Immunoaffinity Purification and Characterization of 5-[[[(Iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic Acid Derivatized Calmodulin†

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ABSTRACT: Fluorescent 5-[[[(iodoacetyl)amino]ethyl]-amino]naphthalene-1-sulfonic acid derivatized bovine brain calmodulin (AEDANSCaM) was purified to homogeneity and characterized. Analysis of a tryptic digest of AEDANSCaM separated by high-performance liquid chromatography revealed 70–80% of the fluorescent dye was covalently bound to the C-terminal tryptic peptide of calmodulin (residues 127–148). Purification of AEDANSCaM was accomplished by immunoaffinity chromatography. Rabbit immunoglobulin obtained from antisera prepared against 5-[[[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid derivatized bovine serum albumin was coupled to a solid agarose support. The immunoaffinity column was used to specifically separate fluorescent-labeled calmodulin from residual unreacted calmodulin remaining after the fluorescent-modification reaction. Ca^{2+}-dependent interactions between purified AEDANSCaM and troponin I or the calmodulin-sensitive phosphodiesterase were quantitated by fluorescence anisotropy. Removal of unmodified calmodulin from AEDANSCaM preparations resulted in significant changes in its apparent affinity for the phosphodiesterase and troponin I. The respective dissociation constants for the $n Ca^{2+}$–AEDANSCaM–troponin I and $n Ca^{2+}$–AEDANSCaM–phosphodiesterase complexes were reduced from 60 nM to 15 nM and 10 nM to 3 nM by removal of unmodified calmodulin from the preparation. In addition, removal of nonmodified CaM increased the half-maximal activation constant for AEDANSCaM activation of the CaM-sensitive phosphodiesterase from 280 pM to 650 pM. Purified AEDANSCaM was used for the determination of the dissociation constant for the native $4Ca^{2+}$–calmodulin–troponin I complex. The dissociation constant for the native complex was 3 nM, 20-fold lower than that previously observed with fluorescent calmodulin. The immunoaffinity-purified preparation of fluorescent calmodulin is more suitable for the rigorous quantitation of calmodulin–enzyme interactions than those previously available. This method may be generally used to purify chemically modified calmodulin from residual unreacted calmodulin.

Calmodulin (CaM),[1] a small 16 700-dalton protein, binds 4 mol of calcium and is thought to mediate the Ca^{2+} dependency of a large number of cellular activities [for reviews, see Wang & Waisman (1979), Means & Dedman (1980), Cheung (1980), and Klee et al. (1980)]. Ca^{2+}-dependent binding of CaM to CaM-binding proteins has been demonstrated by a variety of nonequilibrium techniques that are not well suited to studying the weaker interaction of Ca^{2+}-independent CaM-binding interactions. Recently, quantitative kinetic models have been proposed for Ca^{2+} and CaM interactions with the CaM-sensitive phosphodiesterase (PDE) (Huang et al., 1981; Cox et al., 1981) and myosin light chain kinase (MLCK) (Blumenthal & Stull, 1980). Equilibrium-binding techniques have yielded the Ca^{2+}-dependent dissociation constants for fluorescent AEDANSCaM interactions with troponin I (TnI) and the CaM-sensitive PDE (LaPorte et al., 1981), the Ca^{2+}-dependent affinity constant for [^{3}H]CaM interaction with MLCK (Crouch et al., 1981), and the Ca^{2+}-independent dissociation constant for fluorescent *N*-dansylaziridine-derivatized TnI interaction with CaM (Olwin et al., 1982).

In a previous study, we described the preparation of a fluorescent-labeled CaM (AEDANSCaM) and reported the dissociation constant for the Ca^{2+}-dependent interaction of AEDANSCaM with TnI and PDE. The preparation of fluorescent AEDANS-labeled CaM contained residual nonlabeled CaM and was unreliable for accurate determinations of dissociation constants. The half-maximal Ca^{2+}-dependent activation constant reported for AEDANSCaM activation of PDE was inaccurate (LaPorte et al., 1981). We describe a technique for the preparation of AEDANSCaM free of residual nonlabeled CaM that maximally stimulated the CaM-sensitive PDE, retained Ca^{2+}-dependent affinity for PDE and TnI, and may be generally suitable for purification of chemically modified CaM.

