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

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Structure-activity studies of tetramethinemerocyanine 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, 1982; Manalan and Klee, 1984; Van Eldik and Watterson, 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 (Hamaguchi and Isawa, 1980; Keith *et al.*, 1983; Welsh, 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 Wittenauer, 1983; Olwin and Storm, 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 Harteshorne, 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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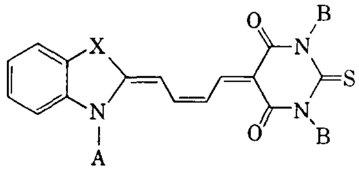
TABLE I

Effect of dye structure on calcium-calmodulin binding

Calmodulin (3.3 equivalents) was added to 2.5 µM dye dissolved in 1 mM $CaCl_2$, 10 mM Tris, pH 7.0. The effects of calmodulin 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.



Dye	X	A	B	CaM binding
O-TBA dyes				
Mc4.10	O	$-CH_3$	$-(CH_2)_3CH_3$	+
Mc4.11	O	$-(CH_2)_3I$	$-(CH_2)_3CH_3$	+
Mc4.12	O	$-(CH_2)_3N^+(CH_3)_3$	$-(CH_2)_3CH_3$	++++
Mc4.13	O	$-(CH_2)_3SO_3^-$	$-(CH_2)_3CH_3$	+
Mc4.14	O	$-(CH_2)_3CH_3$	$-CH_3$	–
Mc4.15	O	$-(CH_2)_3I$	$-CH_3$	–
Mc4.16	O	$-(CH_2)_3N^+(CH_3)_3$	$-CH_3$	–
S-TBA dyes				
Mc4.17	S	$-(CH_2)_3I$	$-(CH_2)_3CH_3$	–
Mc4.18	S	$-(CH_2)_3N^+(CH_3)_3$	$-(CH_2)_3CH_3$	++++
Mc4.19	S	Final reactive dye[a]		++++

[a] A, $-(CH_2)_3N^+(CH_3)_2-(CH_2)_3NCS$; B, $-(CH_2)_3CH_3$.

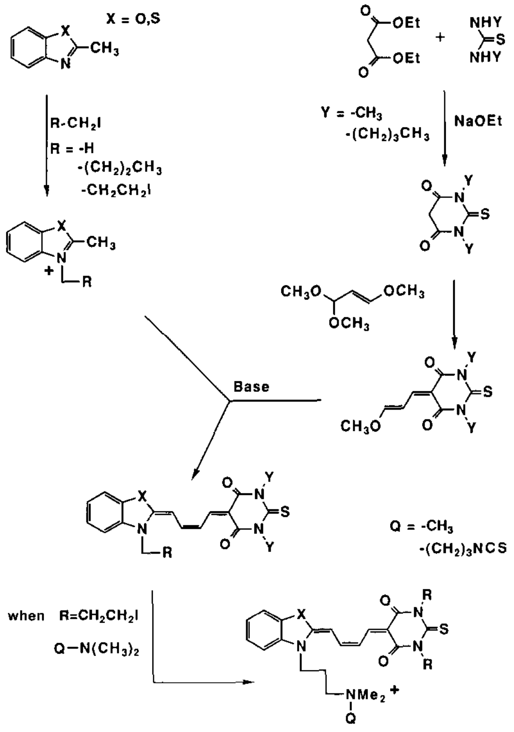


FIG. 1. **Dye synthesis.** This convergent synthesis was well suited to variation of the dye structures. Terminal groups were synthesized separately and linked in different combinations. The sensitive alkyl isothiocyanate group was introduced in the final step through a mild quaternization reaction.

solvent polarity, was covalently attached to calmodulin. 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[1]

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.[2] 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 Me_2SO before addition to aqueous solutions. Dye concentrations in Me_2SO 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,

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[1] Part of "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: S, benzothiazole end group; O, benzoxazole; BA, barbituric acid; TBA, thiobarbituric acid; I, 3,3-dimethylindolenine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CaM, calmodulin; MeroCaM, covalent adduct of calmodulin and dye Mc4.19; Me_2SO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FAB MS, fast atom bombardment mass spectrum.

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, amino acid composition, and ultraviolet absorption spectrum. It was stored at −80 °C, either as a lyophilized powder or in frozen aqueous solution.

Gel Electrophoresis—SDS-PAGE of calmodulin and MeroCaM was carried out in 12% gels using the method of Laemmli (1970). Native PAGE was carried out in 15% gels using the same method but with omission of SDS throughout. The sample buffer was made 10 mM in calcium chloride or EGTA to control calcium concentration.

Protein Concentrations and MeroCaM Dye/Protein—The concentration of calmodulin and MeroCaM in aqueous stock solutions was determined from amino acid analysis data (carried out by the University of Pittsburgh Amino Acid Analysis Facility). The concentration of dye in aqueous solutions was determined by dissolving an aliquot of the solution in Me_2SO, such that total aqueous volume was less than 6%, and measuring the absorbance of the dye. The concentration of melittin was determined using a molar extinction coefficient of $\epsilon_{280\,\text{nm}} = 5470\ \text{M}^{-1}\ \text{cm}^{-1}$ (Cox *et al.*, 1985). For MeroCaM with known dye/protein, the concentration of aqueous solutions was determined by adding a solution aliquot to Me_2SO and measuring dye absorbance.

