A Calcium-sensitive Fluorescent Analog of Calmodulin Based on a Novel Calmodulin-binding Fluorophore*

Klaus M. Hahn†, Alan S. Waggoner, and D. Lansing Taylor

From the Department of Biological Sciences and Center for Fluorescence Research in the Biomedical Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213.

Structure-activity studies of tetramethinemercocyanine fluorophores enabled the synthesis of novel dyes which showed spectral changes during reversible, calcium-dependent association with calmodulin. These spectral changes were greatly enhanced in dyes with a quaternary nitrogen and specifically placed hydrophobic chains. One such dye was covalently attached to calmodulin, producing a calmodulin analog with calcium-sensitive fluorescence. The analog, MeroCaM, showed a calcium-induced 3.4-fold increase in excitation ratio (608/532 nm excitation, 623 nm emission), which was fully reversed by lowering free calcium levels. MeroCaM’s excitation ratio showed a half-maximal change at 300–400 nM calcium, below calcium concentrations reported to produce half-maximal saturation of calcium-calmodulin binding. However, the calcium dependence of MeroCaM’s phosphodiesterase activation paralleled that of calmodulin. MeroCaM’s fluorescence changes therefore appear to reflect primarily calcium binding to high affinity sites. MeroCaM’s maximal phosphodiesterase activation was 30–40% that of calmodulin. In myosin light chain kinase activation, MeroCaM and calmodulin displayed indistinguishable maximal activation levels and concentration dependence of activation. Changes in MeroCaM’s calcium affinity induced by magnesium, phosphodiesterase, and melittin were similar to those reported for calmodulin. Experiments with melittin revealed that target protein interaction could alter the fluorescence changes produced by calcium binding. MeroCaM showed promising brightness and photostability when imaged in individual living fibroblasts. The long excitation and emission wavelengths of MeroCaM, and the strong dependence of its excitation ratio on calcium concentrations, suit it well for use as a probe of calmodulin-dependent calcium signaling in living cells, as well as for experiments in vitro.

Temporal and spatial fluctuations of intracellular free calcium ion concentration have been shown to play important roles in the regulation of many cellular processes (Campbell, 1983). Calmodulin mediates calcium’s effects on numerous chemical reactions involved in these processes by sensing the elevation of free calcium ion concentration and then activating specific proteins (Klee and Vanaman, 1989; Manalan and Klee, 1984; Van Eldik and Watterston, 1985; Cohen and Klee, 1988). Correlating the intracellular temporal and spatial dynamics of the free calcium ion concentration, calmodulin and the specific effector proteins could help to define the molecular basis of cell regulation.

It has been possible to explore the chemistry of living cells through the combination of quantitative light microscopy with use of a variety of fluorescent probes (Taylor and Wang, 1989; Wang and Taylor, 1989). Fluorescent indicators of the free calcium ion concentration are continuing to evolve, but new information about both temporal and spatial signaling has already emerged (Tsien, 1989). In addition, fluorescent analogs of specific macromolecules have been used to define the distribution, mobility, and activity of selected cellular components (Taylor et al., 1984; Wang, 1989). Ratio imaging has become an important technique for quantifying these reagents (Bright et al., 1989; DeBiasio et al., 1988). The continued evolution of these methods should permit the use of living cells as “microcuvettes” for intracellular chemical and molecular analyses.

A number of investigators have sought the dynamic link between changes in the free calcium ion concentration and the regulation of biochemical reactions. Fluorescent analogs of calmodulin have been prepared which are not sensitive to calcium binding but have yielded information on the distribution and mobility of the calmodulin in living cells (Hama-guchi and Isawa, 1980; Kitch et al., 1983; Welch, 1983; Zavortink et al., 1983; Luby-Phelps et al., 1985; Stemple et al., 1988). Calcium-sensitive fluorescent analogs of calmodulin and troponin C have also been prepared and have been used in solution spectroscopic studies (Johnson et al., 1978; Johnson and Wittmer, 1983; Oiwin and Surnm, 1983; Mills et al., 1988) and with cell models (Zot et al., 1986).

In this paper, we describe a new fluorescent calmodulin analog with fluorescence properties strongly dependent on calcium binding. It is well suited for use in living cells, with excitation and emission wavelengths longer than 500 nm and calcium-dependent changes in excitation suitable for ratio imaging. It should enable direct analysis of the relationship between calcium-calmodulin binding and calmodulin behavior.

The calmodulin analog was designed on the basis of previous studies which indicate that the binding of calcium to calmodulin is accompanied by a conformational change leading to exposure of a hydrophobic region on calmodulin (LaPorte et al., 1980; Manalan and Klee, 1984; Cohen and Klee, 1988). This region has been shown to bind a range of specific hydrophobic organic molecules in a calcium-dependent manner (Hidaka and Hartshorne, 1985). A novel hydrophobic dye which bound to calmodulin in a calcium-dependent manner, and had fluorescence properties highly sensitive to

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Calcmodulin (3.3 equivalents) was added to 2.5 μM dye dissolved in 1 mM CaCl₂, 10 mM Tris, pH 7.0. The effects of calcmodulin addition on each dye's absorbance spectrum were observed. ++++, shift in absorbance maximum greater than 24 nm accompanied by strong change in peak shape. —, no appreciable change: shift in absorbance maximum of less than 5 nm and change in extinction coefficient less than 6%. +, lesser changes in peak shape: greater than 25% increase in extinction coefficient not accompanied by a shift in absorbance maximum and/or increase in intensity of a shoulder.

<table>
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<tr>
<th>Dye</th>
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<th>A</th>
<th>B</th>
<th>CaM binding</th>
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<td>Mc4.11</td>
<td>O</td>
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<td>-(CH₂)₂CH₂</td>
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<tr>
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<tr>
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<tr>
<td>Mc4.19</td>
<td>S</td>
<td>Final reactive dye</td>
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S-A, -(CH₂)₂N⁺(CH₃)₂-(CH₂)₂NCS; B, -(CH₂)₂CH₂.

