ensure that equilibrium had been established. Fluorescence intensity was then determined with excitation at 405 nm and emission intensity was monitored at 515 nm. Intensity values were corrected for the inner filter effect (Parker, 1968). This correction was less than 30% at the highest concentration of 9AC employed.

Fluorometric titration of 9AC·CaM with troponin I was performed by addition of troponin I to samples consisting of 20 nmol of 9AC and 5.2 nmol of CaM in 2 mL of buffer A containing 0.1 mM $CaCl_2$ or 0.25 mM EGTA. Fluorescence intensity was then determined as described above.

Equilibrium Dialysis. CaM was prepared for these experiments by extensive dialysis against buffer A containing either 0.1 mM $CaCl_2$ or 0.25 mM EGTA. Following this, protein was determined by the method of Peterson (1977) using CaM as the standard. A Spectropore membrane with a molecular weight cutoff of 6000 to 8000 was used for equilibrium dialysis. The two opposing dialysis chambers contained either 100 μL of the CaM solution or 100 μL of the ligand in the same buffer. Dialysis was carried out for 4 h at 25 °C, since it was determind that free ligand equilibrated across the membrane in 1.5 h. 9AC concentration was determined by dilution of an appropriate volume of sample into 2 mL of 6 M urea and 5 mM EGTA, pH 8.0, followed by measurement of fluorescence intensity with excitation at 381 nm and isolation of emitted light with a Schott KV 470 filter. Standard curves were determined simultaneously with each binding study. Under these conditions, CaM did not effect 9AC fluorescence and 95% of the added ligand could be accounted for. Ans concentrations were determined by absorbance at 360 nm following dilution of the sample into 5 mM EGTA, pH 8.0. This wavelength is the isosbestic point for Ans binding to myoglobin (Stryer, 1965). The absorbance was insensitive to CaM under these conditions and virtually 100% of the added ligand could be accounted for.

Phosphodiesterase Assay. Phosphodiesterase was assayed by a modification of a previously described procedure (LaPorte et al., 1979). The final assay mixture contained 50 μM [^{3}H]cAMP (60 000 cpm), [^{14}C]AMP (6000 cpm), 0.1 mM $CaCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mg/mL bovine serum albumin, enzyme, and other additions as indicated in a total volume of 0.5 mL of buffer A. Basal activity was determined under the same conditions but with the addition of 1 mM EGTA and 1 mM $MgCl_2$. Incubations were for 15 min at 30 °C, at which point 50 μL of 50 mM cAMP was added and the sample was heated at 100 °C for 2 min. One unit of enzyme activity is defined as that which will hydrolyze 1 nmol of cAMP in 15 min in the presence of excess CaM.

Phosphodiesterase Binding to CaM–Sepharose. CaM–Sepharose was prepared by the published method (Westcott et al., 1979) except that 8 μg of CaM was coupled per mL of cyanogen bromide activated Sepharose 4B. The assay mixture for binding of phosphodiesterase to CaM–Sepharose consisted of 65 units of enzyme, 0.1 mg/mL bovine serum albumin, 0.1 mM $CaCl_2$, 100 μL of CaM–Sepharose, and, where indicated, various effectors, in a total volume of 350 μL of buffer A. Triplicate samples were mixed for 20 min at 4 °C, and the resin was sedimented by centrifugation. Two 15-μL samples were withdrawn from the supernatant for phosphodiesterase assays, and 30 μL of 23 mM EGTA in buffer A, with the appropriate effectors, was added. The samples were mixed for 5 min, the resin was sedimented, and two 15-μL aliquots were withdrawn and assayed for phosphodiesterase activity. Ca^{2+}-dependent binding of the phosphodiesterase to CaM was determined from the difference in supernatant activity found before and after EGTA treatment, taking into account the enzyme removed for the first set of assays.

CaM Binding to Troponin I–Sepharose. Troponin I–Sepharose was prepared by the method of Head et al. (1977). The reaction mixture contained 2 mg of troponin I/g of dry resin. Binding of CaM to this resin was determined by chromatography of 70 μg of CaM on troponin I–Sepharose columns (3 × 0.4 cm) equilibrated in buffer A with 0.1 mM $CaCl_2$ and the appropriate effector, where indicated. Samples were diluted 10-fold into the equilibration buffer and applied to the column, and the column was washed with the equilibration buffer at 4 mL/h. CaM remaining bound to the resin was then eluted with the equilibration buffer containing 2 mM EGTA. Protein was determined by the method of Bradford (1976) using CaM, in the equilibration buffer, as the standard. In one experiment the column was equilibrated with buffer A containing 2 mM EGTA prior to sample loading. Fraction size was 1.2 mL.