Materials and Methods

Fluorescent 1,5-IAEDANS was obtained from Molecular Probes, Inc. All other chemicals were reagent grade or better.

Protein Preparations. CaM was prepared as previously described (LaPorte et al., 1979). TnI was prepared from rabbit skeletal muscle by the method of Wilkinson (1974). Bovine heart PDE was prepared as previously described by LaPorte et al. (1979). AEDANSCaM was prepared as described previously (LaPorte et al., 1981), and preparation of immunoaffinity-purified AEDANSCaM is described under Results. Protein concentrations were determined by the methods of Bradford (1976) and Lowry et al. (1951) or spectrophotometrically with $E^{1\%}_{280nm} = 3.97$ for TnI (Wilkinson, 1974) and $E^{1\%}_{277nm} = 1.8$ for CaM (Watterson et al., 1976). Where molar concentrations for CaM, TnI, or the monomer of PDE were

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[1] Abbreviations: HPLC, high-performance liquid chromatography; 1,5-IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; CaM, calmodulin; AEDANSCaM, 5-[[[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid derivatized calmodulin; PDE, phosphodiesterase; TnI, troponin I; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; IgG, immunoglobulin G.

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reported, the respective molecular weight values of 16 723 (Vanaman et al., 1977), 23 000 (Wilkinson, 1974), and 57 000 (LaPorte et al., 1979) were used.

Fluorescence Measurements. All fluorescence measurements were made on an SLM 4800 spectrofluorometer. The buffer used in fluorescence measurements was 10 mM Mops (pH 7.2), 150 mM KCl, 1 mM $MgCl_2$, and 2.0 mM dithiothreitol (buffer A) with additions as indicated. Fluorescent impurities were removed from the titration buffers by passing the buffer over an activated charcoal/Celite 1:2 (v/v) column, and removal of small carbon particles was by Millipore ultrafiltration. The fluorescence intensity and anisotropy of the prepared buffer is low and reduces the background fluorescence intensities. Complex formation between CaM and CaM-binding proteins was detected by fluorescence anisotropy, corrected for background, and the fraction of AEDANSCaM bound was calculated as previously described (LaPorte et al., 1981).

The K_d for Ca^{2+}-dependent interaction of native CaM with TnI was determined by competition of the nCa^{2+}–AEDANSCaM–TnI complex with native $4Ca^{2+}$–CaM. Competition of the Ca^{2+}-dependent AEDANSCaM–TnI complex with native $4Ca^{2+}$–CaM and formation of the $4Ca^{2+}$–CaM–TnI complex were quantitated by the decrease in the fraction of TnI bound to AEDANSCaM (β). If the concentration of TnI is the limiting species and the concentration of the nCa^{2+}–AEDANSCaM–TnI complex was in excess of the K_d, the free-CaM concentration was readily calculated from β and the total concentrations of TnI and CaM, respectively. If one assumes a value for the native $4Ca^{2+}$–CaM–TnI dissociation constant, the expected values of free CaM concentration as a function of β were calculated from

$$[CaM]_{free} = \frac{K_d([AEDANSCaM]_T/[TnI]_T - \beta)[CaM]_T}{K_d'\beta + K_d([AEDANSCaM]_T/[TnI]_T - \beta)} \quad (1)$$

where β is the fraction of TnI bound to AEDANSCaM, K_d is the dissociation constant for the $4Ca^{2+}$–CaM–TnI complex, and K_d' is the dissociation constant for the nCa^{2+}–AEDANSCaM–TnI complex. $[AEDANSCaM]_T$ is the total concentration of AEDANSCaM, $[TnI]_T$ is the total concentration of TnI, and $[CaM]_T$ is the total-added CaM concentration corrected for dilution. Comparison of the calculated and experimental values of free-CaM concentration as a function of β yields the native dissociation constant. For all fluorescence experiments, excitation was at 340 nm, and the excitation monochrometer band-pass and slit widths were at 4 nm. Emitted light was isolated with Schott KV 470 filters. Sample temperature was maintained at 25 ± 0.1 °C.