**Fig. 1. Dye synthesis.** This convergent synthesis was well suited to variation of the dye structures. Terminal groups were synthesized separately and linked in different combinations. The sensitive alkyl isothiocyanate group was introduced in the final step through a mild quaternization reaction.

Solvent polarity, was covalently attached to calcmodulin. Calcium binding to the derivatized protein induced a strong change in fluorescence, presumably due to interaction between the hydrophobic pocket and the solvent-sensitive fluorophore.

**MATERIALS AND METHODS**

**Nomenclature**—The dyes described in this paper have been cataloged using a system of code numbers which encompasses all dyes from ongoing programs in the Waggoner laboratory (Ernst et al., 1989). The structure and code numbers of each dye are shown in Table I. All dyes in this paper are composed of two heterocyclic end groups linked by a 4-carbon bridge, as shown in the structural formula of Table I. To simplify discussion, dye types will be denoted by abbreviations referring to these end groups. Dyes will be named using two end group abbreviations separated by a hyphen. Thus, structure Mc4.10 in Table I, containing benzoxazole and thiobarbituric acid end groups, is an O-TBA dye.

**Synthesis**—Dye synthesis is outlined in Fig. 1. Detailed synthetic procedures and spectral data are provided in the Miniprint Supplement.

**Spectroscopy**—Absorption spectra were recorded on a Hewlett-Packard HP 8452 diode array spectrophotometer. Excitation and emission spectra were obtained using a Spex Fluorolog-2 system, with fluorescence intensities corrected for the intensity of the exciting light and sensitivity of the detection system. The data presented in Fig. 8 were obtained using an SLM 8000 spectrofluorimeter, corrected for the intensity of the exciting light.

**Dye Manipulations**—Dyes were dissolved in Me₂SO before addition to aqueous solutions. Dye concentrations in Me₂SO were determined using extinction coefficients of 165,000 M⁻¹ cm⁻¹ for S-TBA dyes and 111,000 M⁻¹ cm⁻¹ for O-TBA dyes. These extinction coefficients were determined using dyes Mc4.12 and Mc4.18.

**Quantum Yield Determinations**—Quantum yields were measured relative to methanol solutions of 3,3'-diethylthiadicarbocyanine, which have been assigned a quantum yield of 0.33 (Roth and Craig, 1989).
1974). Dye absorbance measurement and fluorescence excitation were carried out on a short wavelength shoulder of the main absorption band. The absorbance of the sample was kept below 0.02.

**Calmodulin Preparation**—Calmodulin was isolated from bovine brain using the method of Burgess et al. (1980). Its identity and purity were established by its calcium-dependent mobility in SDS and native PAGE, activation of myosin light chain kinase and cAMP phosphodiesterase with calcium and EGTA concentrations required to produce a given free calcium concentration. EGTA concentrations were either 0.5 or 0.2 mM. Calcium/EGTA buffer systems—Solutions were buffered with Tris-HCl, pH 7.0. Each assay contained 1.4 mM MgSO₄, 47 mM KCl, and 105 mM MOPS-KOH, pH 7.0. Each point was obtained using 3 mM calmodulin or MeroCaM. Increasing phosphodiesterase levels were used in the presence of 0.3 mM calcium until no further increase in rate was observed. The phosphodiesterase level in the actual assay was held constant at 30% above the level producing maximal hydrolysis. Solutions were preheated to 55 °C at 30 °C before addition of cAMP. The assay mixture contained 2.75 mg/ml phosphodiesterase (0.018 unit/mg, 1 unit = conversion of 1 μmol of cAMP/min). All proteins were calmodulin deficient grade from Boehringer Mannheim. Adenosine deaminase and alkaline phosphatase were from calf intestine, cAMP phosphodiesterase was from beef heart, and cAMP was from Sigma.

**Myosin Light Chain Kinase Activation Assay**—The dependence of myosin light chain kinase activity on calmodulin or MeroCaM concentration was assayed by a modification of the procedure of Adelstein and Klee (1981). Reactions were incubated 5 min, using 0.2 mM CaCl₂ and 1 mM myosin light chain kinase. The reaction was terminated by spotting the reaction mixture on a circle of filter paper and developing this in the trichloroacetic acid/sodium pyrophosphate solution described in the original procedure. After the papers were washed for a total of 45 min in three changes of this solution (10 ml/paper), the papers were counted in 10 ml of Du Pont Biofluor scintillation mixture, in a Beckman LS1701 scintillation counter. Control reaction mixture was zero time points to assure that the rate of phosphate incorporation was linear at 5 min. Myosin light chain kinase and calmodulin-free light chains were a generous gift from Robert Adelstein and James Sellers (National Institutes of Health). [γ-³²P]ATP was purchased from Du Pont-New England Nuclear.

**RESULTS**

**Effect of Dye Structure on Solvent-dependent Fluorescence**—An important first step in the design of the calmodulin analog was selection of a fluorophore with the required spectral properties. A high spectral sensitivity to solution polarity was required so that the dye, when covalently linked to calmodulin, would respond to calcium-induced changes in the hydrophobicity of its protein environment. Of the many fluorophores known to show solvent-sensitive spectral changes, the merocyanine dyes were chosen because of their long excitation and emission wavelengths, and the feasibility of synthetically modifying side chains to affect calmodulin binding (Hamer, 1984; Sturmer, 1987; Waggoner et al., 1989).

In order to identify a specific merocyanine with high solvent sensitivity, merocyanines with different heteroatoms in the fluorophore structure were compared. Tetramethinemerocyanine dyes of type S-TBA, O-TBA, I-TBA, and I-BA were selected for screening based on their availability, excitation and emission wavelengths, expected ease of derivatization, and the magnitude of solvent-induced shifts predicted by previous studies (Brooker et al., 1951; Hamer, 1984; Sturmer, 1987; Waggoner and Grinvald, 1977). Each fluorophore was dissolved in a series of increasingly hydrophobic solvents, and solvent-dependent changes in their absorbance properties were monitored.

