a higher affinity for the right side of the active site (compare E_{TOT} for conformers 3 and 4 of Table II), is a better substrate for lysozyme than is $(GlcNAc)_6$ (Imoto et al., 1972), which has a higher affinity for the left side of the active site cleft (Pincus & Scheraga, 1979).

Those oligomers, therefore, that bind preferentially to the right side of the cleft may be better substrates. Our studies suggest that substrates with a blocked 3-OH group of the sugar residue in site F (as occurs with the 3-*O*-lactyl group of a MurNAc residue in this site) will bind preferentially to the right side of the cleft. Such substrates should undergo more rapid catalysis than should corresponding substrates that contain a free 3-OH group. Because $(GlcNAc\text{-}MurNAc)_2$-$(GlcNAc)_2$ has a preference for "left-side" binding (compare E_{TOT} for conformers 5 and 6 of Table II), its *apparent* specific rate of catalysis, k_{cat}, measured by classical Michaelis–Menten analysis (Imoto et al., 1972), would be expected to be lower than that for $(GlcNAc\text{-}MurNAc)_3$. Because the affinity of $(GM)_2GG$ for the left side of the cleft relative to its affinity for the right side is the same as the corresponding relative affinity of $(GlcNAc)_6$ for the same binding sites (Pincus & Scheraga, 1979), i.e., "left-side" binding is favored for both oligomers by 6 kcal/mol, the values of *apparent* k_{cat} for both of these oligomers should be the same.

Equilibrium and relaxation studies on the binding of $(GlcNAc)_6$ to lysozyme (Holler et al., 1975) have suggested three productive binding forms in which all six saccharide units contact the enzyme. One of these forms is thought to be the actual reactive form. In our prior calculations (Pincus & Scheraga, 1979), we found three low-energy structures, one left side, one right side, and one right side with a distorted D ring. If these calculated species are the same as those inferred experimentally, then one would expect that relaxation studies of the binding of $(GlcNAc\text{-}MurNAc)_3$ to lysozyme would show *two* rather than three productive binding modes. This result would follow from the inability of the alternating copolymer to bind to the left side of the active site.

Acknowledgments

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Preparation of a Fluorescent-Labeled Derivative of Calmodulin Which Retains Its Affinity for Calmodulin Binding Proteins†

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ABSTRACT: Calmodulin was derivatized with 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid to fluorescently label the protein. This derivative (AEDANS·CaM) stimulated the Ca^{2+}-sensitive cyclic nucleotide phosphodiesterase and formed Ca^{2+}-dependent complexes with troponin I and the phosphodiesterase. Association between AEDANS·CaM and these proteins was directly monitored by changes in fluorescence anisotropy. The dissociation constants for the AEDANS·CaM–troponin I and AEDANS·CaM–phosphodiesterase complexes were 60 nM and 4 nM, respectively. This fluorescent derivative of calmodulin appears suitable for direct monitoring of the complexes between calmodulin and calmodulin binding proteins. Rotational diffusion of AEDANS·CaM was also measured with fluorescence anisotropy. These measurements indicated that the shape of calmodulin in solution is best approximated by a prolate ellipsoid.

Since the discovery of calmodulin (CaM)[1] by (Cheung, 1970), it has been determined that this regulatory protein mediates Ca^{2+} stimulation of several enzymes including the Ca^{2+}-sensitive phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970; Wang et al., 1975), brain adenylate cyclase

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[1] Abbreviations used: CaM, calmodulin; PDE, 3′:5′-cyclic nucleotide phosphodiesterase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 4-morpholinopropanesulfonic acid; R_S, Stokes radius; R_{app}, apparent Stokes radius; $\langle\rho\rangle$, average rotational relaxation time; AEDANS·CaM, 5-[[(acetylamino)ethyl]amino]-1-naphthalenesulfonic acid labeled CaM; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol.

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(Brostrom et al., 1975), human erythrocyte Ca^{2+}–Mg^{2+} ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), myosin light chain kinase (Dabrowska et al., 1978), and phosphorylase kinase (Cohen et al., 1978). In addition, CaM also interacts with troponin I (Amphlett et al., 1976) and calcineurin (Wang & Desai, 1977; Klee & Krinks, 1978). Formation of complexes between calmodulin and these proteins in the presence of Ca^{2+} has been demonstrated by gel filtration (Teshima & Kakiuchi, 1974), electrophoresis on nondenaturing gels (Amphlett et al., 1976; LaPorte & Storm, 1978), cross-linking of [^{125}I]CaM using bifunctional cross-linking reagents (LaPorte et al., 1979), and CaM–Sepharose affinity chromatography (Watterson & Vanaman, 1976; Klee & Krinks, 1978; Westcott et al., 1979).

In order to quantitatively characterize interactions between CaM and these proteins, it is necessary to develop methods to directly monitor complex formation between CaM and CaM binding proteins. In this study, we describe the preparation of a fluorescent-labeled derivative of CaM that retains its Ca^{2+}-dependent affinity for troponin I and the Ca^{2+}-sensitive phosphodiesterase. This derivative is suitable for directly quantitating CaM–protein interactions using fluorescence anisotropy. In addition, the fluorescent properties of the derivative provide information concerning the shape and hydrodynamic properties of CaM in solution.

Materials and Methods

Materials. ^3H_2O was obtained from New England Nuclear. Bovine serum albumin (BSA), bovine heart cytochrome *c*, sperm whale myoglobin, soybean trypsin inhibitor, and chicken egg ovalbumin were products of Sigma. 5-[[[(Iodoacetyl)-amino]ethyl]amino]-1-naphthalenesulfonic acid was obtained from Molecular Probes, Inc. All other reagents were the highest grade available.

Protein Preparations. CaM was purified from bovine brain as described previously (LaPorte et al., 1979). Troponin I was purified from rabbit muscle by the method of Wilkinson (1974). CaM-sensitive phosphodiesterase was purified from bovine heart by the method of LaPorte et al. (1979).