Tryptic Digestion of CaM and AEDANSCaM. CaM and AEDANSCaM (1%) were digested for 3 h in 0.1 M NH_4HCO_3, pH 8.0, at 37 °C with TLCK-treated trypsin (Worthington) at 1% (w/w) of the total CaM concentration. Peptide separation was by reverse-phase high-performance liquid chromatography (HPLC) on a Syn-Chropak RP·P column. The mobile phase was 0.1% trifluoroacetic acid, and the mobile-phase modifier was acetonitrile as described by Sasagawa et al. (1982). Fluorescent peptides and the extent of labeling were identified by fluorescence intensity on an SLM 4800 spectrofluorometer.

Production of Antisera to AEDANS-Labeled Proteins. Ten milligrams of bovine serum albumin (Pentex grade, Miles Laboratories) in 1.0 mL of 0.2 M sodium acetate (pH 5.0) was made 10 mM in 1,5-IAEDANS and allowed to react at room temperature for 15 h in the dark. Free 1,5-IAEDANS was separated from AEDANS-labeled BSA (AEDANSBSA) on Sephadex G-25 in 0.02 M sodium acetate, pH 5.0, 0.2 M NaCl, and 8 M urea (freshly deionized), followed by separation on Sephadex G-25 in 10 mM Mops (pH 7.2) and 150 mM KCl. Typical incorporation of 4 mol of AEDANS dye/mol of BSA was obtained by assumption of a molar extinction coefficient of 6000 for bound AEDANS dye (Hudson & Weber, 1973). Two rabbits were injected intradermally on several sites of the back with 1 mg of AEDANSBSA emulsified in an equal volume of Freund's complete adjuvant. After 3 weeks, titer against AEDANSBSA was determined by Ouchterlony diffusion analysis. Both rabbits were reinjected as before with complete adjuvant around sites of initial injection. Two weeks later, antisera was obtained, and IgG was purified on protein A–Sepharose (Pharmacia). Rabbit antisera (2 mL) was applied to a protein A–Sepharose column (0.5 mL) in 0.01 M Mops, pH 7.2, and 0.15 M NaCl. Rabbit immunoglobulin was specifically eluted with 0.05 M sodium citrate, pH 2.5, and 0.15 M NaCl. Purified IgG was used to determine serum titer quantitatively by fluorescence anisotropy (data not shown). Where reported, concentrations were determined by using $A^{1\%}_{280nm} = 14.5$ (Hudson & Fay, 1976) and a molecular weight of 150 000.

The anti-AEDANS affinity column was prepared by coupling 25 mg of purified IgG (purified on protein A–Sepharose) in 0.1 M Mops, pH 7.2, to 2.0 mL of Affi-Gel 10 (Bio-Rad Laboratories) for 12 h at 4 °C. The affinity resin was washed with 0.01 M Mops, pH 7.2, 0.15 M KCl, and 8 M urea (freshly deionized) and equilibrated in 10 mM Mops (pH 7.2), 150 mM KCl, and 0.2 mM $CaCl_2$ before application of AEDANSCaM. The column was not coupled with affinity-(AEDANS) purified IgG specific for the AEDANS dye due to large losses in antibody-binding activity that occurred under these coupling conditions (data not shown).

Amino Acid and Sequence Analyses. Amino acid analyses were performed with a Dionex D-500 analyzer. Automated sequence analyses were performed on a Beckman sequence Model 890, and phenylthiohydantoin derivatives of amino acids were identified by HPLC as described by Sasagawa et al. (1982).

Results

Purification of AEDANSCaM by Immunoaffinity Chromatography. AEDANSCaM was prepared as described by LaPorte et al. (1981). Briefly, 2 mg of CaM in 1.2 mL of 0.2 M sodium acetate (pH 5.0) and 2.0 mM $CaCl_2$ was made 10 mM in 1,5-IAEDANS and allowed to react for 9 h in the dark. The preparation was desalted on Sephadex G-25 into 10 M sodium acetate (pH 5.0), 8 M urea, 0.3 M NaCl, and 3.0 mM EGTA (to remove noncovalently bound dye) and then desalted on Sephadex G-25 into 10 mM Mops (pH 7.2), 150 mM KCl, and 0.2 mM $CaCl_2$. AEDANSCaM was further purified on troponin I–Sepharose (Head et al., 1979) to remove inactive, modified CaM. The preparation yields approximately 0.3 mg of active AEDANSCaM. The purification of AEDANSCaM from unmodified CaM was by application of 0.3 mg of active AEDANSCaM to the anti-AEDANS immunoaffinity column. To determine whether unmodified CaM would bind to the immunoaffinity column, the column was purposefully overloaded with a 3-fold excess of AEDANSCaM over the column capacity. Prior to application of AEDANSCaM to the column, 481 000 cpm of ^{125}I-labeled CaM (LaPorte & Storm, 1978) was added to monitor the chromatography of unmodified CaM. Chromatography of AEDANSCaM was monitored by a hand-held ultraviolet light and quantitated by relative fluorescence measurements with an SLM 4800