The S-TBA and O-TBA fluorophores were selected for further study because they showed far stronger spectral changes than the other dyes tested. Both these dyes underwent a greater than 20 nm shift to longer absorbance wave length and a greater than 1.8-fold increase in extinction coefficient when dissolved in n-decane rather than water. Previous studies have shown that S-TBA and O-TBA dyes have fluorescence quantum yields of greater than 0.5 in octanol, and that both show a similar inverse dependence of quantum yield on solvent polarity. Quantum yield determinations described below for the S-TBA dye Mc4.18 supported these results.

Further characterization of the S-TBA and the O-TBA fluorophores revealed that their solvent-dependent absorbance changes, although promising, were quite complex. Absorbance spectra were sensitive to the concentration of KC1 in aqueous dye solutions, and to the concentration of dye. Each dye showed markedly different solvent-dependent changes in the shape of its absorbance spectrum.

**Effect of Dye Side Chains on Calmodulin Binding**—It was necessary to derivatize the fluorophore with properly placed charged and nonpolar groups to induce calmodulin binding. The dye had to bind calmodulin in a calcium-dependent
manner, reversibly, and with an orientation producing strong spectral changes. To find the proper derivative, a series of O-TBA analogs bearing nonpolar and charged side chains were synthesized and tested for absorbance changes induced by calmodulin binding.

Each dye's absorbance spectrum was recorded in aqueous calcium chloride, before and after the addition of excess calmodulin. Results of these assays are given in Table I, and representative spectra are shown in Fig. 2. (The S-TBA dyes included in Table I were assayed in later studies, which will be described. Their behavior largely reinforced the structure-activity relationships initially determined for the O-TBA dyes.)

Some of the O-TBA dyes showed no change in absorbance throughout the procedure, whereas others showed only the formation of shoulders on the long wavelength side of the absorbance peak and/or an increase in extinction coefficient. Dye Mc4.12, however, showed a dramatic shift to longer absorbance maximum and increase in extinction coefficient on addition of calmodulin (see Fig. 2).

This very responsive dye was examined further. The reversibility and calcium dependence of its calmodulin binding were tested by adding an additional step to the calmodulin binding assay. After calmodulin binding had been observed, the assay solution was made 10 mM in EGTA and additional spectra were taken. Addition of EGTA caused the absorbance spectrum of the dye to revert to that seen before the addition of calmodulin (see Fig. 2). On addition of calmodulin, the dye showed an increase in fluorescence emission of between 10- and 25-fold, depending on the excitation wavelength. This fluorescence increase was also reversed by the addition of EGTA.

The qualitative distinctions in dye behavior were great enough to delineate clear structure-activity relationships. Only dyes bearing a butyl group specifically on the thiobarbituric acid moiety (position B in Table I) showed any response to calmodulin. Although all but one dye with a butyl group in that position did show at least a slight response, only the structures with a positively charged group attached at a second position were strongly affected by calmodulin.

**Design of the Reactive Merocyanine Dye.—**The structure-activity studies described in the preceding sections guided synthesis of a reactive dye for calmodulin conjugation. In this structure, a reactive alkyl isothiocyanate group was added to the side chain configuration which had given optimal calmodulin binding. The alkyl isothiocyanate group forms stable covalent linkages with primary or secondary amines, including the side chain amine of lysine, but is relatively less reactive than the more commonly used active esters or aromatic isothiocyanates. Its lower reactivity enhanced selective attachment based on lysine reactivity and favored calmodulin conjugation through an affinity labeling process. Conjugation was performed under high calcium conditions which favored binding of the dye to calmodulin prior to covalent linkage, thus producing attachment at positions giving access to the calmodulin-dependent binding site.

Although the O-TBA fluorophore had been used in structure-activity studies of calmodulin binding, difficulties in synthesis of a reactive O-TBA derivative led to a reexamination of the S-TBA fluorophore. Before proceeding with attempts to incorporate reactive functionality in an S-TBA dye, an S-TBA analog with the side chain configuration that had produced optimal calmodulin binding in the O-TBA fluorophore was synthesized (dye Mc4.18). As shown in Table I, it too displayed the promising calmodulin binding characteristics of the O-TBA analog.

The fluorescence of this S-TBA analog was examined. The emission maximum was 610 nm in water, 621 nm in butanol, and 640 nm in Me2SO. The excitation maximum shifted from 570 nm in water to 599 nm in Me2SO, and its quantum yield increased from 0.35 in butanol to 0.54 in octanol.

Comparison of the dye's aqueous absorbance and excitation spectra strongly suggested the existence of two or more absorbing species, as the fluorescence quantum yield appeared to be much lower at shorter wavelengths. Irradiation of aqueous dye solution at different exciting wavelengths had little effect on the emission maximum or shape of the aqueous emission spectrum, suggesting the presence of only one fluorescent species. Comparison of absorbance and excitation spectra in solvents of different polarity indicated that lower solvent polarity favored formation of the fluorescent species, which absorbed at longer wavelengths. Consistent with this, the proportion of the more fluorescent species was also increased in aqueous solutions of lower ionic strength.

An S-TBA analog bearing the reactive isothiocyanate group and side chains producing the desired calmodulin binding was synthesized (Table I, dye Mc4.19). In this structure, the side chains which had produced optimum calmodulin binding in dyes Mc4.12 and Mc4.18 were retained, but one methyl group on the quaternized nitrogen was replaced by a propyl isothiocyanate chain. When assayed for binding to calcium-calmodulin, the reactive dye showed the same favorable properties seen previously in dyes Mc4.12 and Mc4.18 (see Table I).