Except where indicated, protein was determined by the method of Peterson (1977) with bovine serum albumin as the standard. CaM was determined spectrophotometrically using 1 mg/mL, A_{277nm} = 0.18 (Watterson et al., 1976). Molar concentrations of CaM and troponin I were calculated using molecular weights of 16 723 (Vanaman et al., 1977) and 23 000 (Wilkinson, 1974) for CaM and troponin I, respectively.

Results

Effect of CaM and Ca^{2+} on the Fluorescence of 9AC, Ans, and N-Phenyl-1-naphthylamine. 9AC is a cationic amphiphile, which, in an aqueous medium, displayed a broad emission spectrum with a maximum at ca. 514 nm (Figure 1A). In the presence of EGTA, CaM caused a 1.5-fold enhancement in the integrated fluorescence intensity with a shift in the emission maximum to 505 nm. In the presence of Ca^{2+}, however, CaM produced a 24-fold enhancement in 9AC fluorescence intensity with a blue shift in the maximum to 486 nm. Trifluoperazine (TFP), which is known to bind to CaM in a Ca^{2+}-dependent manner (Levin & Weiss, 1977), reversed the enhancement of 9AC fluorescence, with 50% reversal occurring at 4 μM TFP. Equilibrium dialysis confirmed that TFP competitively displaced 9AC from CaM.

Ans, an anionic amphiphile, had an emission maximum at 540 nm in the absence of CaM (Figure 1B). In the presence of EGTA, CaM caused a 1.5-fold increase in the fluorescence intensity, accompanied by an emission maximum shift to 520 nm. With Ca^{2+}, CaM produced a 27-fold enhancement in fluorescence intensity and a blue shift to 488 nm. Attempts to reverse this fluorescence enhancement with TFP were unsuccessful because Ans formed a coprecipitate with TFP, thereby greatly decreasing the solubility of the latter.

CaM had similar effects on the fluorescence of *N*-phenyl-1-naphthylamine, which is an uncharged hydrophobic ligand (Figure 1C). Ca^{2+} alone did not affect the fluorescence of 9AC, Ans, or *N*-phenyl-1-naphthylamine. For all three probes, these Ca^{2+}-induced spectral changes were reversed by the addition of excess EGTA. It is notable that these three fluorescent ligands have different charges, but they are all relatively hydrophobic compounds.

Low concentrations of the fluorophores were employed for determination of these spectra, so that addition of Ca^{2+} would produce the largest possible effect on emission intensity. At 1 mM 9AC or Ans, the spectrum of the bound ligand, determined by difference, had essentially the same wavelength of maximum emission as at the lower concentrations. Further

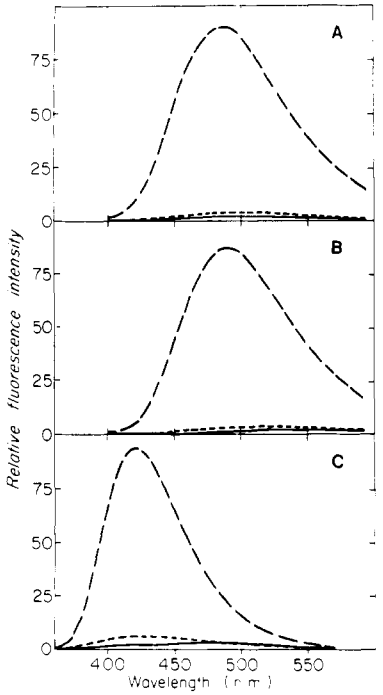

FIGURE 1: Effect of CaM and Ca^{2+} on the fluorescence emission spectra of 9AC, Ans, and *N*-phenyl-1-naphthylamine. Samples consisted of the indicated concentration of fluorophore in buffer A in addition to 100 μM CaM and 0.1 mM $CaCl_2$ (——); 100 μM CaM and 0.25 mM EGTA, (- -); or without CaM (—). The resolution of both monochromaters was set at 4 nm. Following acquisition of each set of spectra, we determined a blank and subtracted it from the spectra. The blank was less than 10% of the sample intensity in all cases. Spectra were corrected for instrument response as described previously (LaPorte et al., 1980): (A) 6 μM 9AC, excitation at 361 nm; (B) 12 μM Ans, excitation at 360 nm; (C) 4 μM *N*-phenyl-1-naphthylamine, excitation at 350 nm.

experimentation with *N*-phenyl-1-naphthylamine was not pursued because of its poor aqueous solubility at higher concentrations.