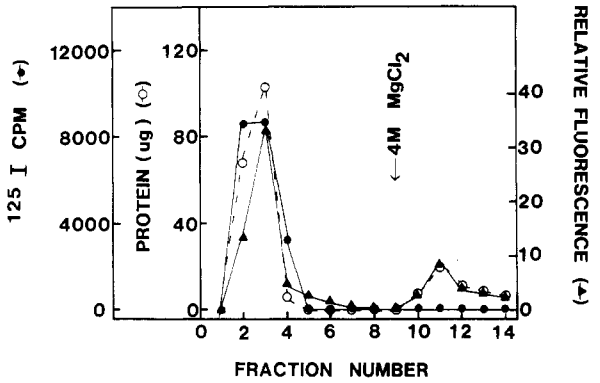

FIGURE 1: Immunoaffinity purification of AEDANSCaM on anti-AEDANS-agarose. Profiles of protein concentration (○), ^{125}I-labeled CaM (●), and fluorescence intensity (▲) demonstrate separation of residual nonlabeled CaM from AEDANSCaM. Approximately 400 000 cpm of ^{125}I-labeled CaM was added to 0.30 mg of AEDANSCaM [(prepared as described by LaPorte et al. (1981)] and applied to a 2.0-mL column of anti-AEDANS IgG (25 mg) coupled to Affi-Gel 10 as described under Materials and Methods. The column was washed with 8.0 mL of 10 mM Mops, pH 7.2, 150 mM KCl, and 0.2 mM $CaCl_2$ to remove residual nonlabeled CaM. Bound AEDANSCaM was specifically eluted with 4 M $MgCl_2$ in 5 mM Mops, pH 7.2. Each 1.0-mL fraction was assayed for protein content by the method of Bradford (1976) and counted for [^{125}I]CaM cpm, and the fluorescence intensity was measured as described under Materials and Methods. The capacity of the 2.0-mL immunoaffinity column is approximately 60 μg of AEDANSCaM. Fractions of AEDANSCaM were pooled by fluorescence intensity, concentrated on DEAE-Sephacel, and stored at −70 °C.

Table I: Amino Acid Composition of Fluorescent Peptide FP-2

residue	FP-2[a]	C-terminal peptide residues 127–148[b]
Asx	4.1 (4)	4
Thr	1.4 (1)	1
Ser	0.9 (0)	0
Glx	5.0 (5)	5
Pro	0 (0)	0
Gly	2.9 (2)	2
Ala	2.3 (2)	2
Val	2.1 (2)	2
Met	0.9 (2)	2
Ile	1.7 (1)	1
Leu	0.9 (1)	0
Tyr	0.4 (1)	1
Phe	0.8 (1)	1
His	0 (0)	0
Lys	1.0 (1)	1
Arg	0 (0)	0
total no. of residues	23	22

[a] Residues/molecule determined by amino acid analysis for FP-2. [b] Residues/molecule determined by amino acid analysis or from the sequence (Watterson et al., 1980; Sasagawa et al., 1982).

spectrofluorometer. Following application of the sample, the column was washed with application buffer until the fluorescence intensity and [^{125}I]CaM cpm returned to zero (Figure 1). All ^{125}I-labeled CaM was eluted in the void volume with residual, nonlabeled CaM. The column was eluted with 4 M $MgCl_2$ in 5 mM Mops, pH 7.2, yielding fluorescent AEDANSCaM free of CaM. AEDANSCaM was immediately desalted into buffer A containing 0.2 mM $CaCl_2$, concentrated on DEAE-Sephacel, and stored at −70 °C in 10 mM Mops, pH 7.2, 0.5 M KCl, and 0.2 mM $CaCl_2$. Approximately 60 μg of immunoaffinity-purified AEDANSCaM was obtained from elution of the 2.0-mL immunoaffinity column. Immunoaffinity-purified AEDANSCaM was subsequently used to determine the K_d for Ca^+-dependent interaction of AEDANSCaM with TnI and PDE.