Calmodulin Binding Studies—A description of these studies can be found in the legend to Table I. To assay the reversibility of dye Mc4.12 binding to calmodulin, 400 ml of aqueous calmodulin was first added to a dye solution, producing final concentrations of 2.3 mM dye and 7.4 mM calmodulin in 2.4 ml of 41 mM Tris-HCl, pH 7.0. Next, EGTA (250 ml of a 0.1 M, pH 7.0, solution) was added, and absorption and fluorescence spectra were monitored throughout.

Preparation of MeroCaM—Calmodulin (9 mg, 0.53 μmol) was dissolved in 2.5 ml of 2 mM $CaCl_2$, 10 mM sodium borate, pH 8.3. The solution was clarified for 2 min at 20,000 × *g* and then incubated at 37 °C in the dark throughout the reaction with the dye. The reactive dye Mc4.19 was added as a 9 mM solution in Me_2SO. A total of 120 μl of dye solution (1.1 μmol, 2 equivalents) was added as 10 aliquots of approximately equal volume over a period of 4–5 h. The reaction mixture was vortexed during dye addition to prevent precipitation. The reaction mixture was incubated for 21 h after completion of dye addition, allowed to cool to room temperature, and made 10 mM in calcium chloride through addition of 0.1 M calcium chloride. It was then loaded on a 7 × 2-cm phenyl-Sepharose column (Sigma) which had been equilibrated with 0.1 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5. The column was eluted with 40 ml of 0.1 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, followed by a 100-ml gradient of 0–5 mM EGTA in 50 mM Tris-HCl, pH 7.0. The fraction size was 1 ml. Almost all protein eluted during the EGTA gradient as a single band with absorption at both 278 and 550 nm. This was MeroCaM, which was frozen, lyophilized to a volume of 1 ml or less, and then passed through a 17 × 1.5-cm column of Sephadex G-25 fine (Pharmacia LKB Biotechnology Inc.) equilibrated with 200 mg/liter ammonium bicarbonate. The purple protein, eluted in the void volume, was aliquoted, lyophilized to remove ammonium bicarbonate, and stored at −80 °C.

Calcium/EGTA Buffer Systems—Solutions were buffered with EGTA to maintain selected free calcium concentrations. The program of Robertson and Potter (1984) was used to calculate the total concentrations of EGTA and calcium required to produce a given free calcium concentration. EGTA concentrations were either 0.5 or 0.2 mM. Buffer pH was adjusted after the addition of all reagents. Buffers were made using high purity chemicals from Fluka: calcium chloride dihydrate, microselect grade; MOPS, microselect grade; EGTA, purissima grade.

cAMP Phosphodiesterase Activation Assay—The procedure of Schiefer (1986) was modified as follows: The assay buffer contained 1.4 mM $MgSO_4$, 47 mM KCl, and 105 mM MOPS-KOH, pH 7.0. Each point was obtained using 3 nM 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 phosphodiestrase level in the actual assay was held constant at 30% above the level producing maximal hydrolysis. Solutions were incubated for 5 min 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 $CaCl_2$ and 1 nM myosin light chain kinase. The reaction was terminated by spotting the reaction mixture on a circle of filter paper and dropping 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 mixtures were assayed at various 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-^{32}P]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, 1964; Sturmer, 1977; 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, 1964; Sturmer, 1977; 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 wavelength and a greater than 1.8-fold increase in extinction coefficient when dissolved in *n*-decanol 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.[3] 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 KCl 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

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[3] A. Waggoner, unpublished observations.

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 calcium-dependent binding site.

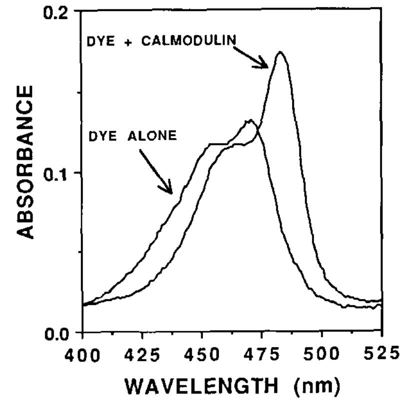

FIG. 2. **Response of dye Mc4.12 to calcium-calmodulin.** Calmodulin (3.3 equivalents) was added to a solution of 2.5 mM dye Mc4.12 in 1 mM $CaCl_2$, 10 mM Tris, pH 7.0. A 24-nm shift in absorbance maximum and 36% increase in extinction coefficient were observed. These spectra were taken as part of the structure-activity studies presented in Table I. The calmodulin-induced changes were fully reversed by the addition of EGTA to the assay medium.

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 Me_2SO. The excitation maximum shifted from 570 nm in water to 599 nm in Me_2SO, 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 fluorescing 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_a 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 passed down the phenyl-Sepharose column, calmodulin 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_2SO and taking the visible absorbance spectrum. Dissolving MeroCaM in Me_2SO 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_2SO solution had no effect on the spectrum, and the shape of the spectrum was indistinguishable from that of the free dye in Me_2SO.