Calcium-Dependent Binding of 9AC and Ans to CaM. Binding of 9AC to CaM was directly measured by equilibrium dialysis in the presence of Ca^{2+} or EGTA (Figure 2). For the purposes of this discussion, total binding is defined as binding in the presence of Ca^{2+}, and differential binding is the difference between binding in the presence and absence of Ca^{2+}. Analyses of both types of binding are presented, since it is not possible to unambiguously define either class of binding sites as representing the calcium-sensitive sites. Scatchard plots of both sets of data derived from Figure 2 were concave upward, indicating heterogeneity of binding sites. The average dissociation constant, $\bar{K}_d$, could be estimated from the slope of the plot at high saturation (Dahlquist, 1978). Extrapolation of the final slope to the abscissa gave an estimation of the number of 9AC binding sites. Total binding was characterized by a $\bar{K}_d$ of 440 μM, with 5.5 binding sites for 9AC. The $\bar{K}_d$ for differential binding was 440 μM for 3.9 binding sites. Although very weak binding sites may have been omitted from this analysis, CaM appears to have four to six Ca^{2+}-sensitive binding sites for 9AC with a $\bar{K}_d$ of 440 μM.

The Ca^{2+}-dependent binding of Ans to CaM was also characterized (data not shown). Again, Scatchard plots for both sets of data were concave upward, suggesting binding site heterogeneity. Differential binding was characterized as 2.1 Ans binding sites with a $\bar{K}_d$ of 490 μM. The $\bar{K}_d$ for total binding was 1 mM for 2.8 Ans binding sites.

Inhibition of Phosphodiesterase–CaM Interactions. The fluorescence and binding data discussed above suggest that

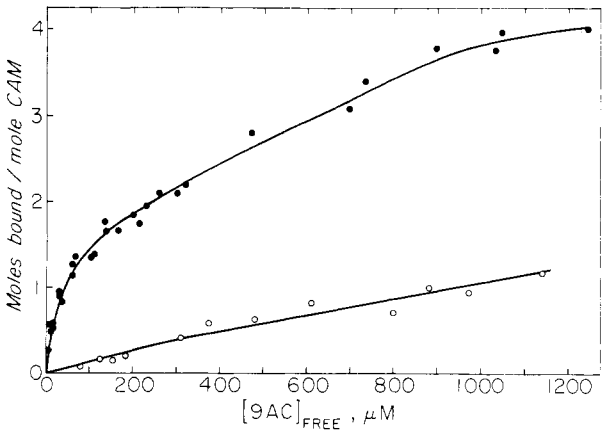

FIGURE 2: Binding of 9AC to CaM. Binding of 9AC to CaM was determined by equilibrium dialysis, as described under Materials and Methods. Protein samples consisted of 96 μM CaM in buffer A with either 0.1 mM $CaCl_2$ (●) or 0.25 mM EGTA (○). CaM samples were dialyzed for 4 h against various concentrations of 9AC in the same buffer.

the fluorescent ligands bind to a hydrophobic domain of CaM when the protein binds Ca^{2+}. If the CaM hydrophobic binding sites expressed in the presence of Ca^{2+} are part of the interface for association with the Ca^{2+}-sensitive phosphodiesterase, then 9AC should inhibit interactions of the enzyme with CaM. In agreement with previous reports (Weiss et al., 1974), TFP, which also binds to CaM in a Ca^{2+}-dependent manner, inhibited CaM stimulation of the phosphodiesterase. At 4 μM TFP, the activation constant, K_{act}, for CaM stimulation increased from 120 to 600 pM. Similarly, 100 μM 9AC increased the K_{act} to 720 pM CaM, although it had no effect on basal phosphodiesterase activity. Ans strongly inhibited basal enzyme activity at concentrations well below where an effect on CaM stimulation would be expected.