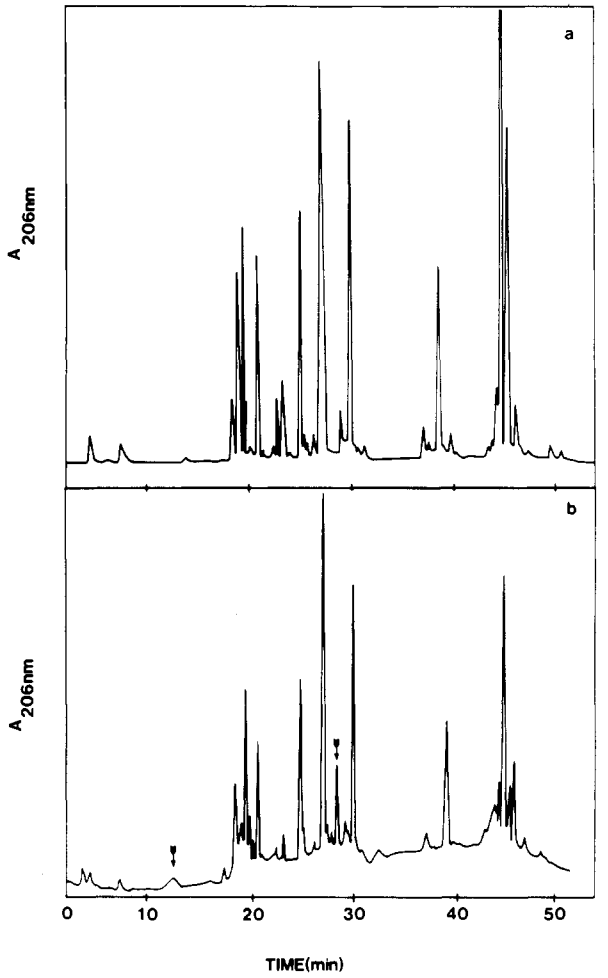

FIGURE 2: Tryptic digests of native CaM and AEDANSCaM. Separation of tryptic digests of bovine brain CaM (a) and AEDANSCaM (b) by HPLC. Tryptic digests of CaM (12 nmol) and AEDANSCaM (12 nmol) were separated on a Syn-Chropak RP-P column (250 × 4.1 mm). The mobile phase was 0.1% trifluoroacetic acid with an increasing gradient of 0–45% acetonitrile as described under Materials and Methods. AEDANSCaM was prepared as previously described by LaPorte et al. (1981). Arrows refer to peaks present in the HPLC profile of trypsin-digested AEDANSCaM peaks not present in the CaM profile. Peaks FP-1 and FP-2 with respective retention times of 12 and 28 min were found to contain 20% and 80% of the respective total fluorescence intensity. Peak FP-1 was not identifiable by amino acid analysis, and peak FP-2 was the derivatized C-terminal tryptic peptide of CaM (residues 127–148).

Tryptic Digestion and Isolation of AEDANSCaM Peptides. Fluorescent AEDANSCaM was characterized by tryptic digestion, and separation of those digests was by HPLC. Two AEDANSCaM preparations purified through the troponin I–Sepharose step (0.7 mg) were digested with trypsin and chromatographed by reverse-phase HPLC. The overall yield of FP-2 was 5%; however, 50% or less of the applied AEDANSCaM preparation was labeled with dye. Because of the low yield of FP-2 and the significant time involved in preparation of AEDANSCaM and FP-2, the sequence analysis was not pursued further.