Phosphodiesterase Assay. Phosphodiesterase activity was assayed as described previously (LaPorte et al., 1979). The assay mixture contained 0.05 mM [^{3}H]cAMP (~60 000 cpm), 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, 0.1 mM $CaCl_2$, 5′-[^{14}C]AMP (~6000 cpm), 0.1 mM $PhCH_2SO_2F$, 0.1 mg/mL BSA, 2 mM DTT, and varying amounts of CaM in a total volume of 0.5 mL.

Preparation of Fluorescent Calmodulin. The 5-[[(acetyl-amino)ethyl]amino]-1-naphthalenesulfonic acid derivative of CaM (AEDANS·CaM) was prepared with conditions similar to those employed by Walsh & Stevens (1977) for modification of the methionines of CaM with iodoacetate. The reaction mixture contained 2 mg of CaM, 200 mM sodium acetate, pH 5.0, 1 mM $CaCl_2$, and 10 mM 5-[[[(iodoacetyl)amino]-ethyl]amino]-1-naphthalenesulfonic acid (Hudson & Weber, 1973) in a final volume of 1.2 mL. After incubation in the dark for 9 h at 20 °C, unreacted reagent was removed by passage of the sample through a Sephadex G-25 column (1 × 20 cm) equilibrated in 10 mM sodium acetate, 100 mM KCl, pH 5.0, 8 M deionized urea, and 1 mM EGTA. The sample was then applied to a Sephadex G-25 column (1 × 20 cm) equilibrated in 10 mM Mops, pH 7.2, 100 mM KCl, 0.2 mM $CaCl_2$, and 10% sucrose (w/v). The desalted sample was applied to a 1.2 × 25 cm troponin I–Sepharose column (Head et al., 1979) equilibrated in 10 mM Mops, pH 7.2, 100 mM KCl, and 0.2 mM $CaCl_2$ and washed with the same buffer at 6 mL/h for 12 h. Bound CaM was then eluted with 10 mM Mops, pH 7.2, 100 mM KCl, and 2 mM EGTA. Peak fractions were pooled and passed through a Sephadex G-25 column (1 × 20 cm) equilibrated in 10 mM Mops, pH 7.2, 100 mM KCl, and 0.2 mM $CaCl_2$. AEDANS·CaM was stored in aliquots at −60 °C. The extent of incorporation of the probe into CaM was determined spectrophotometrically with the molar extinction coefficient of the probe taken to be 6000 M^{-1} cm^{-1} at 337 nm (Hudson & Weber, 1973) and protein determined by the method of Lowry (Lowry et al., 1951). The extent of incorporation was typically ~0.8 mol of probe/mol of CaM. Amino acid analysis of samples of performic acid–oxidized CaM and AEDANS·CaM also indicated incorporation of 0.8 mol of probe/mol of CaM, through modification of methionine residues (Vithayathil & Richards, 1960).

Fluorescence Measurements. Fluorescence measurements were made with an SLM 4800S spectrofluorometer. Sample temperatures were maintained at 25 ± 1 °C. The buffer used in all experiments was 10 mM Mops, pH 7.2, 150 mM KCl, and 1 mM $MgCl_2$ (buffer A) with other additions as indicated. Fluorescence emission spectra were acquired and corrected for background signal and instrument response, as described previously (LaPorte et al., 1980). Excitation was at 340 nm, and resolution of both monochrometers was set at 4 nm.

Anisotropy is defined as

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescent intensities observed parallel and perpendicular to the polarization of the exciting light, respectively, and A is anisotropy. The measurements were made in the "T" format, with instrumental error being nulled with the excitation polarizer in the horizontal position. Excitation was at 340 nm, unless otherwise indicated, and emitted light was isolated with Schott KV470 filters. Resolution of the excitation monochromater was set at 4 nm.

The limiting anisotropy, A_0, and the apparent Stokes radius, R_{app}, were determined from anisotropy measurements by using a method essentially the same as that described by Perrin (1929) and Weber (1953):

$$A^{-1} = A_0^{-1}\left(1 + \frac{3k\tau}{4\pi R_{app}}\frac{T}{\eta}\right) \tag{2}$$

where k is Boltzmann constant, τ is the fluorescent lifetime, T is absolute temperature, and η is viscosity. This equation is only rigorously valid for spheres or randomly labeled particles. In other cases, the value of R_{app} is sensitive to the orientation of the probe with respect to the protein and will not yield the true Stokes radius. Furthermore, for dye–protein conjugates, the value of A_0 in this equation is often less than the true limiting anisotropy of the dye as a result of motion of the dye with respect to the protein. This motion is typically fast and is reflected only in the value of A_0. Samples consisted of 20 nmol of AEDANS·CaM in an initial volume of 1.5 mL of buffer A. The sample temperature was maintained at 25 ± 1 °C and T/η was varied by addition of buffer A containing 60% sucrose (w/v). The background intensity did not exceed 2% of the sample intensity. A_0 and R_{app} were then determined from a plot of $1/A$ vs. T/η by using eq 2. The data were then replotted as A_0/A vs. T/η (Withold & Brand, 1970).

The average rotational relaxation time, $\langle\rho\rangle$, was calculated from a rearrangement of one form of the Perrin relation:

$$\langle\rho\rangle = \frac{3\tau A}{A_0 - A} \tag{3}$$

where the variables are those defined above and A is the anisotropy determined before addition of sucrose.