The calcium dependence of MeroCaM's spectral characteristics was next investigated. MeroCaM spectra were taken in two solutions buffered with EGTA to *p*Ca 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 *p*Ca 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 wavelength. 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 (*p*Ca 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 *p*Ca 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

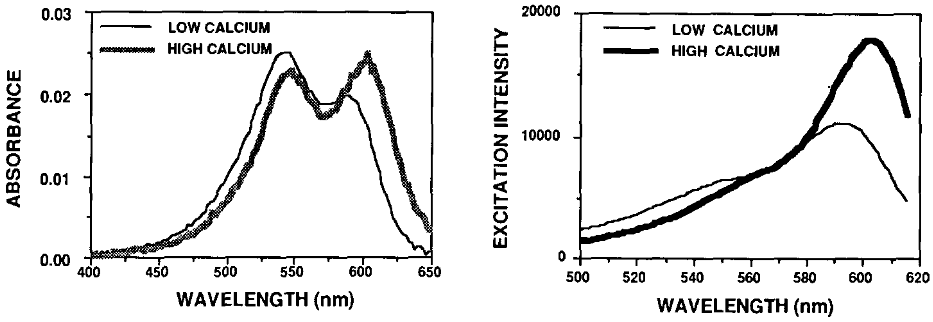

FIG. 3. **Calcium-dependent changes in MeroCaM absorbance and in fluorescence excitation.** MeroCaM was dissolved in Ca/EGTA buffers of *p*Ca 4.0 and 9.0 at pH 7.0 and 0.1 M ionic strength. Absorbance and excitation of 623 nm emission were monitored. The drop in apparent quantum yield at shorter wavelengths 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.

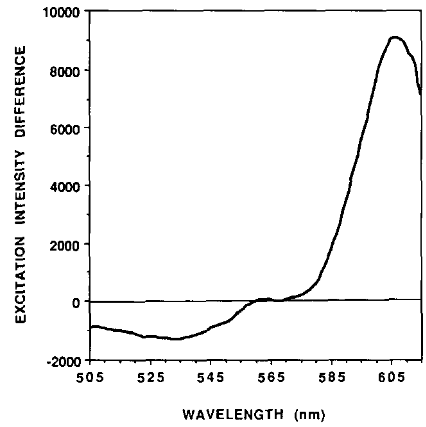

FIG. 4. **Difference in MeroCaM *p*Ca 4.0 and 9.0 excitation spectra.** The above plot shows the wavelength-dependent difference between MeroCaM excitation spectra taken in buffers of *p*Ca 9.0 and 4.0, at pH 7.0 and 0.1 M ionic strength. Emission was measured at 623 nm. The largest calcium-induced differences 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.

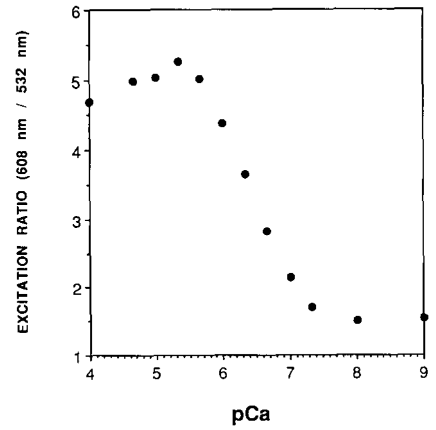

FIG. 5. **Calcium dependence of the MeroCaM excitation ratio.** The 608/532 nm ratio of MeroCaM's excitation intensities was measured at different calcium concentrations (with emission at 623 nm). MeroCaM was dissolved in EGTA/calcium buffers at pH 7.0 and 0.1 M ionic strength. The sigmoidal dependence 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.

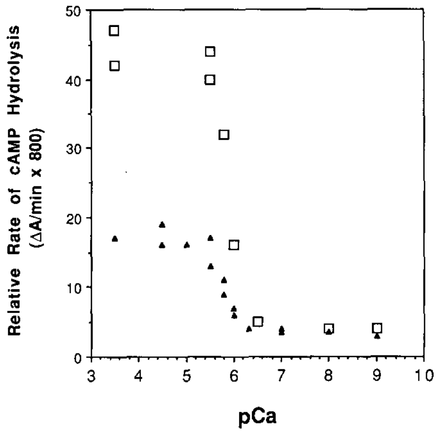

FIG. 6. **Calcium dependence of calmodulin and MeroCaM activation of cAMP phosphodiesterase.** A fixed concentration of either calmodulin or MeroCaM was incubated, at differing calcium concentrations, with excess cAMP phosphodiesterase. Rates of cAMP hydrolysis at different calcium concentrations are 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