**Calmodulin Labeling.—**Calmodulin was covalently labeled with the reactive merocyanine dye (Mc4.19) using the affinity labeling conditions described above. Dye was added in small aliquots over several hours to maximize the proportion of dye bound and minimize hydrolysis of the reactive group. The concentration of buffer salts was kept low to minimize the formation of weakly fluorescent dye species, which could be dye aggregates or other species with altered calmodulin binding properties. It has been shown that the differential reactivity of calmodulin's lysines is greatly enhanced at high calcium and lower pH (Giedroc et al., 1985; Mann and Vanaman, 1987, 1989). Labeling was performed at pH 8.3, well below the solution pK, of lysine's ε-amino group.

An absorbance spectrum taken after addition of the first dye aliquot, with calmodulin present in great excess over dye, closely resembled spectra of the dye in organic solvents. After longer incubation times and addition of more dye, absorption spectra of the reaction mixture showed the appearance of a
prominent short wavelength shoulder, typical of aqueous dye spectra. An absorbance spectrum taken when the reaction was halted showed that greater than 85% of the fluorophore remained intact.

The reaction mixture was passed through a hydrophobic phenyl-Sepharose column using a gradient of decreasing calcium concentration. Thus, labeled calmodulin retaining calcium-dependent hydrophobic binding was separated from other protein species and from unreacted dye. The labeled protein fraction which had bound to the phenyl-Sepharose column in a calcium-dependent fashion was named MeroCaM. It was desalted by passage through Sephadex equilibrated with volatile buffer and lyophilized prior to storage. The calcium-dependent binding of calmodulin to phenyl-Sepharose has been used previously for calmodulin purification and for other investigations of calmodulin biochemistry (Vogel et al., 1983; Gopalakrishna and Anderson, 1985a, 1985b; Battey and Venis, 1988).

In a control experiment, calmodulin was incubated with the unreactive dye Mc4.18 in conditions shown during structure-activity experiments to produce dye-calmodulin binding. The side chains on this unreactive dye were similar to those on the reactive merocyanine. When the noncovalent complex was eluted from the column, but all dye remained fixed as a purple band at the origin. This purple band could not be removed even by washing with 6 M urea. When calmodulin which had been subjected to the covalent labeling reaction was passed through phenyl-Sepharose, a purple band of unreacted dye was also left on the column. These observations indicated that the phenyl-Sepharose column was removing noncovalently attached dye from the protein, and that all the absorption of the protein fractions at greater than 500 nm was due to covalently attached dye. Control experiments showed that a desalting column equilibrated with EGTA could not remove noncovalently attached dye from the protein.

SDS and native gel electrophoresis of MeroCaM showed greater than 95% of the protein in one band, which was visualized by its fluorescence when excited at 200–300 nm, by inspection of the dye in the gel, and by Coomassie staining. The mobility of this material was altered by the addition of calcium or EGTA to the sample buffer, exactly as was native calmodulin.

As a further demonstration that free dye was not present in the MeroCaM, tracking dye was omitted from the gel sample buffer so that unattached dye running with very low apparent molecular weight could be seen. Only dye which coincided in position with the Coomassie-stained protein band was observed. The validity of this procedure was demonstrated by incubating calmodulin with the unreactive dye Mc4.14 under conditions shown in structure-activity studies to promote binding. When this complex was subjected to gel electrophoresis, all dye was seen to run with very low apparent molecular weight, well clear of the protein band. The results of the control were the same with high and low protein loads. Similar results were obtained by paper chromatography.

MeroCaM Characterization—MeroCaM was found to have a dye/protein ratio of 1.0. The concentration of dye and calmodulin in aqueous solutions of MeroCaM was determined and used to calculate this value. Protein concentration was determined by amino acid analysis of a lyophilized sample, and dye concentration by adding a small amount of the solution to Me$_2$SO and taking the visible absorbance spectrum. Dissolving MeroCaM in Me$_2$SO minimized possible interaction of the dye with protein and allowed the use of an accurate dye extinction coefficient not likely affected by the presence of multiple dye species. Boiling the Me$_2$SO solution had no effect on the spectrum, and the shape of the spectrum was indistinguishable from that of the free dye in Me$_2$SO.

The calcium dependence of MeroCaM's spectral characteristics was next investigated. MeroCaM spectra were taken in two solutions buffered with EGTA to pCa 4.0 or 9.0 (0.2 or 0.5 mM EGTA, 100 mM KCl, 10 mM MOPS-KOH, pH 7.0, 25 °C). The MeroCaM emission maximum remained essentially constant at 620 nm in either solution. Changing the excitation wavelength to 500, 550, or 570 nm had no appreciable effect on the position of the emission maximum or the shape of the emission curve.

Large calcium-dependent differences in both excitation and absorbance were observed, as shown in Fig. 3. At both pCa 4.0 and 9.0, nonsuperimposable absorbance and excitation spectra indicated the presence of more than one form of the dye. This was consistent with a calcium-induced increase in the proportion of a more fluorescent dye species absorbing at longer wavelengths. Other explanations, including the presence of analogs labeled in different sites, are possible but less likely in light of the dye/protein ratio of 1 and the homogeneity of the labeled protein in SDS and native PAGE.

At higher calcium concentrations (pCa 4.0 versus 9.0), the excitation spectra showed a drop in intensity at short wavelengths, an increase in long wavelength intensity, and a 10 nm shift to longer excitation maximum. A difference spectrum of the high and low calcium excitation spectra (Fig. 4) revealed that the maximum calcium-induced excitation decrease was at 532 nm, and the maximum increase at 608 nm. A ratio of excitation at these two wavelengths therefore provided a more calcium-sensitive spectral parameter than any single wavelength measurement.