Protein–protein complex formation was monitored by using anisotropy measurements during the titration of AEDANS·CaM with troponin I or phosphodiesterase. This is possible because the observed anisotropy in a mixture of emitting species is the weighted average of the individual anisotropies. For a binary mixture the solution is

$$A = f_1A_1 + f_2A_2 \quad (4)$$

where f_1 and f_2 are the fractions of total fluorescent intensity due to the first and second species, respectively, and A_1 and A_2 are their anisotropies. Equation 4 can be shown to yield a relation useful for the calculation of α, the fraction of AEDANS·CaM bound,

$$\alpha = \frac{A - A_F}{A(1 - q) + q(A_B) - A_F} \quad (5)$$

where A_F and A_B are the anisotropies of AEDANS·CaM, free and bound, respectively, and q is the ratio of the fluorescent intensity of the bound species over that of the free species. A sample consisting of (2–5) × 10^{-8} M AEDANS·CaM and 0.5 mM dithiothreitol in 1.5 mL of buffer was titrated with troponin I or phosphodiesterase in parallel with a buffer blank. The sample temperature was maintained at 25 ± 1 °C. Following each addition of troponin I or phosphodiesterase, 10 min was allowed for the system to achieve equilibrium. The intensities and anisotropies were then determined as described above, with total intensity taken to be $I_{\parallel} + 2I_{\perp}$. The anisotropy of the sample was corrected for background fluorescence by application of eq 4, taking A, A_1, and A_2 to be the anisotropy of the sample, the blank, and the corrected sample, respectively, and calculating f_1 and f_2 from the measured intensity. The corrected sample anisotropy is the value that would have been determined in the absence of background interference. A_F, A_B, and q were determined at the limits of the titration, and α was calculated from eq 5 by using the corrected anisotropy.

Fluorescent lifetimes were determined by the method of Spencer & Weber (1969). Excitation was at the indicated wavelength, with incident light modulated at 18 MHz. Emitted light was isolated with a Schott KV470 filter. A polarizer, oriented at 35° from the vertical, was placed in the excitation pathway in order to eliminate the influence of Brownian rotation on the measured lifetimes (Spencer & Weber, 1970). Lifetime values determined by phase shift and demodulation differed from each other by less than 10%, so the average of the two values is reported. Samples consisted of 1 × 10^{-5} M AEDANS·CaM in buffer A and were maintained at 25 ± 1 °C.

Determination of the Stokes Radius of CaM by Gel Filtration. A Sephadex G-150 column (1.5 × 90 cm) was prepared and equilibrated in buffer A containing either 0.1 mM $CaCl_2$ or 0.25 mM EGTA. Samples, consisting of 2 mg of CaM, 0.15 mg of AEDANS·CaM, and 2 μCi of ^3H_2O in 4 mL of the equilibration buffer, were applied, and the column was eluted at 12 mL/h. Fraction size was 1 mL. Following chromatography, protein was determined by the method of Lowry (Lowry et al., 1951). Fluorescence intensity was determined with excitation at 340 nm and emitted light isolated with a Schott KV470 filter. A buffer blank, found to be less than 3% of the peak intensity, was determined and was subtracted from each sample intensity.

The Sephadex G-150 column was standardized in separate experiments. The void and included volumes were measured with blue dextran and ^3H_2O, respectively. The Stokes radius of CaM was determined by the method of Laurent & Killander (1964). The standards were the following: bovine serum albumin, 35.5 Å; bovine serum albumin dimer, 43 Å; sperm whale myoglobin, 20.7 Å; chicken egg ovalbumin, 27.3 Å (Andrews, 1970); bovine heart cytochrome *c*, 17.4 Å; soybean trypsin inhibitor, 22.5 Å (Brewer et al., 1974).

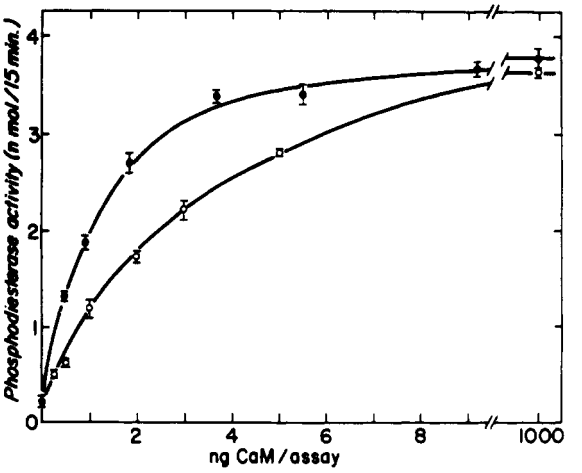

FIGURE 1: Stimulation of purified Ca^{2+}-sensitive phosphodiesterase by CaM (●) and by AEDANS·CaM (○). The phosphodiesterase was purified as described by LaPorte et al. (1979) through the second DEAE-cellulose column step and was assayed as a function of CaM or AEDANS·CaM as described under Materials and Methods. Each assay contained 1.7 mU of phosphodiesterase. Error bars show the standard error of the mean of triplicate determinations.

General Techniques. Protein was determined by the method of Lowry (Lowry et al., 1951) by using bovine serum albumin as the standard. Purified CaM was determined spectrophotometrically by using $E_{1\%}$ = 1.8 at 277 nm (Watterson et al., 1976). Where molar concentrations are reported for CaM or troponin I, molecular weight values of 16 723 (Vanaman et al., 1977) or 23 000 (Wilkinson, 1974), respectively, were used.

Results

Stimulation of the Phosphodiesterase by AEDANS·CaM. Fluorescent-labeled CaM retained its ability to stimulate the CaM-sensitive cyclic nucleotide phosphodiesterase from bovine heart (Figure 1). The concentrations of CaM and AEDANS·CaM required for half-maximal stimulation of phosphodiesterase activity were 120 and 280 pM, respectively. CaM and AEDANS·CaM both maximally stimulated the enzyme approximately 15-fold. Stimulation of the phosphodiesterase could have been due to unmodified CaM present in the preparation; however, AEDAN·CaM formed Ca^{2+}-dependent complexes with the phosphodiesterase which were detectable by changes in fluorescence anisotropy (discussed below).