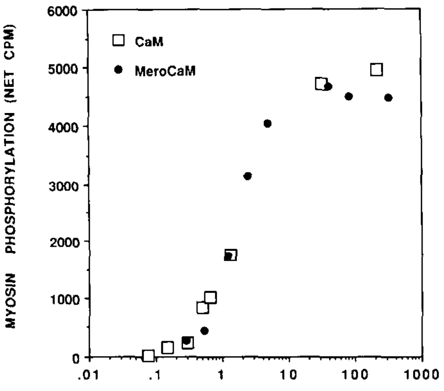

FIG. 7. **Calmodulin and MeroCaM activation of myosin light chain kinase.** Calmodulin or MeroCaM was incubated with myosin light chain kinase (*MLCK*), myosin light chains, and [^{32}P]ATP. The level of ^{32}P incorporation into the light chains was measured, at a fixed time interval and kinase concentration,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

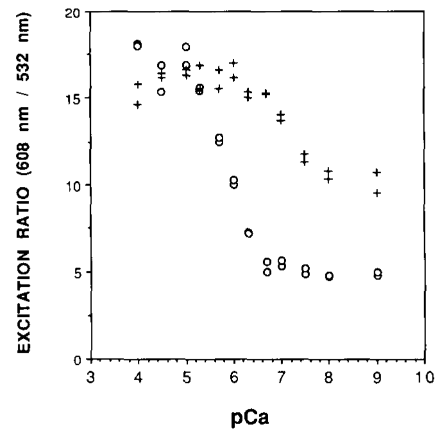

FIG. 8. **Effect of melittin on the calcium dependence of MeroCaM's excitation ratio.** The excitation ratio of 123 nM MeroCaM (608/532 nm; 623 nm emission) was measured at differing calcium concentrations with and without 378 nM melittin, in 1.4 mM Mg^{2+} at pH 7.0 and 0.1 M ionic strength. ○, no melittin; +, with melittin. The half-maximal change was at 50–150 nM 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 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, 1964). In initial trials, 1,3,3-trimethoxypropene or malonaldehyde dianil was combined with the benzoxazole 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-iodopropyl group introduced through reaction with 1,3-diiodopropane 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_2SO, 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 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 ratioing. Ratioing 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 (Waggoner, 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 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 alkyl isothiocyanate 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_d 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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Supplemental Material to:

A CALCIUM SENSITIVE FLUORESCENT CALMODULIN ANALOG

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General Synthetic. Unless otherwise noted, reagents and chromatographic media used for organic synthesis were obtained from the Aldrich Chemical Co (Milwaukee, WI). 1,3,3-Trimethoxypropene was from Kodak Chemical Co. (Rochester, NY). TLC[1] plates were obtained from the Analtech Co. (Newark, DE). Silica gel GF plates and RPS-F reversed phase plates were used. Flash column chromatography was performed according to Still et al. (1978), using 60 angstrom, 230-400 mesh silica gel. Cellulose 20 micron powder was used for cellulose chromatography.

Infrared spectra were obtained with a Nicholet Model 5DXB FT-IR, and NMR spectra with an IBM 300 FT-NMR. Mass spectra were obtained for us at the University of Pittsburgh mass spectral facility. NMR spectra are reported in ppm relative to tetramethylsilane (s = singlet, d = doublet, t = triplet, q=quartet, m = multiplet, b = broad). Coupling constants are in Hertz.

Dyes discussed under the heading "Effect of Dye Structure on Solvent Dependent Fluorescence" were synthesized for previous studies in the Waggoner laboratory. Their structure and purity were confirmed by TLC, and by UV-Vis and NMR spectroscopy. The side chains on these dyes varied by at most one carbon in chain length, allowing meaningful comparison of spectral properties.

1,2-Dimethylbenzoxazolium iodide (I). A mixture of 2-methylbenzoxazole (1 g) and methyl iodide (4.3 g) was placed in a sealed tube and heated in a steam bath for 80 minutes. During this period a heavy white precipitate was formed. The tube was removed from the steam bath and allowed to cool to room temperature. Ether was then added to the reaction mixture, resulting in the formation of additional white precipitate. The solid was thrice suspended in ether and collected by centrifugation, then evaporated and dried under vacuum to yield pure product (414 mg, 20%). Yields were not optimized because this reaction was used only to synthesize material for structure-activity studies. NMR (deuterochloroform, trace trifluoroacetic acid) 7.8 (4H, overlapping multiplets), 4.2 (3H, s), 3.2 (4H, s).

1-Butyl-2-methylbenzoxazolium iodide (II). A mixture of 2-methylbenzoxazole (2.9 g) and butyl iodide (4.4 g) was heated to 110° C with vigorous stirring under nitrogen for 18 hours. The reaction mixture was allowed to cool to room temperature under nitrogen. Ether was then added, resulting in heavy precipitation. Some of this precipitate was recrystallized from ethanol-ether and dried under vacuum to give N-butyl-2-methylbenzoxazole, a tan solid (427 mg, 6%). Yields were not optimized because this reaction was used only to synthesize material for structure-activity studies. NMR (deuterochloroform) 8.0 (1H, m), 7.85 (1H, m), 7.75 (2H, overlapping multiplets), 4.75 (2H, t, J=7), 3.4 (3H, s), 2.0 (2H, m), 1.5 (2H, m), 1.0 (3H, t, J=6).