At 532 nm, where the maximum calcium-induced drop in excitation occurred, signal intensity was low. Fortunately, the drop showed a similar amplitude at wavelengths extending in a wide range above and below 532 nm. This would permit light collection using a broad bandwidth filter, which could increase the signal/noise ratio without great detriment to dynamic range.

Fig. 5 shows the MeroCaM 608/532 nm excitation ratio as a function of calcium concentration. MeroCaM was dissolved in a series of buffers with pCa ranging from 4.0 to 9.0 (0.2 or 0.5 mM EGTA, 100 mM KCl, 10 mM MOPS-KOH, pH 7.0, 25 °C). The excitation ratio showed a 3.4-fold change, with a sigmoidal dependence on calcium concentration similar in shape to the reported calcium-binding isotherm of calmodulin, and a half-maximal change at 300–400 nM calcium. Spectral changes induced by calcium were completely reversed by the addition of buffered EGTA to the dye solution. A region between the two calcium-sensitive portions of the excitation spectrum was minimally affected by calmodulin (see Figs. 3 and 4), but no clearly defined isosbestic point was observed.

The calcium dependence of CAMP phosphodiesterase activation by calmodulin and MeroCaM were compared. At differing calcium concentrations, a constant amount of either MeroCaM or calmodulin was incubated with excess phosphodiesterase, and the rate of CAMP hydrolysis was measured. Phosphodiesterase was present in sufficient excess to assure that the rate of phosphodiesterase-catalyzed CAMP hydrolysis would reflect the extent of activation of calmodulin by calcium, and not the affinity of calcium-calmodulin for phosphodiesterase. Results of these experiments, shown in Fig. 6, indicate a very similar calcium response for native and derivatized protein. The half-maximal change in rate was 1–5 μM for both calmodulin and MeroCaM, and both curves showed a similar sigmoidal dependence on calcium concentration. The
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Fig. 3. Calcium-dependent changes in MeroCaM absorbance and in fluorescence excitation. MeroCaM was dissolved in Ca/EGTA buffers of pCa 4.0 and 9.0 at pH 7.0 and 0.1 M ionic strength. Absorbance and excitation of 623 nm emission were monitored. The drop in apparent quantum yield at shorter wavelengths indicated the presence of multiple dye species. The calcium-induced shift in the spectra can be attributed to changes in the proportion and/or spectral properties of a more fluorescent species.

Fig. 4. Difference in MeroCaM pCa 4.0 and 9.0 excitation spectra. The above plot shows the wavelength-dependent difference between MeroCaM excitation spectra taken in buffers of pCa 9.0 and 4.0, at pH 7.0 and 0.1 M ionic strength. Emission was measured at 623 nm. The largest calcium-induced differences in excitation were the increase at 608 nm and the decrease at 532 nm. The ratio of these two wavelengths was a more calcium-sensitive parameter than any single wavelength measurement.

Fig. 5. Calcium dependence of the MeroCaM excitation ratio. The 608/532 nm ratio of MeroCaM's excitation intensities was measured at different calcium concentrations (with emission at 623 nm). MeroCaM was dissolved in EGTA/calcium buffers at pH 7.0 and 0.1 M ionic strength. The sigmoidal dependence of excitation ratio on calcium concentration resembled calmodulin's calcium binding isotherm. The change in excitation ratio from its lowest to highest point was 3.4-fold, with half-maximal change occurring at 300-400 nM calcium.

Fig. 6. Calcium dependence of calmodulin and MeroCaM activation of cAMP phosphodiesterase. A fixed concentration of either calmodulin or MeroCaM was incubated, at differing calcium concentrations, with excess cAMP phosphodiesterase. Rates of cAMP hydrolysis at different calcium concentrations are plotted. ○, calmodulin; ▲, MeroCaM. The half-maximal change in rate was at 1–5 μM calcium for both MeroCaM and calmodulin. MeroCaM produced 30–40% of the maximal activation of calmodulin.

Similar calcium binding behavior of calmodulin and MeroCaM indicated that the known phosphodiesterase-induced increase in calmodulin's calcium affinity was extant in MeroCaM. Phosphodiesterase increases the affinity of calmodulin's high affinity sites 2–10-fold, and of the low affinity sites 100-fold (Klee, 1988). MeroCaM's maximal activation of the phosphodiesterase was 30–40% that of native calmodulin.

MeroCaM retained calmodulin's ability to activate myosin light chain kinase. The phosphorylation of myosin light chains by myosin light chain kinase was assayed as a function of calmodulin and MeroCaM concentration. The results of these assays, shown in Fig. 7, indicate that MeroCaM was able to activate myosin light chain kinase to the same maximal level as did calmodulin. Furthermore, the curves for concentration dependence of activation, and hence the binding affinities for the kinase, were indistinguishable for calmodulin and MeroCaM.

In order to assess the possible effects of target protein binding on MeroCaM fluorescence, the calcium dependence of the fluorescence ratio was measured in the presence of melittin. This peptide has been studied as a model for the binding domains of calmodulin target proteins (Comte et al., 1983; Maulet and Cox, 1983; Cox et al., 1985; Seeholzer et al., 1986; Cohen and Klee, 1988). The experiment was carried out in the phosphodiesterase assay buffer to simplify comparison...
Magnesium-induced decrease in calmodulin's calcium affinity.

The probe was sufficiently photostable, bright, and nontoxic to permit multiple measurements at acceptable light levels and intracellular concentrations. Experiments using MeroCaM in vivo will be published elsewhere.