Titration of AEDANS·CaM with Troponin I. Fluorescence anisotropy was used to monitor the binding of AEDANS·CaM to troponin I (Figure 2A). As would be expected from the increase in molecular volume, the AEDANS·CaM–troponin I complex had a considerably increased anisotropy relative to that of free AEDANS·CaM. This was reversed by addition of a large excess of unlabeled CaM. The troponin I induced increase in anisotropy had an absolute requirement for Ca^{2+} at troponin I concentrations below ~1 × 10^{-6} M. At troponin I concentrations greater than 1 × 10^{-6} M, an increase in anisotropy was observed even in the presence of excess EGTA. This was not characterized because troponin I is poorly soluble at concentrations above ~1 × 10^{-5} M (Greaser & Gergely, 1973).

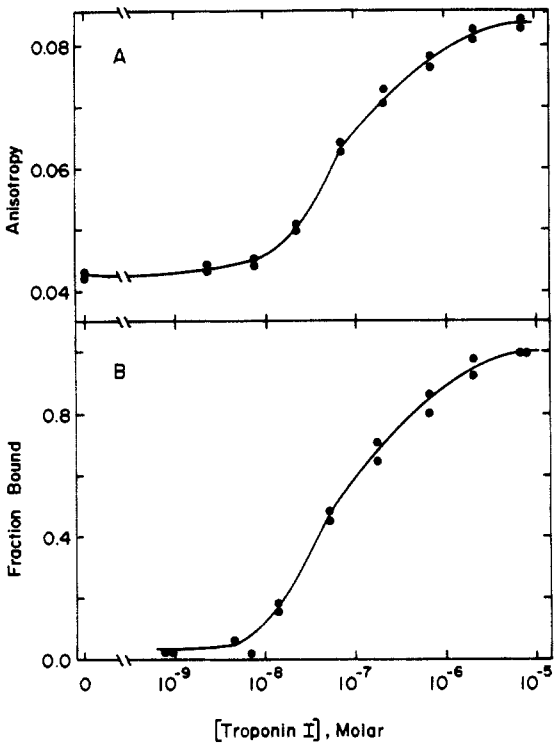

FIGURE 2: Titration of AEDANS·CaM with troponin I. The sample consisted of 75 pmol of AEDANS·CaM in 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, and 0.1 mM $CaCl_2$ in a starting volume of 1.5 mL. The sample was titrated in parallel with a buffer blank, allowing 10 min after each addition before measurement of intensity and anisotropy, as described under Materials and Methods. Excitation was at 340 nm, and emitted light was isolated with Schott KV470 filters. The final sample volume was 1.7 mL. The anisotropy of the sample was corrected for background fluorescence, as described under Materials and Methods. This correction did not exceed 15%. The data represent two independent experiments. (A) Anisotropy was plotted as a function of total troponin I concentration. (B) The fraction of AEDANS·CaM in complex with troponin I was plotted as a function of free troponin I. This was calculated by using eq 5 as described in the text.

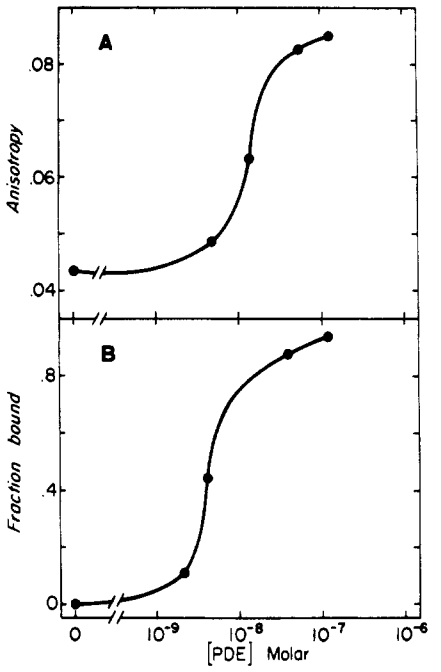

FIGURE 3: Titration of AEDANS·CaM with PDE. The sample consisted of 38 pmol of AEDANS·CaM in 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, and 2.5 mM $CaCl_2$ in a starting volume of 1.5 mL. The sample was titrated in parallel with a buffer blank, allowing 5 min after each addition before measurement of intensity and anisotropy. Excitation was at 340 nm, and emitted light was isolated with Schott KV 470 filters. The final sample volume was 2.06 mL. The anisotropy of the sample was connected for background fluorescence. PDE was prepared as described by LaPorte et al. (1979). (A) Anisotropy is plotted as a function of total PDE concentration. (B) The fraction of AEDANS·CaM in complex with PDE was plotted as a function of free PDE. This was calculated as described under Materials and Methods.

The limits of the titration were used to obtain estimates for the anisotropies of free AEDANS·CaM (A_F) and the AEDANS·CaM–troponin I complex (A_B) as well as the relative intensity change upon complex formation (q). By use of values of 0.0428, 0.0834, and 1.20 for A_F, A_B and q, respectively, eq 4 under Materials and Methods was used to calculate the fraction of AEDANS·CaM complexed to troponin I for each point in the titration. From this, the concentration of free troponin I was readily obtained, and the binding data were plotted by the method of Bjerrum (1923) Figure 2B). These values for A_B and q were probably underestimated because the AEDANS·CaM was not fully saturated with troponin I. Full saturation was not possible because troponin I is poorly soluble at higher concentrations. Nevertheless, the shape of the titration curve indicates that saturation was fairly closely approached.

The binding curve shown in Figure 2 yielded a dissociation constant of 6×10^{-8} M. The logarithmic interval of free troponin I concentration from 0.1 to 0.9 fractional saturation of AEDANS·CaM was ~2.1. This interval provides an estimate of affinity heterogeneity, with an ideal system exhibiting an interval of 1.9 for a single class of noninteracting sites (Weber, 1975).