1-(3-Iodopropyl)-2-methylbenzoxazolium iodide (III). A mixture of 2-methylbenzoxazole (4.5 g) and 1,3-diiodopropane (20 g) was heated with vigorous stirring under nitrogen at 120° C for 5 hours. A cake of solid formed in the reaction mixture during heating. Isopropanol was poured into the reaction mixture. The resulting precipitate and the previously formed cake were ground together into a fine powder, collected by filtration, washed with isopropanol, and dried under vacuum to give 7.02 g (48%) of solid product. NMR (deuterochloroform, trace trifluoroacetic acid) 8.0 (1H, m), 7.85 (1H, m), 7.8 (2H, overlapping multiplets), 4.9 (2H, t, J=7), 3.4 (5H, overlapping t and s), 2.6 (2H, m).

3-(3-Iodopropyl)-2-methylbenzothiazolium iodide (IV). A mixture of 2-methylbenzothiazole (1 g) and 1,3-diiodopropane (7.9 g) was heated at 120° C with vigorous stirring under nitrogen for 9 hours. During the reaction a cake of solid formed. The reaction mixture was cooled to room temperature and diethyl ether was added, producing a heavy precipitate. This precipitate and the cake were ground together into a fine powder, which was triturated several times with diethyl ether. After trituration the solid was collected first by centrifugation and in the final step by vacuum filtration. It was washed with methanol, and dried under vacuum to produce 1.7 grams (57%) of solid product. NMR (deuterated dimethylsulfoxide) 8.45 (1H, d, J=8), 8.35(1H, d, J=8), 7.9 (1H, d of d, J=8, 9), 7.8 (1H, d of d, J=8,9), 4.75 (2H, t, J=9), 3.4 (5H, overlapping t and s), 2.4 (2H, m).

N,N'-Dibutylthiourea (V). 1-Butylamine (3.02 g) was added slowly to a solution of 1-butylisothiocyanate (4.4 g) in 15 ml dry ether with stirring in a reflux apparatus. The rate of addition was kept slow enough to prevent vigorous boiling. The reaction mixture was allowed to cool and stirred for 4 hours at room temperature, during which time a heavy white precipitate formed. Evaporation of the reaction mixture and vacuum drying produced 6.2 g (83%) of the product, a white solid. NMR (deuterochloroform) 5.7 (2H, broadened), 3.4 (4H, broadened), 1.6 (4H, m), 1.4 (4H, m), 0.95 (6H, t, J=9).

1,3-Dibutylthiobarbituric acid (VI). This procedure was a modification of that used by Brooker et al. (1951) to produce 1,3-diethylthiobarbituric acid. The reaction was carried out under nitrogen, using flame dried apparatus and dry solvents and reagents, with vigorous stirring throughout. Sodium methoxide (26 ml of a 25% solution in methanol) was added to a refluxing solution of N, N'-dibutylthiourea (10 g) and diethyl malonate (17.2 g) in 25 ml methanol. The mixture was refluxed for 24-36 hours under nitrogen. Distilled water (50 ml) was added and the solution was partially evaporated to remove methanol. Any white precipitate forming during evaporation was removed by filtration (melting point indicated this to be N,N'-dibutylthiourea). The solution was diluted with distilled water, chilled in an ice water bath and acidified to pH 1-2 with concentrated HCl. The resulting precipitate was collected and dried under vacuum to produce 10.6 g (78%) of the product as a light yellow powder. NMR (deuterochloroform) 4.3 (4H, t, J=8), 3.7 (2H, s), 1.6 (4H, m), 1.35 (4H, m), 0.95 (6H, t, J=7).

1,3-Dimethylthiobarbituric acid (VII). This compound was previously synthesized for other studies in the Waggoner laboratory. After recrystallization from ethanol, its structure and purity were confirmed by NMR spectroscopy. NMR (deuterochloroform, trace trifluoroacetic acid) 4.0 (2H, s), 3.7 (6H, s).

5-(3-Methoxylallylidene)-1,3-dimethylthiobarbituric acid (VIII). This procedure was carried out using flame dried glassware and anhydrous solvents and reagents. 1,3-Dimethylthiobarbituric acid (358 mg) was dissolved in 50 ml methanol. The solution was warmed briefly on a steam bath to produce complete solubilization. While stirring vigorously, trifluoroacetic acid (0.5 ml) was added, immediately followed by 1,3,3-trimethoxypropene (1.5 g) added all in one portion. A heavy yellow precipitate appeared within 30 seconds. After stirring an additional 10 minutes, the precipitate was filtered and dried under vacuum to give 353 mg (71%) of the product. NMR (deterochloroform) 8.15 (1H, d, J=12), 7.5 (2H, overlapping m), 4.0 (3H, s), 3.7 (6H, s); UV-Vis absorbance maximum in dichloromethane = 374 nm.