Synthesis of Dyes—All dyes were made using the generalized procedure presented in Fig. 1. This convergent synthesis allowed the ready production of dyes with a wide variety of side chains. The two heterocyclic rings comprising each end of the merocyanine dyes were synthesized separately and then linked together with a polymethine bridge derived from 1,3,3-trimethoxypropene. Others have used similar methodology to synthesize merocyanine and cyanine dyes, but the polymethine bridge was most often derived from a malonaldehyde dianil (Hamer, 1984). In initial trials, 1,3,3-trimethoxypropene or malonaldehyde dianil was combined with the benzoaxazole or thiobarbituric acid intermediates, but initial attachment of 1,3,3-trimethoxypropene to the thiobarbituric acid proved to be a superior approach. The thiobarbituric acid-trimethoxypropene condensation product was produced with high purity and yield simply by adding excess trimethoxypropene to a methanolic solution of thiobarbituric acid, and collecting the resulting precipitate.

The high temperatures required for quaternization of 2-methylbenzothiazole or 2-methylbenzoxazole restricted the functionality which could be present at this step, but the 3-iiodopropyl group introduced through reaction with 1,3-diiodopropene could itself be used to quaternize an alkyl amine under much milder conditions. This milder quaternization reaction allowed introduction of the sensitive isothiocyanate group as the last step in the synthesis, through reaction of dye Mc4.17 with N,N-dimethyl-3-aminopropylisothiocyanate.

Coupling of the thiobarbituric acid trimethoxypropene condensation product with quaternized 2-methylbenzoxazoles using weaker bases such as triethylamine, pyridine, or sodium acetate produced a very small yield of O-TBA dye. Reasonable yields were obtained only by using sodium ethoxide. In contrast, dyes made from 2-methylbenzothiazoles formed readily with pyridine. The milder base used for S-TBA formation allowed greater flexibility in the introduction of sensitive functionality. This fluorophore was therefore emphasized during development of a reactive dye for calmodulin conjugation, and was used in MeroCaM. The methods developed for incorporation of the sensitive isothiocyanate group into the S-TBA dye should, however, be adaptable to the synthesis of reactive dyes based on other fluorophores, including O-TBA.

Although many of the dyes were readily purified by silica gel chromatography, those bearing both a quaternary alkyl amine and hydrophobic butyl groups presented unusual difficulties. These dyes remained fixed at the origin on both silica and reversed phase media in any solvent tested. Purification was accomplished by chromatography on cellulose. Reaction and purification of some dyes with 1,3-dimethylthiobarbituric acid end groups (dyes Mc4.15 and Mc4.16) were complicated by their unusually low solubilities in a wide range of solvents. These dyes were practically insoluble in all solvents tested except Me$_2$SO, in which they were only very sparingly soluble.

**Discussion**

Although the interaction of fluorescent dyes with proteins has been used extensively to study protein conformational changes in vitro, the dyes and methods employed have not been optimal for monitoring conformational changes in living cells. Fluorescent analogs of cellular proteins observed in living cells have utilized fluorescent probes in the visible wavelengths, with the largest possible product of extinction coefficients and quantum yields. An absence of environmental

![Figure 7](image-url) Calmodulin and MeroCaM activation of myosin light chain kinase. Calmodulin or MeroCaM was incubated with myosin light chain kinase (MLCK), myosin light chains, and [32P]ATP. The level of [32P]incorporation into the light chains was measured, at a fixed time interval and kinase concentration, for differing concentrations of calmodulin or MeroCaM. The concentration dependence and maximal kinase activation were very similar for calmodulin and MeroCaM.

![Figure 8](image-url) Effect of melittin on the calcium dependence of MeroCaM's excitation ratio. The excitation ratio of 123 nM MeroCaM (505/532 nm, 623 nm emission) was measured at differing calcium concentrations with and without 378 μM melittin, in 1.4 mM Mg$^{2+}$ at pH 7.0 and 0.1 M ionic strength. O, no melittin; +, with melittin. The half-maximal change was at 80–150 μM in the presence of melittin and at 1–5 μM without it. Melittin caused an increase in the excitation ratio of MeroCaM at low calcium.

with other experiments. Fig. 8 shows the excitation ratio of 123 nM MeroCaM as a function of calcium concentration, in the presence and absence of 378 nM melittin. The half-maximal change in excitation ratio was shifted to lower calcium by melittin (1–5 μM versus 50–150 nM), as is calmodulin’s calcium dissociation constant (Maulet and Cox, 1983). The plateau in MeroCaM’s excitation ratio at lowest calcium levels occurred at a higher value in the presence of melittin.

The effect of magnesium on the calcium dependence of MeroCaM's excitation ratio can be seen by comparing Figs. 5 and 8. The experiments shown in these two figures were performed under very similar conditions, except that magnesium was included in the buffer used to produce Fig. 8. Magnesium shifted the half maximal change in excitation ratio to a higher calcium level, consistent with the reported magnesium-induced decrease in calmodulin's calcium affinity (Haiech et al., 1981; Klee, 1988). Preliminary studies involving microinjection and imaging of MeroCaM in living fibroblast cells gave promising indications that the probe was sufficiently photostable, bright, and
sensitivity has usually been sought to enable the tracking of the analogs by imaging methods (Simon and Taylor, 1986; Wang, 1989). Mapping the distribution of a protein's conformational states in vivo could reveal information about protein function and regulation inaccessible to in vitro studies.

In MeroCaM, we have sought to fulfill the unique requirements of a fluorescent protein analog to be used in live cells. Although the potential for improvement is clear, we believe MeroCaM is a useful "first generation" analog, which can provide meaningful information about calcium-calmodulin interaction in live cells, and which exemplifies the feasibility of designing conformationally sensitive fluorescent analogs for live cell studies.

Published studies and empirical screening led to selection of the S-TBA fluorophore for use in MeroCaM. This fluorophore showed not only the strong spectral sensitivity required to report protein conformational change, but also possessed several other properties uniquely required of a dye to be used in living cells. It showed solvent-sensitive fluorescence changes suitable for the application of fluorescence ratiometry. Ratiometry is highly desirable for eliminating the effects of cell thickness, probe concentration, and other factors on interpretation of probe emission (Bright et al., 1989). The S-TBA dye's long excitation and emission wavelengths obviated serious problems known to be associated with the use of shorter wavelengths that coincide with cellular absorbance and autofluorescence. Autofluorescence can interfere with signal acquisition and interpretation (Taylor and Salmon, 1989), and absorbance by cellular components can lead to cell damage (Waggone, 1986). Other important characteristics of the dye were judged during preliminary observations of MeroCaM in live cells, as will be discussed.