Titration of AEDANS·CaM with the Ca^{2+}-Sensitive Phosphodiesterase. Binding between the Ca^{2+}-sensitive phosphodiesterase and AEDANS·CaM was also monitored by fluorescence anisotropy (Figure 3). The titration curve shown in Figure 3 is quite steep, suggesting cooperativity. However, more extensive binding data are required in order to make definitive conclusions concerning cooperativity for CaM–phosphodiesterase interactions. Titration of AEDANS·CaM with the phosphodiesterase in the presence of Ca^{2+} resulted in significant anisotropy changes which were reversed by addition of excess unlabeled CaM. Using the methods described above, with the same limitations, it was possible to estimate the dissociation constant for this interaction. Under these conditions, the K_d for the phosphodiesterase–AEDANS·CaM complex appears to be 4 nM. This dissociation constant was significantly higher than the concentration of AEDANS·CaM required for half-maximal stimulation of the enzyme; however, the apparent affinity of the phosphodiesterase for CaM determined by activity measurements is dependent upon assay conditions (Wolff & Brostrom, 1974; Teo et al., 1973; Brostrom & Wolff, 1976). Furthermore, the data reported in Figure 1 were determined in the presence of cAMP. Since CaM lowers the apparent K_m for cAMP (Brostrom & Wolff, 1976) and cAMP decreases the concentration of CaM required for half-maximal stimulation of the phosphodiesterase, one would expect the K_d directly determined in the absence of cAMP to be greater than the apparent K_d determined by activity measurements. The concentrations of CaM required for half-maximal stimulation of the phosphodiesterase are, of course, not true dissociation constants.

Effect of Ca^{2+} on the Emission Spectrum of AEDANS·CaM. In the presence of EGTA, AEDANS·CaM exhibited a featureless emission spectrum with a maximum at 494 nm (Figure 4, Table I). Addition of $CaCl_2$ in excess of EGTA

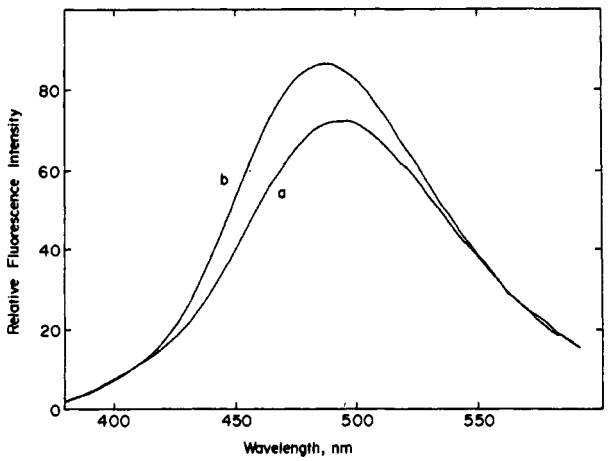

FIGURE 4: Effect of Ca^{2+} on the emission spectrum of AEDANS-CaM. (a) The sample consisted of 10 μM AEDANS-CaM, 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, and 0.25 mM EGTA in a volume of 1.5 mL. Excitation was at 340 nm, and the resolution of both monochromaters was set at 4 nm. The spectrum was acquired and corrected for background intensity and instrument response as described under Materials and Methods. (b) $CaCl_2$ was added to the same sample to a final concentration of 0.35 mM, and the spectrum was acquired and processed in the same manner. Dilution was 0.2%.

Table I: Fluorescence Properties of AEDANS·CaM

parameter	excitation wavelength (nm)	+$CaCl_2$ [a]	+EGTA [b]
emission maximum (nm)	340	485 (2) [c]	494 (1)
relative intensity [d]	340	1.00	0.84 (0.02)
limiting anisotropy (A_0)	310	0.107 (0.002)	0.114 (0.002)
	340	0.131 (0.005)	0.130 (0.002)
	380	0.214 (0.001)	0.214 (0.001)
lifetime (ns) [e]	310	14.5 (0.5)	13.0 (0.3)
	340	14.7 (0.4)	13.5 (0.3)
	380	14.8 (0.5)	13.5 (0.2)

[a,b] Parameters determined in buffer A containing 0.1 mM $CaCl_2$ or 0.25 mM EGTA, respectively. [c] Standard error. [d] Determined from integrated emission spectra, taking the integral of the spectrum determined in the presence of $CaCl_2$ to be 1.00. [e] Determined by the method of Spencer & Weber (1969), as described under Materials and Methods.

produced a 19% increase in the integrated intensity and shifted the emission maximum to 485 nm. This was accompanied by an increase in fluorescence lifetime from 13.5 to 14.7 ns (Table I).

Hydrodynamic Properties of AEDANS·CaM. Fluorescence anisotropy was employed to study the rotational diffusion of AEDANS·CaM in the presence or absence of Ca^{2+}, as described under Materials and Methods (Perrin, 1929; Weber, 1953).

Anisotropy (A) was determined with excitation at 310, 340, or 380 nm. Temperature (T) was maintained at 298 K while viscosity (η) was varied by addition of sucrose. Following eq 2, each set of data was plotted as $1/A$ vs. T/η, and the limiting anisotropy (A_0) was determined from the intercept with the ordinate (Table I). Although A_0 varied with excitation wavelength, this parameter was insensitive to Ca^{2+}. The fluorescence lifetimes, determined for each sample, were independent of excitation wavelength but were increased in the presence of Ca^{2+}, as mentioned above.

Having obtained A_0 from the Perrin plots, these data were replotted as normalized Perrin plots (Figure 5) (Withold & Brand, 1970). By doing so, the slope of the plot was no longer influenced by A_0, and differences in slope reflected differences