5-(3-Methoxylallylidene)-1,3-dibutylthiobarbituric acid (IX). This procedure was carried out using flame dried glassware and anhydrous solvents and reagents. 1,3-Dibutylthiobarbituric acid (2 g) was dissolved in 20 ml methanol. While stirring vigorously, 1,3,3-trimethoxypropene (1.5 g) was added, all in one portion. A heavy yellow precipitate appeared within 30 seconds. Methanol (10 ml) was added and the thick slurry was swirled by hand for an additional 2 minutes. The precipitate was filtered, washed with methanol, and dried under vacuum to give 1.93 g (76%) of the product. Exposure to moisture produces a pink impurity which could be removed by washing with methanol. NMR (deuterochloroform) 8.1 (1H, d, J=11), 7.5 (2H, overlapping m), 4.4 (4H, m), 1.7 (4H, m), 1.4 (4H, m), 0.96 (6H, t with additional fine splitting, J=9); UV-Vis absorbance maximum in dichloromethane = 380 nm.

3-(Dimethylamino)propylisothiocyanate (X). Oven dried glass-ware, and anhydrous solvents and reagents were used. Thiophosgene (14 g) was added carefully to a vigorously stirring solution of 3-(dimethylamino)propylamine (10 g) in 350 ml acetone. Caution was exercised during this addition both because the reaction is highly exothermic and because of the toxicity of thiophosgene. An oversize vessel was used as a precaution against spattering, and because the formation of a heavy precipitate during the reaction necessitated very vigorous stirring. After stirring for 10 minutes, the reaction mixture was poured into a beaker containing 1 L of rapidly stirring ether. Additional precipitation resulted. Three 150 ml quantities of ether were used to wash the remaining solid from the walls of the reaction vessel into the large ether suspension. The combined precipitate was collected by vacuum filtration and washed with 100 ml ether. It was dissolved in water, which was brought to pH 10 with saturated sodium carbonate solution. The aqueous solution was extracted with ether, and the ether solution was dried over sodium sulfate and evaporated. The evaporation residue was dried under vacuum to produce 6 g (38%) of 3-(dimethylamino)propylisothiocyanate as a yellow oil. The product was further purified by vacuum distillation at 107°C and pressure of 10 mm Hg. NMR (deuterochloroform) 3.55 (2H, t, J=6), 2.35 (2H, t, J=6), 2.2 (6H, s), 1.8 (2H, m) IR (neat on KCl plate) 2770, 2187, 2112, 1461cm^{-1}.

Dye Mc4.10. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and anhydrous solvents. 1,2-Dimethylbenzoxazolium iodide (80 mg) was added to a solution of 5-(3-Methoxylallylidene)-1,3-dibutylthiobarbituric acid (41 mg) in 15 ml of 3:1 dichloromethane : methanol. Sodium methoxide (16 mg) was added and the solution was stirred under nitrogen for an additional 1.5 hours. The reaction mixture was evaporated to dryness, and the product was isolated by flash column chromatography on silica gel eluting with methylene chloride. Column fractions were assayed by silica TLC with 2.5% methanol in methylene chloride (v/v). Only very pure fractions were combined, leading to isolation of 9 mg (16%) of product. This material was required only for structure-activity studies so yields were not optimized. NMR (deuterochloroform) 8.05 (1H, d, J=12), 7.9 (2H, m), 7.45 (1H, d, J=9), 7.35 (2H, complex overlapping m), 7.15 (1H, d, J=9), 5.6 (1H, d, J=12), 4.5 (4H, t, J=9), 3.6 (3H, s), 1.75 (4H, m), 1.4 (4H, m), 1.0 (6H, t, J=6); UV-Vis absorbance maximum in dimethylsulfoxide = 562 nm; FAB MS 439.

Dye Mc4.11. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and anhydrous solvents. 5-(3-Methoxylallylidene)-1,3-dibutylthiobarbituric acid (170 mg) was dissolved in 30 ml of 1:1 methanol : dichloromethane. 1-(3-Iodopropyl)-2-methylbenzoxazolium iodide (378 mg) was added, followed by sodium methoxide (50 mg). The reaction mixture was stirred an additional 1.5 hours and then evaporated. The product was purified from the evaporation residue using silica flash chromatography eluting with 2.5% methanol in dichloromethane (v/v). The first red-purple band eluting was the product, weighing 62 mg (20%) after vacuum drying. Silica TLC of the reaction product using the column solvent showed only one material. NMR (deuterochloroform) 8.1 (1H, d, J=12), 7.9 (2H, m), 7.45 (1H, d, J=9), 7.35 (3H, complex overlapping multiplets), 5.6 (1H, d, J=12), 4.45 (4H, t, J=7), 4.1 (2H, t, J=6), 3.25 (2H, t, J=6), 2.35 (2H, m), 1.75 (4H, m), 1.4 (4H, m), 1.0 (6H, t, J=6); UV-Vis absorbance maximum in dimethylsulfoxide = 566 nm; FAB MS 593, 594.