To reflect calcium-induced changes in calmodulin conformation, the dye had to bind to the protein in a calcium-dependent manner, reversibly, and with a configuration producing strong spectral change. Side chains on the fluorophore were shown to have a strong effect on its calmodulin binding properties. Structure-activity studies of dyes with different side chain configurations led to the development of an analog showing strong, reversible, calcium-dependent spectral changes in the presence of calmodulin. The structure-activity relationships observed in these studies reflected the reported binding requirements of other calmodulin ligands. A hydrophobic domain, like the butyl chains on the optimized merocyanine dye, is common to almost all known ligands. As with the merocyanine, the binding of many molecules is greatly enhanced by the presence of a positive group (Gietzen et al., 1981; Cox et al., 1985; Hidaka and Harteshorne, 1985; Xu and Zhang, 1986). The orientation of the charged residue relative to the hydrophobic domain has also been shown to be important (Inagaki et al., 1983; Hidaka and Harteshorne, 1985).

Covalent attachment of dye to protein is required in the complex environment within a live cell, and enables intracellular localization of the analog. The S-TBA analog which had shown optimal calmodulin binding was modified to include the lysine-reactive alkyli thiocyanate group for dye-calmodulin conjugation. Conditions of the conjugation reaction were chosen to encourage affinity labeling of the calcium-dependent dye binding site, and to enhance selective labeling based on lysine reactivity. MeroCaM isolated using hydrophobic affinity chromatography had a dye/protein ratio of 1, and appeared homogeneous by native and SDS-PAGE run in high or low calcium. This strongly suggested that a single species had been isolated. Highly selective labeling of calmodulin lysines has previously been achieved using affinity labels and hydrophobic probes (Jackson and Puett, 1984; Faust et al., 1987), including an alkyl isothiocyanate derivative of a hydrophobic calmodulin binding drug (Newton et al., 1983; Newton and Klee, 1989).

The point of dye attachment and nature of the covalent linkage can strongly affect the dye's response to protein conformational change. Calcium concentration changes did not induce spectral shifts in MeroCaM as great as those seen when the fluorophore alone was dissolved in different solvents, or when the free dyes Mc4.12 or Mc4.18 were exposed to calcium-calmodulin. MeroCaM did, however, show a greater than 3-fold change in its 608/532 nm excitation ratio when exposed to 10 nM versus 6 μM calcium. These changes were shown to be fully reversible. The excitation and emission maxima of MeroCaM in high or low calcium were close to those of the free dye in organic solvents, and never approached the shorter wavelength maxima of free dye in aqueous solutions. This suggests that changes in calcium concentration induced movement of the dye between two hydrophobic protein environments, rather than between a protein site and the aqueous solution. Increasing the water solubility of the dye could enhance the spectral response.

Although MeroCaM's excitation ratio showed a sigmoidal dependence on calcium concentration similar in shape to calmodulin's calcium binding isotherm, it was clear that MeroCaM's excitation ratio changes did not precisely parallel calmodulin's calcium binding behavior. The half-maximal change in excitation ratio occurred at calcium levels well below those reported to produce half-maximal calcium-calmodulin binding under similar conditions. Under the conditions used to produce Fig. 5, where the half-maximal change in excitation ratio occurred at 300-400 nM calcium, calcium-calmodulin binding has been shown to reach half-maximal saturation at 5-10 μM calcium (Klee, 1988).

A shift to tighter calcium binding in MeroCaM might be expected as a result of free energy differences produced by dye-protein binding. Alternately, the fluorescent dye on MeroCaM might respond differentially to the filling of each calcium site, showing greatest sensitivity to initial calcium binding. To distinguish between these possibilities, MeroCaM-calcium binding was investigated using a method that did not rely on fluorescence measurements. The calcium binding of calmodulin and MeroCaM was compared by observing the calcium dependence of their cAMP phosphodiesterase activation (Fig. 6). In this assay, the calcium binding properties of MeroCaM and native calmodulin were indistinguishable. Thus attachment of the dye did not greatly perturb MeroCaM's calcium binding properties. Most of MeroCaM's calcium dependent fluorescence changes apparently occur during calcium binding to the high affinity sites. The calcium binding constants of the two high affinity sites have been determined (Haiech et al., 1981), and are close to the calcium concentrations producing half-maximal fluorescence change in MeroCaM.

Several experiments shed light on the ability of MeroCaM to interact with target proteins and allosteric effectors. MeroCaM's maximal activation of phosphodiesterase was 30-40% that of native calmodulin. In an assay of myosin light chain kinase activation, both the dependence of kinase activity on activator concentration and the level of maximal activation were the same for MeroCaM and the native protein. Changes in MeroCaM's calcium affinity induced by phosphodiesterase, melittin, and magnesium were similar to those reported for calmodulin.

MeroCaM excitation ratios measured in phosphodiesterase assay buffer, but without phosphodiesterase (Fig. 8), showed a half-maximal change at the same calcium concentration...
that induced the half-maximal hydrolysis rate in the phosphodiesterase assay (Fig. 6). Had phosphodiesterase not affected the calcium affinity of calmodulin and MeroCaM, the half-maximal hydrolysis rate would have occurred at a higher calcium level than the half-maximal change in excitation ratio (see above).