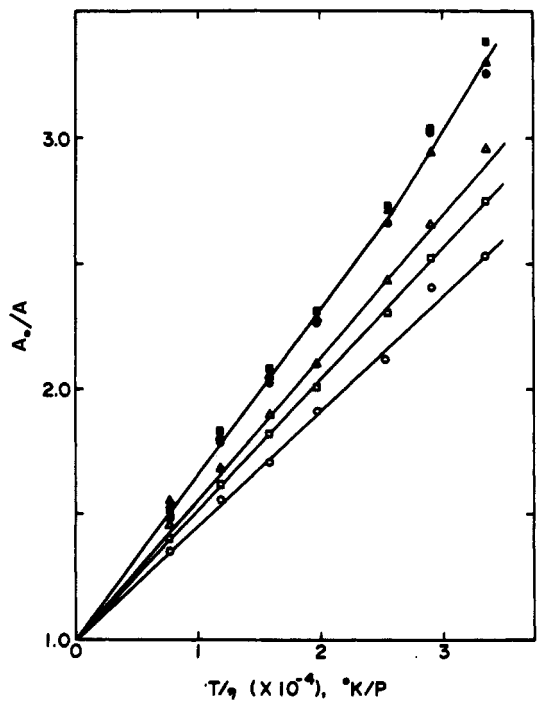

FIGURE 5: Normalized Perrin plots of AEDANS·CaM. The sample consisted of 15 nmol of AEDANS·CaM in a starting volume of 1.5 mL of 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, and either 0.1 mM $CaCl_2$ (○, □, △) or 0.25 mM EGTA (●, ■, ▲). Temperature (T) was maintained at 298 K and viscosity (η) was varied by addition of 60% sucrose (w/v) in the same buffer. Anisotropy (A) was determined after each addition. Excitation was at 310 (○, ●), 340 (□, ■), or 380 nm (△, ▲), and emitted light was isolated with Schott KV470 filters. Resolution of the excitation monochromater was set at 4 nm. The limiting anistropy (A_0) was determined from a plot of $1/A$ vs. T/η, as described under Materials and Methods. The data were then replotted as normalized Perrin plots (Withold & Brand, 1970).

Table II: Hydrodynamic Properties of AEDANS·CaM and Unmodified CaM

parameter	excitation wavelength (nm)	+$CaCl_2$ [a]	+EGTA [b]
$\langle\rho\rangle$ (ns) [c]	310	28.2 (1.1) [d]	17.7 (0.7)
	340	23.8 (1.2)	17.6 (0.5)
	380	22.6 (0.4)	17.4 (0.2)
R_{app} (Å) [e]	310	22.0 (0.4)	18.7 (0.1)
	340	20.6 (0.4)	18.8 (0.2)
	380	20.4 (0.1)	18.8 (0.2)
R_s (AEDANS·CaM) (Å) [f]	NA [h]	23.5 (0.3) [i]	24.1 (0.3)
R_s (CaM) (Å) [g]	NA	23.5 (0.3)	23.5 (0.3)

[a,b] Parameters determined in buffer A containing 0.1 mM $CaCl_2$ or 0.25 mM EGTA, respectively. [c] Average rotational relaxation time, calculated with eq 3. [d] Standard error. [e] Apparent Stokes radius, calculated with eq 2. [f,g] Stokes radius of AEDANS·CaM and unmodified CaM, respectively, determined by gel filtration, as described under Materials and Methods. [h] Not applicable. [i] Values quoted for the standard error of the Stokes radius reflect the error in estimation of the elution position only. The correlation coefficient of the standard curve was 0.996.

in the apparent Stokes radius (R_{app}) or the fluorescence lifetime. In the presence of EGTA, the normalized Perrin plots were independent of excitation wavelength. The average rotational relaxation time ($\langle\rho\rangle$) and R_{app} were ~18.8 ns and ~17.6 Å, respectively, at all three excitation wavelengths (Table II). In marked contrast, in the presence of Ca^{2+}, the normalized Perrin plots were quite dependent on excitation wavelength. R_{app} was found to be 22.0, 20.6, and 20.4 Å when excitation was at 310, 340, and 380 nm, respectively, while

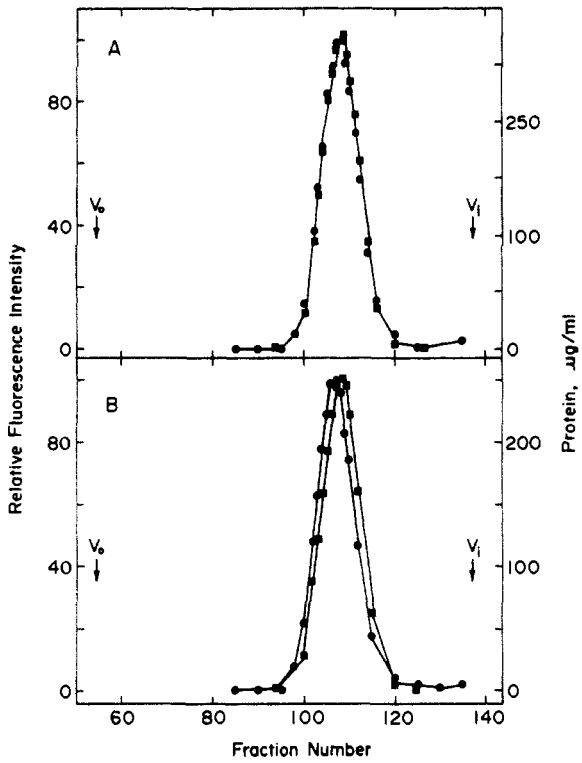

FIGURE 6: Sephadex G-150 elution profile for CaM and AEDANS-CaM. A Sephadex G-150 column (1.5 × 90 cm) was equilibrated in 10 mM Mops, pH 7.2, 1 mM $MgCl_2$, 150 mM KCl, and either (A) 0.1 mM $CaCl_2$ or (B) 0.25 mM EGTA. Samples, consisting of 2 mg of CaM, 0.15 mg of AEDANS-CaM and 2 μCi of ^3H_2O in 4 mL of the equilibration buffer, were applied, and the column was eluted at 12 mL/h. Fraction size was 1 mL. Following chromatography, protein (■) and fluorescence intensity (●) were determined as described under Materials and Methods. The included volume, V_i, was determined for each experiment with ^3H_2O. The void volume, V_o, was determined in a separate experiment with blue dextran.

$\langle\rho\rangle$ was 28.2, 23.8, and 22.6 ns at these wavelengths. It should also be noticed that $\langle\rho\rangle$ and R_{app} were substantially greater when determined in the presence of $CaCl_2$ rather than EGTA.