Dye Mc4.12. Dye Mc4.11 (50 mg) was dissolved in 25% methanolic trimethylamine. The reaction mixture was refluxed under nitrogen with stirring, and was monitored by silica TLC using 2.5% and 20% methanol in dichloromethane (v/v). TLC showed essentially complete conversion of starting material to a product of much lower R_f after 1.5 hours. The reaction mixture was allowed to cool to room temperature, ether was added, and the resulting purple precipitate was collected by centrifugation. The solid was repeatedly triturated with ether until no yellow color was visible in the ether. The solid was then dissolved in hot methanol and precipitated with a minimum of ether. The precipitated product was collected by centrifugation, triturated with ether, and dried under vacuum, giving 10 mg (18%) of product. NMR (deuterated dimethylsulfoxide) 8.05 (1H, m), 7.8 (4H, overlapping m), 7.55 (2H, overlapping m), 6.4 (1H, d, J=13), 4.4 (4H, broad t),3.2-3.5 (13H, large s overlaps multiplets), 2.25 (2H, m), 1.6 (4H, m), 1.3 (4H, m), 0.9 (6H, t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 566 nm; FAB MS 526.

Dye Mc4.13 This dye, known as "merocyanine 540", has been studied extensively by other investigators (Waggoner and Grinvald, 1977, and references cited therein). The sample used in this study was synthesized previously in the Waggoner laboratory. Its purity and structure were substantiated by TLC, and by spectral characterization. NMR (deuterated dimethylsulfoxide) 7.95 (1H, m), 7.75 (4H, overlapping m), 7.45 (2H, overlapping m), 6.5 (1H, d, J=13), 4.45 (2H, broad t), 4.3 (4H, broad t), 3.35 (2H, broad t), 2.6 (2H, t, J=6), 2.1 (2H, m), 1.6 (4H, broad m), 1.3 (4H, broad m), 0.9 (6H, t, J=6); UV-Vis absorbance maximum in dimethylsulfoxide = 564 nm; FAB MS 547, 570 (+Na).

Dye Mc4.14. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and anhydrous solvents. Sodium methoxide (80 mg) was added to a solution of 5-(3-Methoxylallylidene)-1,3-dimethylthiobarbituric acid (93 mg) and 1-butyl-2-methylbenzoxazolium iodide (210 mg) in 15 ml of 5:1 dichloromethane : methanol. A heavy, purple precipitate appeared within 3 minutes. After the reaction was stirred for an additional 30 minutes, the reaction mixture was poured into ether to induce further precipitation, and the precipitate was collected by centrifugation. The precipitated product was purified by trituration with ether. Silica TLC of the product with 2.5% methanol in dichloromethane (v/v) showed only one material. NMR (deuterochloroform) 8.05 (1H, d, J=12), 7.9 (2H, m), 7.5 (1H, d, J=7), 7.3 (3H, overlapping m), 5.7 (1H, d, J=12), 4.0 (2H, t, J=7), 3.75 (6H, s), 1.85 (2H, m), 1.5 (2H, m), 1.05 (3H, t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 560; FAB MS 398.

Dye Mc4.15. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and anhydrous solvents. 5-(3-Methoxylallylidene)-1,3-dimethylthiobarbituric acid (1.44 g) was dissolved in 250 ml of 1:9 methanol : dichloromethane. Sodium methoxide (378 mg) was added, followed by 1-(3-Iodopropyl)-2-methylbenzoxazolium iodide (3 g). The reaction mixture was stirred for 15 minutes, during which time a purple precipitate formed. The reaction mixture was poured into 300 ml ether and the precipitate was collected by filtration. The filtrate was added to an additional 400 ml ether, chilled , and more precipitate was collected. The mass of the combined precipitates was 1 g. This material showed extremely poor solubility in all solvents tested, including dimethylsulfoxide. Small amounts could be purified using flash column chromatography on silica. The crude product was loaded on the column in 1:20 methanol:dichloromethane and eluted with the same solvent. Dye purified by this means showed one spot on silica TLC (run with the column solvent). The extremely low solubility of this compound hindered NMR spectroscopy, and only low mass fragments were seen in the mass spectrum. Structure assignment was based on successful conversion of this product to dye Mc4.16, and on UV-Vis absorbance data. The presence of the O-TBA chromophore was indicated by the product's absorbance maximum. Lack of substantial impurities was indicated by the products extinction coefficient, which matched that of other O-TBA dyes (see Materials and Methods). The spectrum of a dilute NMR sample was obscured by trace solvent impurities and noise, but the following peaks could be clearly discerned: NMR (deuterated dimethylsulfoxide) 7.95 (d of d, J=12,13), 6.7-7.7 (multiple overlapping peaks), 6.45 (d, J=13), 4.35 (t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 562 nm;

Dye Mc4.16. The reaction was performed under nitrogen. Dye Mc4.15 (25 mg) was added to 50 ml dimethylsulfoxide, and undissolved solid was removed by filtration. This solution was added to 40 ml of refluxing 25% methanolic trimethylamine (Kodak Co.) over 45 minutes. An additional 50 ml of methanolic trimethylamine was then added, and reflux continued for 45 minutes. Silica TLC using 2.5% and 50% methanol in dichloromethane (v/v) showed essentially complete conversion of starting material to a single product of much lower R_f. The reaction mixture was evaporated to low volume, added to 500 ml ether, and chilled. Precipitated solid was dissolved in hot methanol and precipitated with a minimum of ether. The precipitated product was collected by centrifugation and washed with ether. Silica TLC showed only one spot. The yield was less than 5%. Yields were not optimized as this material was required only for structure-activity studies. The solubility of this dye was very poor in all solvents tested, but saturated dimethylsulfoxide solutions were sufficiently concentrated to produce a clear NMR. NMR (deuterated dimethylsulfoxide) 8.1 (1H, d of d, J=13, 12), 7.85 (4H, overlapping m), 7.6 (2H, overlapping m), 6.45 (1H, d, J=13), 4.4 (2H, broad m), 3.55 (6H, s), 3.3 (9H, broad s), 3.0-3.6 (peaks obscured by large singlets), 2.25 (2H, broad m); UV-Vis absorbance maximum in dimethylsulfoxide = 564 nm.