TFP, 9AC, and Ans all inhibited binding of the phosphodiesterase to CaM–Sepharose in the presence of Ca^{2+}. In the absence of effectors, 48 units of the 65 phosphodiesterase units were absorbed to CaM–Sepharose in the presence of Ca^{2+}. TFP at 25 or 500 μM 9AC reduced phosphodiesterase absorption to 1 ± 1 and 4 ± 2 units, respectively. Ans seriously inhibited phosphodiesterase activity. Although this made it difficult to quantitate the effect of Ans on phosphodiesterase binding to CaM–Sepharose, 2 mM Ans completely inhibited binding of the enzyme to the resin.

Since troponin I forms a Ca^{2+}-dependent complex with CaM (Amphlett et al., 1976; Dedman et al., 1977b), this interaction was used as a model system for experiments described below. Troponin I competitively inhibited CaM stimulation of phosphodiesterase activity (Figure 3). With 6×10^{-10} M CaM, 50% inhibition of CaM stimulation occurred at 3×10^{-8} M troponin I. At 4×10^{-7} M CaM, only slight inhibition of CaM stimulation was evident at the highest troponin I concentrations, suggesting competition between the binding of troponin I and the phosphodiesterase to CaM.

Inhibition of CaM Binding to Troponin I–Sepharose. As previously reported (Head et al., 1979), CaM·Ca^{2+} was absorbed to troponin I–Sepharose (Figure 4). When the column was equilibrated with EGTA, nearly all of the applied CaM was nonabsorbed and appeared in the first fraction. TFP (25 μM), 500 μM 9AC, and 2 mM Ans all inhibited CaM absorption, and the protein was distributed between the first two fractions. The differences in distribution of CaM in the presence of Ca^{2+} and these effectors compared to that in the presence of EGTA indicate that CaM–troponin I interactions were not totally blocked. This is the expected behavior if the

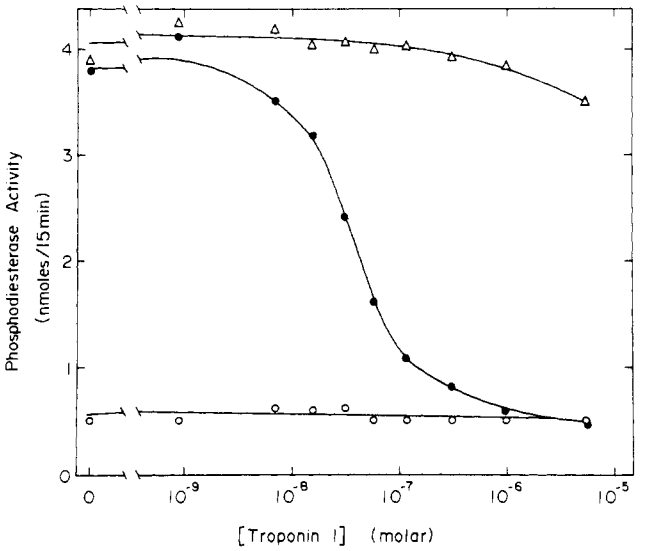

FIGURE 3: Inhibition of CaM stimulation of phosphodiesterase by troponin I. Phosphodiesterase was assayed as described under Materials and Methods. The assay mixture contained 4 units of enzyme, the indicated concentration of troponin I, standard reagents, and the following additions: 4×10^{-7} M CaM (△); 6×10^{-10} M CaM (●); or 1 mM EGTA and 1 mM $MgCl_2$ (○).