The possibility that the difference in R_{app} determined in the presence of $CaCl_2$ rather than EGTA was due to a Ca^{2+}-induced change in the Stokes radius, R_S, was investigated by gel filtration (Figure 6, Table II). The Stokes radius of unmodified CaM was found to be 23.5 Å and was not influenced by Ca^{2+}. In the presence of Ca^{2+}, the AEDANS-CaM peak was superimposable on that of unmodified CaM (Figure 6A, Table II). In the presence of EGTA, however, the Stokes radius of AEDANS-CaM was found to be 24.1 Å, an increase of 2–3% over that determined for AEDANS-CaM in the presence of Ca^{2+} and that determined for unmodified CaM.

Discussion

We have developed a technique for the preparation of a fluorescent derivative of CaM, AEDANS-CaM, which retained its ability to stimulate the Ca^{2+}-sensitive phosphodiesterase. This derivative formed Ca^{2+}-dependent complexes with troponin I and the phosphodiesterase, which were directly detectable by increases in fluorescence anisotropy.

The dissociation constant of the AEDANS-CaM–troponin I complex in the presence of Ca^{2+} was $\sim 6 \times 10^{-8}$ M. Formation of a Ca^{2+}-independent complex between AEDANS-CaM and troponin I was detected above 1×10^{-6} M troponin I, but was not characterized because of the poor solubility of troponin I at higher concentrations (Greaser & Gergely, 1973). If the free-energy coupling for Ca^{2+} and troponin I binding to AEDANS-CaM is similar to that found for unmodified CaM (Keller et al., 1980), the Ca^{2+}-independent dissociation constant for the AEDANS-CaM–troponin I would be predicted to be $\sim 4 \times 10^{-5}$ M. Thus, the Ca^{2+}-independent formation of the AEDANS-CaM–troponin I complex was detectable by fluorescence anisotropy in approximately the concentration range where it would be expected. The dissociation constant for AEDANS-CaM–phosphodiesterase in the presence of Ca^{2+} was $\sim 4 \times 10^{-9}$ M. Therefore, the affinity of AEDANS-CaM for the phosphodiesterase was approximately 20 times greater than for troponin I.

The binding of Ca^{2+} to AEDANS-CaM produced a 9-nm blue shift of the emission maximum of the probe and increased the integrated intensity of the emission spectrum by ~20%. This was accompanied by an increase in the fluorescence lifetime from ~13.3 to ~14.7 ns. These changes suggest that, in the presence of Ca^{2+}, some population of the probe underwent a substantial reduction in solvent exposure. Despite these environmental changes, the freedom of motion of the probe with respect to the protein surface did not change, as reflected in the insensitivity of A_0 to Ca^{2+}.

Randomness of probe incorporation into dye–protein conjugates and the shape of the protein can be analyzed by construction of "normalized Perrin plots" at different excitation wavelengths (Withold & Brand, 1970). This approach is based on the assumption that each excitation wavelength chosen will yield a different average angle between the absorption and emission vectors. This angle influences the extent to which the various rotational motions of the protein are reflected in the observed rotational diffusion and thus in the normalized Perrin plots (Weber & Anderson, 1969; Withold & Brand, 1970). No differences in these plots would be observed for spheres or oblate ellipsoids, because the rotational rates around the principal axes of such particles are not sufficiently different (Weber, 1953). In the case of a randomly labeled prolate ellipsoid, the various labeling positions would be compensatory so that no dependence on excitation wavelength would be observed in this case either. However, labeling may be compensatory without being truly random. The normalized Perrin plots should exhibit a dependence on excitation wavelength only in the case of nonrandom labeling of a prolate ellipsoid.

The apparent rotational diffusion observed for AEDANS-CaM in the presence of $CaCl_2$ was found to be significantly dependent on the wavelength of excitation. Following the reasoning given above, this implies that AEDANS-CaM can be approximated as a nonrandomly labeled prolate ellipsoid under these conditions. The theory of Withold & Brand (1970) does not, however, consider the possibility of segmental flexibility, which might produce similar results. This seems unlikely in this case, however, because segmental flexibility is very uncommon, especially in small proteins constructed of a single polypeptide chain (Yguerabide et al., 1970).

In contrast, the behavior of AEDANS-CaM in the absence of Ca^{2+} appeared to be more consistent with random labeling of a prolate ellipsoid. Although these results could also be explained by invoking a massive conformational change yielding a spherical or oblate ellipsoidal shape, it seems unlikely that such a change would come about without substantially changing the Stokes radius determined by gel filtration. Thus, the appearance of apparent randomness of orientation of the probes as well as the Ca^{2+}-induced changes in $\langle\rho\rangle$ and R_{app} probably results from a reorientation of some fraction of the probes with respect to the protein. As discussed above, the apparent randomness of probe orientation observed in the absence of Ca^{2+} does not necessarily indicate that the labeling

was random in any rigorous sense.

The Stokes radius of native CaM, determined by gel filtration, was found to be 23.5 Å, both in the presence and in the absence of Ca^{2+}. This is similar to the value obtained for rat testes CaM (Dedman et al., 1977). Although the Stokes radius of AEDANS·CaM was equal to that of native CaM when determined in the presence of Ca^{2+}, an increase of 2–3% was found when the experiment was performed in the presence of EGTA. The origin of this discrepancy is not clear, but it may result, at least in part, from the contribution of the probe itself to the Stokes volume of the conjugate. Addition of such a probe to an unhydrated sphere with a volume equivalent to that of CaM would be expected to increase the Stokes radius by ~1%. The actual effect on the Stokes radius of CaM could differ considerably from 1%, depending on the placement and the degree of solvent exposure of the probe. The lack of significant influence of the probe on the Stokes radius of CaM determined in the presence of $CaCl_2$ could, then, be explained by a decreased solvent exposure of the probe. Such a decrease in solvent exposure was detected by the Ca^{2+}-induced changes in the emission spectrum and fluorescence lifetimes, as discussed above. Alternatively, a conformational change, induced by the probe in the absence of Ca^{2+}, is not readily ruled out. The lack of effect of Ca^{2+} on A_0, however, suggests that a gross unfolding had not occurred. In any event, this discrepancy in Stokes radius measurements was quite small and was not observed in the presence of Ca^{2+}.