Myosin light chain kinase and cAMP phosphodiesterase activation are among the calmodulin functions most sensitive to calmodulin modification. Unlike activation of some other proteins, these activities are perturbed both by certain calmodulin enzymatic digestion and derivatization procedures (Klee, 1988). It has been proposed that calmodulin interaction with many target proteins requires either one or both of two calmodulin sites (Klee, 1988). Current evidence is consistent with a requirement for binding to both sites by myosin light chain kinase and phosphodiesterase (Klee, 1988). MeroCaM’s activation of these two proteins is therefore a good indication that its activation of other proteins will remain intact. The difference in MeroCaM’s maximal activation of the two proteins is noteworthy in light of their usually similar response to calmodulin alterations (Klee, 1988).

The calcium dependence of MeroCaM’s excitation ratio was examined in the presence and absence of melittin, in order to explore possible effects of target protein binding on MeroCaM fluorescence. The plateau in MeroCaM’s excitation ratio at lowest calcium levels occurred at a higher value in the presence of melittin. This was not consistent with the reported melittin-calmodulin binding constants, which indicate that apocalmodulin would be greater than 95% free of melittin under the experimental conditions (K, for melittin: 3 nm for calcium-calmodulin, 10 μM for apocalmodulin; Maulet and Cox, 1983). These results indicate direct interaction between melittin and the bound dye, or an allosteric alteration of the melittin binding site by the dye. The experiment demonstrates the possibility of environment-specific effects on calcium-induced changes in MeroCaM’s fluorescence.

MeroCaM in its current state should enable the investigation of calcium-calmodulin interactions in living cells and in biochemical reconstitutions. Its strong and reversible calcium-dependent fluorescence changes occur at long wavelengths and permit application of ratio imaging techniques. It retains at least some of calmodulin’s regulatory activity, and preliminary experiments in live cells have shown it to possess the photostability and signal intensity required for observation of individual cells. Its primary disadvantages, in its present form, lie in the environmental sensitivity of its calcium response, and fluorescence changes which reflect primarily calcium binding to the high affinity sites. However, the present analog could provide quantitative measurements of calcium binding after normalization, under controlled conditions in vitro. In living cells, useful observations of calcium-calmodulin binding changes relative to transients in free calcium ion concentration should be possible.

The present MeroCaM fluorescent analog will serve as the starting point for optimizing the ability to “sense” specific calmodulin functions. For example, derivatization of calmodulin fragments, combined with chemical modification, may lead to production of analogs which function as probes of calcium concentration unaffected by the presence of target proteins. In addition, we hope to prepare an analog with sensitivity to target proteins, unaffected by calcium binding.

MeroCaM demonstrates the feasibility of designing fluorescent analogs which report protein conformational changes in living cells. It is hoped that dyes similar to the one developed for this study will enable design of other protein-based indicators for use in living cells. The remarkable binding specificity of proteins provides a unique basis for the design of intracellular reagents sensitive to a range of biologically important molecules.

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REFERENCES


A Calcium-sensitive Fluorescent Analog of Calmodulin


Supplemental Material to:

A CALCIUM-SENSITIVE FLUORESCENT CALMODULIN ANALOG

Klaus M. Hahn, Alan S. Waggoner, D. L. Taylor
A Calcium-sensitive Fluorescent Analog of Calmodulin

5-(3-Methoxyallyliden)-1,3-dibutylthiobarbituric acid (IX). This compound was carried out using flame dried glassware and azobenzene solvents and reagents. 1,3-Dibutylthiobarbituric acid (2 g) was dissolved in 20 mL of methanol while using nitrogen gas to displace all air. After the mixture had been stirred for a few minutes, the mixture was poured into a beaker containing 1 L of rapidly stirring ether. The reaction mixture was then filtered and washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.

Dye Me4.15. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and azobenzene solvents. 3-(3-Methoxyallyliden)-1,3-dibutylthiobarbituric acid (19 mg) was dissolved in 15 mL of 1:9 methanol: dichloromethane. Sodium methoxide (16 mg) was added, followed by sodium methoxide (16 mg). The reaction mixture was allowed to stand at room temperature for 15 minutes. After stirring for an additional 2 minutes, the reaction mixture was poured into 100 mL of dichloromethane. The product was then washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.

Dye Me4.16. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and azobenzene solvents. 5-(3-Methoxyallyliden)-1,3-dibutylthiobarbituric acid (41 mg) was dissolved in 15 mL of 1:9 methanol: dichloromethane. Sodium methoxide (16 mg) was added, followed by sodium methoxide (16 mg). The reaction mixture was allowed to stand at room temperature for 15 minutes. After stirring for an additional 2 minutes, the reaction mixture was poured into 100 mL of dichloromethane. The product was then washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.

Dye Me4.17. Dye Me4.17 (95 mg) was dissolved in 10 mL of methanol and then diluted with 10 mL of dichloromethane. The reaction mixture was allowed to stand at room temperature for 15 minutes. After stirring for an additional 2 minutes, the reaction mixture was poured into 100 mL of dichloromethane. The product was then washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.

Dye Me4.18. Dye Me4.18 (45 mg) was dissolved in 10 mL of methanol and then diluted with 10 mL of dichloromethane. The reaction mixture was allowed to stand at room temperature for 15 minutes. After stirring for an additional 2 minutes, the reaction mixture was poured into 100 mL of dichloromethane. The product was then washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.

Dye Me4.19. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and azobenzene solvents. 3-(3-Methoxyallyliden)-1,3-dibutylthiobarbituric acid (19 mg) was dissolved in 15 mL of 1:9 methanol: dichloromethane. Sodium methoxide (16 mg) was added, followed by sodium methoxide (16 mg). The reaction mixture was allowed to stand at room temperature for 15 minutes. After stirring for an additional 2 minutes, the reaction mixture was poured into 100 mL of dichloromethane. The product was then washed with ether. The remaining solid was then dried under vacuum at room temperature. The product was then dried under vacuum for 96 hours to obtain a pure product. The product was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue. The residue was then dissolved in dimethylsulfoxide and vacuum evaporated to a solid residue.