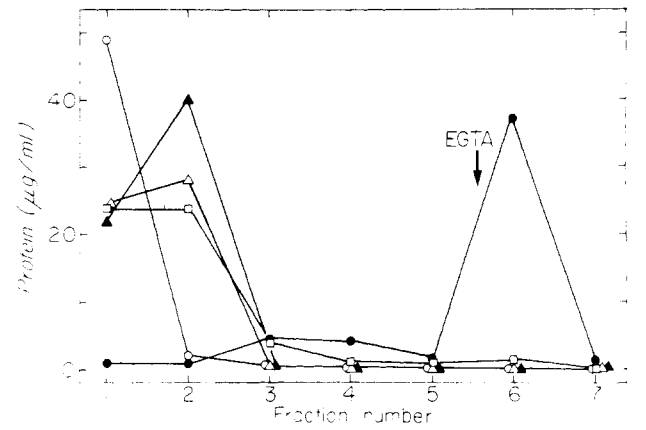

FIGURE 4: Inhibition of CaM binding to troponin I–Sepharose by 9AC, Ans, and TFP. Troponin I–Sepharose columns (3 × 0.4 cm) were equilibrated in buffer A containing either 0.1 mM $CaCl_2$ and the indicated ligand or 2 mM EGTA. Samples, consisting of 70 μg of CaM in the equilibration buffer, were applied, followed by washing of the column with this buffer at 4 mL/h. At the point indicated by the arrow, bound CaM was eluted with the equilibration buffer containing 2 mM EGTA. Protein was determined as described under Materials and Methods. The fraction size was 1.2 mL. In addition to buffer A and 0.1 mM $CaCl_2$, the equilibration buffer contained the following: no addition (●); 2 mM EGTA (○); 2 mM Ans (□); 500 μM 9AC (△); or 25 μM TFP (▲).

mechanisms of inhibition of absorption are competitive in nature.

Effect of Troponin I on Binding of 9AC to CaM. If 9AC binds to the interface between CaM and other proteins, binding of 9AC to CaM should be antagonized by troponin I. 9AC was chosen for these experiments since it does not bind significantly to troponin I. The Ca^{2+}-dependent binding of 9AC to CaM was inhibited by troponin I (Figure 5). The data presented in Figure 5 were determined by monitoring 9AC fluorescence enhancement. Binding studies done by equilibrium dialysis directly confirmed that troponin I inhibited binding of 9AC to CaM. The stoichiometry of this effect was determined by titration of CaM with troponin I in the presence of 10 μM 9AC (Figure 6). Addition of troponin I caused a linear decrease in the Ca^{2+}-dependent 9AC fluorescence enhancement to a troponin I/CaM ratio of 0.9, at which point a sharp break occurred. These data are consistent with the formation of a 1:1 complex between CaM and troponin I, which inhibits the Ca^{2+}-dependent binding of 9AC to CaM. A similar effect was seen at 250 μM 9AC, although the apparent K_d for the CaM–troponin I complex was increased.

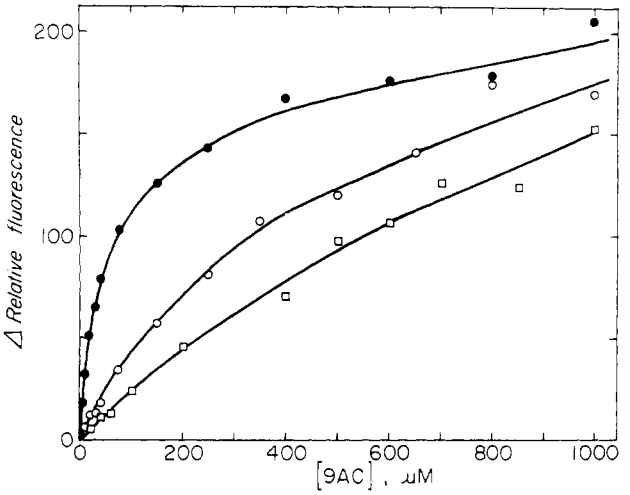

FIGURE 5: Inhibition of the Ca^{2+}-dependent binding of 9AC to CaM by troponin I. Samples contained 1.6 μM CaM, various amounts of troponin I, the indicated concentration of 9AC, and either 0.1 mM $CaCl_2$ or 0.25 mM EGTA in 2 mL of buffer A. After the samples were incubated for 3 h at 30 °C, fluorescence intensity was determined with excitation at 405 nm and emitted light monitored at 515 nm. The Ca^{2+}-dependent fluorescence enhancement, "Δ Relative fluorescence", was determined by comparison of otherwise identical samples containing either 0.1 mM $CaCl_2$ or 0.25 mM EGTA. Intensity measurements were corrected for the inner filter effect (Parker, 1968). This correction was less than 30% at the highest concentration of 9AC employed. Troponin I concentrations were 0.0 μM (●); 1.2 μM (○); or 3.2 μM (□).