Separation of the tryptic digests from bovine brain CaM (Figure 2a) and AEDANSCaM (Figure 2b) identified one minor and one major fluorescent peptide designated FP-1 and FP-2 with respective retention times of 13 and 28 min. The major fluorescent peptide (FP-2) was 78% and 65% of the total measured fluorescence intensity for two separate AEDANSCaM preparations. Amino acid anlysis of FP-2 (Table I) was consistent with the composition of the C-terminal tryptic peptide (residues 127–148) (Watterson et al., 1980; Sasagawa

Table II: Sequence of Fluorescent Peptide FP-2

C-terminal peptide (residues 127-148)[a]	E-A-D-I-D-G-D-G-Q-V-N-Y-E-E-E-F-V-Q-M-M-T-A-K-OH
FP-2[b]	E_{597}-A_{571}-D_{716}-I_{413}-D_{491}-G_{267}-D_{546}-G_{278}-Q_{156}-V_{206}-N_{287}-Y_{196}-E_{116}-E_{90}[c]

[a] Sequence of C-terminal tryptic peptide of CaM (Watterson et al., 1980; Sasagawa et al., 1982). [b] Partial sequence of fluorescent peptide FP-2. [c] Subscripts are picomoles of phenylthiohydantoin (PTH) amino acids identified by HPLC from 2 nmol of FP-2. The PTH-amino acids for Q and N were the respective sums of PTH-Gln and PTH-Glu or PTH-Asn and PTH-Asp.

et al., 1982), but it yielded 0.9 methionine residue/peptide. Since acid hydrolysis of AEDANS-methionine would yield methionine, *S*-(carboxymethyl)homocysteine, homoserine, and its lactone, it was not certain from the composition whether either or both of two adjacent methionine residues in the peptide were labeled with 1,5-IAEDANS. To clarify this problem, FP-2 was subjected to automated sequence analysis. Unfortunately, the yield of FP-2 was low (approximately 2 nmol from 15 nmol of AEDANSCaM), and the analysis at this low amount did not permit identification of the derivatized methionine. It unambiguously confirmed, however, that FP-2 was the C-terminal peptide (Table II), indicating that the AEDANS dye was covalently bound to methionine-144 or -145 or both of CaM.

The minor fluorescent peptide (FP-1) was composed of low concentrations of several amino acids (approximately 0.05 nmol each) not identified as tryptic digests of CaM (data not shown). FP-1 may consist of acid-hydrolyzed free AEDANS dye released during or before HPLC chromatography in 0.1% trifluoroacetic acid. Fluorescence-lifetime data suggest that the labeling of CaM with 1,5-IAEDANS yields a homogeneous product (LaPorte et al., 1981). In addition, Lambooy et al. (1982) prepared AEDANSCaM by the method of LaPorte et al. (1981) and determined the fluorescence-lifetime decay in the presence and absence of Ca^{2+}. The lifetime decays were composed of a single component, strongly suggesting that incorporation of 1,5-IAEDANS is homogeneous.

Stimulation of Bovine Heart PDE by Immunoaffinity-Purified and Nonpurified AEDANSCaM. Purified AEDANSCaM free of native CaM was able to maximally stimulate bovine heart PDE (Figure 3). The half-maximal activation constants (K_a) for native, purified, and nonpurified AEDANSCaM were 120 pM, 650 pM, and 280 pM, respectively (Table II). Bovine heart PDE was purified through the second DEAE column as previously described (LaPorte et al., 1979). Two milliunits of PDE was assayed for 15 min at 30 °C in a total volume of 0.5 mL. Assays were performed as previously described (LaPorte et al., 1981). The removal of residual nonlabeled CaM from the AEDANSCaM preparation resulted in a greater than 2-fold increase in the half-maximal activation constant for AEDANSCaM activation of the phosphodiesterase. Accurate determination of the activation constant for chemically modified CaM is not possible without removal of the residual nonlabeled CaM.

Interaction of Immunoaffinity-Purified and Nonpurified AEDANSCaM with TnI. Ca^{2+}-dependent binding of immunoaffinity purified and nonpurified AEDANSCaM to rabbit skeletal TnI was monitored by fluorescence anisotropy (Figure 4). Binding of TnI to AEDANSCaM was demonstrated by the increase in fluorescence anisotropy from 0.0430 for free AEDANSCaM–$n$$Ca^{2+}$ to 0.0800 for the AEDANSCaM–$n$$Ca^{2+}$–TnI complex. The two binding curves in Figure 4 demonstrate the differences for the interaction of purified and