The extent to which the hydrodynamic behavior of a protein departs from that of an ideal sphere is often expressed as the ratio of the observed frictional coefficient (f) to that calculated for an unhydrated sphere of equivalent volume (f_0). The frictional ratio observed for globular proteins is typically close to 1.2 (Tanford, 1961). Using the value for the Stokes radius of CaM determined by gel filtration, we found f/f_0 to be ~1.4 for this protein, in agreement with other workers (Teo et al., 1973; Dedman et al., 1977). This unusually large value for the frictional ratio appears to result, at least in part, from the elongated shape detected for CaM by the fluorescence experiments described above.

An elongated shape may have a special advantage for CaM in that it would allow each Ca^{2+} site to transmit its influence to the protein–protein interface through relatively independent regions of the protein structure. It has been suggested that only a small amount of energy may be transmitted through a protein as a result of the relatively weak forces maintaining protein structure (Weber, 1975). Thus, transmission of this energy through spatially separated regions might allow for the maximization of total free energy coupling. Some support is lent to this suggestion by the observation that the Ca^{2+} binding sites of calmodulin are noninteracting sites (Dedman et al., 1977; Keller et al., 1980). It is unlikely that this would be the case if these sites transmitted their effects through the same regions of protein structure. There is, however, considerable disagreement concerning the metal ion binding characteristics of calmodulin, and Klee has observed that Ca^{2+} binding to calmodulin exhibits positive cooperativity (Crouch & Klee, 1980).

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Carbon-13 Nuclear Magnetic Resonance Study of Protonation of Methotrexate and Aminopterin Bound to Dihydrofolate Reductase†

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ABSTRACT: Methotrexate, aminopterin, and folate have been synthesized with 90% enrichment of C-2 with ^{13}C. ^{13}C nuclear magnetic resonance has been used to examine the state of protonation of the pteridine ring of these compounds under various conditions and gives much more clear-cut results than most other methods. For the free compounds the following pK values were obtained: methotrexate, 5.73 ± 0.02 (N-1); aminopterin, 5.70 ± 0.03 (N-1); folic acid, 2.40 (N-1) and 8.25 ± 0.05 (N-3, O-4 amide group). The state of protonation of these compounds when complexed to dihydrofolate reductase (isoenzyme 2 from *Streptococcus faecium*) was also studied over the pH range 6–10. The resonance from bound methotrexate showed a constant chemical shift over the whole pH range studied, and it is inferred that in the complex the pteridine ring remains protonated to at least pH 10. The same result was obtained for the binary complex of aminopterin with the reductase and for either methotrexate or aminopterin in ternary complex with reductase and $NADPH_4$. The latter is an inhibitor of the reductase competitive with NADPH. However, folate bound to the reductase in either the binary or the ternary complex shows the same protonation behavior as in the free state. The data indicate that the association constant for binding of methotrexate is increased enough when protonation of N-1 occurs to account for the enhanced binding of methotrexate as compared with folate.

More than 20 years ago methotrexate (4-amino-4-deoxy-10-methylfolic acid, MTX), aminopterin, and related 2,4-diamino heterocycles were shown to be potent inhibitors of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3, DHFR). Typically MTX binds to the enzyme with an association constant considerably greater than that for combination with the substrate, dihydrofolate (DHF) or folate. When MTX combines with the enzyme–NADPH complex, the equilibrium favors the ternary complex so greatly that the association constant frequently cannot be measured with accuracy. In the case of DHFR from *Streptococcus faecium*, for example, this association constant is $>10^9$ (Williams et al., 1979). The clinical usefulness of inhibitors such as MTX and trimethoprim give added significance to this interaction of the inhibitors with DHFR, their primary target.

Almost as soon as it was discovered, Baker (1959) suggested that the tight binding of aminopterin to DHFR is due to the increased basicity of the pteridine ring in this inhibitor, which is a consequence of the replacement of the 4-oxo substituent of folate by a 4-amino group. It was postulated that the increased basicity permits a strong interaction between the protonated species of the inhibitor with an acidic group in the catalytic site of the enzyme. This interaction would not occur in the case of folate or DHF. Subsequently Baker and his colleagues obtained considerable evidence in support of this view [reviewed by Baker (1967)]. They compared the association constants for the binding to DHFR of homologous inhibitors which are protonated, unprotonated, or partially protonated at neutral pH and found that binding increased with degree of protonation. Similarly, Montgomery et al. (1971) found that the more basic 3-deazamethotrexate binds more tightly than 1-deazamethotrexate.

Spectroscopic evidence consistent with this view was first obtained by Erickson & Mathews (1972), who examined the electronic absorption spectrum of MTX and aminopterin. The difference spectrum obtained by comparing the free inhibitor with the complex of inhibitor with DHFR of T4 phage (both at pH 7.0) approximated the difference spectrum obtained by comparing inhibitor at low pH (4.8 or 2.8) vs. inhibitor at pH 7.0. This suggested that enzyme-bound methotrexate is protonated, although, as might be expected, the two difference spectra showed significant deviations. Similar results were obtained by Poe et al. (1974) for DHFR from *Escherichia coli*, by Gupta et al. (1977) for the enzyme from the L1210 murine lymphoma, by Hood & Roberts (1978) for DHFR from *Lactobacillus casei*, and by Subramanian & Kaufman (1978) for chicken liver DHFR. Saperstein et al. (1978) found that Raman spectra were also indicative of protonation of the pteridine ring of MTX bound to *E. coli* DHFR. Subramanian & Kaufman (1978) obtained calorimetric evidence that when MTX binds to the chicken liver enzyme, a proton is abstracted

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