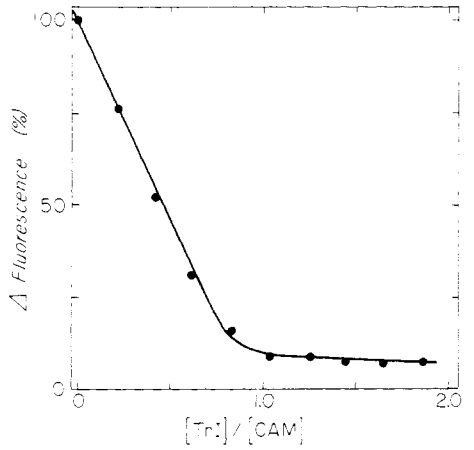

FIGURE 6: Stoichiometry of the troponin I effect on 9AC binding to CaM. Samples consisted of 5.2 nmol of CaM, 20 nmol of 9AC, and either 0.1 mM $CaCl_2$ or 0.25 mM EGTA in 2 mL of buffer A. The fluorescence intensity of these samples was determined with excitation at 405 nm and emitted light monitored at 515 nm. Both samples were then titrated with troponin I, allowing 5 min after each addition before the fluorescence intensity was measured. Fluorescence intensity was corrected for dilution, which was 4% at the conclusion of the titration. Fluorescence intensity is expressed as the difference in intensity between the two samples, with the difference before addition of troponin I taken as 100%. Troponin I concentration is expressed as the molar ratio of troponin I to CaM.

Discussion

It has been proposed that hydrophobic forces are principally responsible for stabilizing protein–protein interactions (Chothia & Janin, 1975; Richards, 1977). These investigators argue

that polar and electrostatic interactions, which play a key role for specificity, do not contribute significantly to the net binding energy. Following these arguments, it might be proposed that binding of Ca^{2+} to CaM may generate a domain with enhanced hydrophobicity which acts as the interface for interactions between CaM and various protein systems. Therefore, we have examined Ca^{2+}-induced conformational changes of CaM using fluorescent dyes which interact with hydrophobic domains of proteins.

The fluorescence intensity of Ans, 9AC, and *N*-phenyl-1-naphthylamine increased dramatically in the presence of Ca^{2+} and calmodulin. With all three ligands there was a significant shift in the emission spectrum to shorter wavelengths. In contrast, CaM had only a small effect on the emission spectra of these probes in the presence of EGTA, and Ca^{2+} alone had no effect on the fluorescence of these ligands. These spectral changes are consistent with removal of the fluorescent dyes from an aqueous environment to hydrophobic binding sites on the protein (Stryer, 1965; Weber & Laurence, 1954; Turner & Brand, 1968; Edelman & McClure, 1968). Direct binding studies revealed that CaM has four to six Ca^{2+}-sensitive binding sites for 9AC and two to three Ca^{2+}-sensitive binding sites for Ans. It is noteworthy that the Ca^{2+}-dependent fluorescence changes were qualitatively similar for a positive, negative, and uncharged probe. Therefore, it seems likely that the major forces contributing to binding of these ligands to CaM are hydrophobic interactions, although polar or electrostatic interactions may make some contribution.

The Kosower *Z* value can be used to estimate the relative hydrophobicity of protein–ligand binding sites (Turner & Brand, 1968). This parameter is determined by comparison of the wavelength of maximum emission of the protein-bound ligand to that of the fluorophore in solvents of known polarity. Such an analysis was carried out for binding of Ans, 9AC, and *N*-phenyl-1-naphthylamine to CaM. In the presence of CaM and Ca^{2+}, all three probes had *Z* values of approximately 79 to 80. Although these values are not necessarily a rigorously quantitative estimation of polarity, since the emission maxima are also affected by the rigidity of the local environment (Ainsworth & Flanagan, 1969), it is interesting that the *Z* values for the three probes were quite similar. This suggests that these probes bind to sites with similar environments.