Dye Mc4.17. This reaction was carried out under nitrogen with constant stirring, using flame dried glassware and anhydrous solvents. Finely powdered 3-(3-iodopropyl)-2-methylbenzothiazolium iodide (385 mg) was added over two minutes to a solution of 5-(3-Methoxylallylidene)-1,3-dibutylthiobarbituric acid (270 mg) in pyridine (10 ml). After stirring for an additional 5 minutes, the reaction mixture was poured into water, producing a purple precipitate. The precipitate was collected by vacuum filtration and removed from the filter paper by washing with dichloromethane. Evaporation of the dichloromethane solution yielded 327 mg of crude purple product which was purified by silica flash chromatography using 1% methanol in dichloromethane (v/v). The product was the first purple band eluted. The reaction and column could be monitored on silica TLC using the same solvent. The reaction produced 202 mg (39%) of the product. NMR (deuterochloroform) 8.0 (1H, s, J=13), 7.85 (1H, d of d, J=12,13), 7.2-7.7 (5H, overlapping m), 6.15 (1H, d, J=13), 4.5 (4H, t, J=8), 4.2 (2H, t, J=8), 3.3 (2H, t, J=6), 2.35 (2H, m), 1.75 (4H, m), 1.4 (4H, m), 1.0 (6H, t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 601 nm; FAB MS 609, 610.

Dye Mc4.18. Dye Mc4.17 (50 mg) was dissolved in 25% methanolic trimethylamine obtained from the Kodak company. The reaction mixture was refluxed under nitrogen with stirring, and was monitored by silica TLC using 2.5% and 20% methanol in dichloromethane (v/v). TLC showed essentially complete conversion of starting material to a product of much lower R_f within 2 hours. The reaction mixture was allowed to cool to room temperature, ether was added, and the resulting purple precipitate was collected by centrifugation. The solid was dissolved in hot methanol and precipitated with a minimum of ether. The precipitated product was collected by vacuum filtration, washed with ether, and vacuum dried to give 44 mg (81%) of product. NMR (deuterated dimethylsulfoxide) 8.1 (1H, d, J=7), 7.75-7.9 (4H, overlapping m), 7.6 (1H, d of d, J=8,9), 7.45 (1H, d of d, J=8,9), 6.8 (1H, d, J=13), 4.5 (2H, t, J=9), 4.35 (4H, t, J=9), 3.55 (2H, m), 3.1 (9H, s), 2.2 (2H, broad m), 1.6 (4H, m), 1.3 (4H, m), 0.95 (6H, t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 602 nm; FAB MS 542.

Dye Mc4.19. This reaction was carried out in oven dried glassware using anhydrous solvents and reagents. 3-(Dimethylamino)propylisothiocyanate (1 g) was added to a solution of dye Mc4.17 (100 mg) in 0.5 ml of dichloromethane. The reaction mixture was stirred at 60° C under nitrogen while monitoring with silica TLC developed with 1% methanol in dichloromethane (v/v). Starting material had an R_f of greater than 0.9 and the product an R_f of 0 in this solvent system. After 90 minutes, some starting material remained but substantial amounts of side products of intermediate R_f were seen. The reaction mixture was poured into 100 ml ether, producing a purple precipitate which was collected by centrifugation. The product was isolated from the precipitate by flash column chromatography on cellulose, eluting with 2% methanol in dichloromethane (v/v). The column fractions were monitored using cellulose TLC developed with 2.5% methanol in dichloromethane. The product appeared as the higher of two closely spaced spots just above the origin. Although the starting material ran much faster than the two product spots on cellulose TLC, it eluted only slightly behind the upper of the two product spots on the column. Residual starting material could be removed by trituration with 5% methanol in dichloromethane. The yield of this dye ranged between was 18 mg (15%). NMR (deuterochloroform, trace deuteromethanol) 7.7 (1H, d, J=9), 7.1-7.6 (6H, overlapping multiplets), 6.5 (1H, d, J=12), 4.55 (2H, broad t), 4.35 (4H, t, J=9), 4.15 (2H, broad m), 3.85 (2H, t, J=6), 3.65 (2H, broad m), 3.0 (6H, s), 2.35 (4H, broad m), 1.7 (4H, broad m), 1.4 (4H, broad m), 0.95 (6H, t, J=7); UV-Vis absorbance maximum in dimethylsulfoxide = 602 nm; FAB MS 626. IR 2956, 2931, 2869, 2194, 2112, 1725, 1625, 1543 cm^{-1}.