A number of observations suggest that these fluorescent ligands bind to a CaM domain which is the interface for interactions with other proteins. Like TFP, 9AC inhibited CaM stimulation of the Ca^{2+}-sensitive phosphodiesterase. For example, 100 μM 9AC increased the K_{act} for CaM from 120 to 720 pM. Although this increase in K_{act} suggests a strong antagonism of CaM–phosphodiesterase complex formation, it does not, by itself, constitute direct proof of this phenomenon. However, binding of the phosphodiesterase to CaM–Sepharose was inhibited by TFP, 9AC, and Ans. These three ligands also inhibited absorption of CaM to troponin I–Sepharose. It could be argued that the inhibition of complex formation between CaM and troponin I or the phosphodiesterase was due to interaction of these ligands with the other protein rather than with CaM. This is not possible for the 9AC-mediated inhibition of CaM–troponin I complex formation because 9AC did not bind significantly to troponin I. Furthermore, a wide variety of cationic amphiphilic compounds have been found to inhibit CaM stimulation of phosphodiesterase, adenylate cyclase, and (Ca^{2+}–Mg^{2+})-ATPase (Brostrom et al., 1977; Kobayashi et al., 1979; Levin & Weiss, 1979). In all these systems, CaM is the only common participant. Finally, an approximately linear relationship has been found between Ca^{2+}-dependent binding of these compounds to CaM and their ability to inhibit CaM stimulation of phosphodiesterase (Levin & Weiss, 1979). These findings strongly argue that these effects are brought about by interaction with CaM itself. If troponin I and 9AC interact with CaM at a common site, it would be predicted that troponin I should affect binding of 9AC to CaM. As predicted, troponin I antagonized binding of 9AC to CaM. This does not mean that these probes are incapable of binding to the complementary hydrophobic domain that would be expected to exist on these other proteins. These findings simply suggest that, for these probes, the interaction with CaM is predominant. Furthermore, not all the Ca^{2+}-sensitive sites for these ligands need to be at the interface domain, although the ability of troponin I to inhibit 9AC binding suggests that most of these sites occur within the same domain.

The model for CaM–protein interactions proposed in this study provides insight for several observations reported in the literature. A number of amphiphilic cationic antipsychotic drugs bind to CaM and inhibit CaM stimulation of the Ca^{2+}-sensitive phosphodiesterase (Weiss & Levin, 1977). Although some of the antipsychotic drugs exhibit stereospecificity for some of their in vitro and in vivo effects, binding to CaM and inhibition of CaM stimulation of phosphodiesterase were not nearly as stereospecific (Levin & Weiss, 1979). The data reported in this study indicate that a variety of hydrophobic ligands may interact relatively nonspecifically with CaM. It might be noted that several of these antipsychotic agents bind to CaM with an affinity significantly greater than that of 9AC or Ans. Differences in affinities would be expected for probes of different structures, and the finding that the antipsychotic drugs bind more strongly does not necessarily address the question of the pharmacological relevancy of this process. Therefore, the physiological significance of the binding of antipsychotic drugs to CaM is questionable.

CaM–Sepharose has been used for the purification of a number of CaM binding proteins. This column binds several proteins in a calcium-dependent manner that do not appear to interact specifically with CaM (Klee & Krinks, 1978; LaPorte et al., 1979). Rechromatography of the samples on CaM–Sepharose removed these proteins, suggesting that their interactions with CaM were much weaker than those between CaM and the phosphodiesterase (LaPorte et al., 1979). The Ca^{2+}-dependent exposure of a hydrophobic binding site on CaM may result in nonspecific binding of proteins to CaM–Sepharose.

It has been reported that the CaM-sensitive phosphodiesterase is stimulated by certain lipids and that this stimulation is competitive with CaM stimulation (Wolff & Brostrom, 1976; Pichard & Cheung, 1977; Hidaka et al., 1978). If, as we have proposed, the interface for CaM–phosphodiesterase interactions is significantly hydrophobic, it would follow that the phosphodiesterase might have a complementary hydrophobic site to which these lipids could bind. The observation that CaM and various lipids competitively interact with the enzyme is consistent with this proposal.

In summary, the data presented in this study indicate that binding of Ca^{2+} to CaM causes the formation of a surface possessing considerable hydrophobic character. In addition, binding of CaM to the Ca^{2+}-sensitive phosphodiesterase or troponin I is antagonized by a variety of amphiphilic ligands that interact with this hydrophobic domain. Therefore, we propose that the hydrophobic domain expressed in the presence of Ca^{2+} may be the interface for CaM–protein interactions. Although hydrophobic bonding may be the driving force for

CaM–protein complex formation, the specificity of these associations is most likely dictated by complementarity of the surfaces and specific electrostatic or polar interactions. Although the data reported in this paper support such a model, rigorous proof must await extensive X-ray crystallographic studies.

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