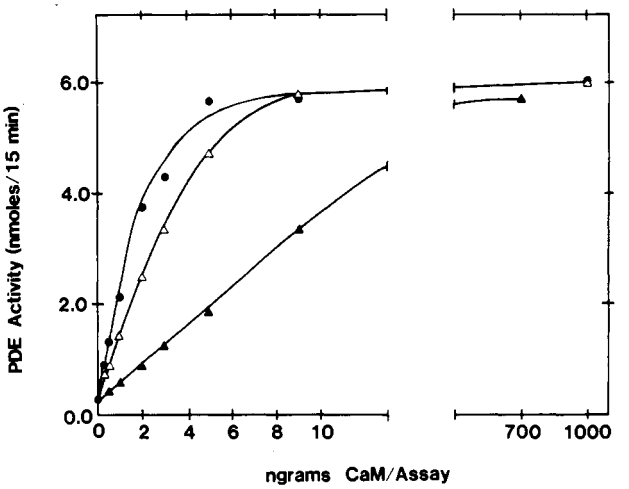

FIGURE 3: Stimulation of bovine heart phosphodiesterase (PDE) by CaM and AEDANSCaM. Stimulation of PDE by native CaM (●), nonpurified AEDANSCaM (△), and immunoaffinity-purified AEDANSCaM (▲) is shown as a function of the nanograms of CaM added to the assay. Basal PDE activity was determined in the absence of CaM. Two milliunits of PDE was assayed for 15 min at 30 °C in a total volume of 0.5 mL. One unit of PDE activity hydrolyzes 1 μmol of cAMP/min in the presence of 20 mM imidazole, saturating cAMP, and saturating CaM concentrations (LaPorte et al., 1979). The PDE was purified as described previously (LaPorte et al., 1979) and assayed by a modification of a method of LaPorte & Storm (1978). Assays contained 10 mM Mops (pH 7.2), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM KCl, 0.1 mg/mL Pentex grade bovine serum albumin, 2 mM dithiothreitol, 0.1 mM phenylmethylanesulfonyl fluoride, 0.05 mM [^{3}H]cAMP (60 000 cpm), [^{14}C]-5′-AMP (6000 cpm), and the indicated concentrations of AEDANSCaM and native CaM.

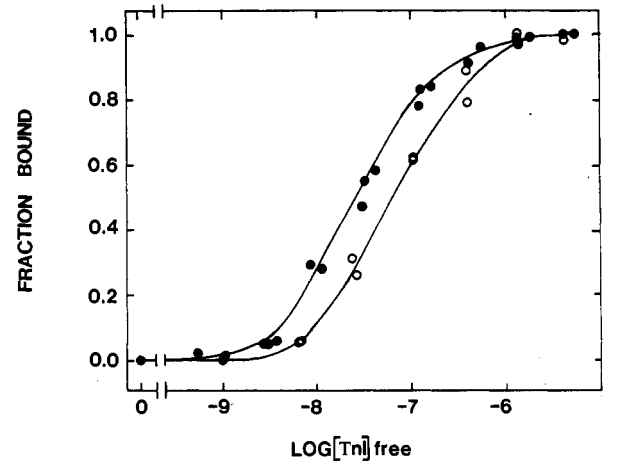

FIGURE 4: Titration of nonpurified and purified AEDANSCaM with troponin I. The fraction of immunoaffinity-purified AEDANSCaM (●) and nonpurified AEDANSCaM (○) bound is plotted as a function of the log of the free molar TnI concentration. Titration of 9.4 pmol of AEDANSCaM was in 0.375 mL of 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2 mM dithiothreitol. Complex formation was monitored by fluorescence anistropy, corrected for background and calculated for fraction bound as described by LaPorte et al. (1981). The sample was titrated in parallel with a buffer blank. The final sample volume was 0.400 mL, and the background fluorescence intensity did not exceed 10%. The respective fluorescence anisotropies of the free and bound species (from the limits of the titration) were 0.0432 and 0.0804. The ratio of the fluorescence intensity of the bound species over that of the free species was 1.03. The tritration curves for immunoaffinity-purified AEDANSCaM and nonpurified AFDANSCaM are the results of the two separate experiments. A similar curve for nonpurified AEDANSCaM was previously published (LaPorte et al., 1981).

nonpurified $n$$Ca^{2+}$–AEDANSCaM binding to TnI. Removal of residual nonlabeled CaM from the AEDANSCaM preparation resulted in a 4.0-fold decrease in the dissociation con-

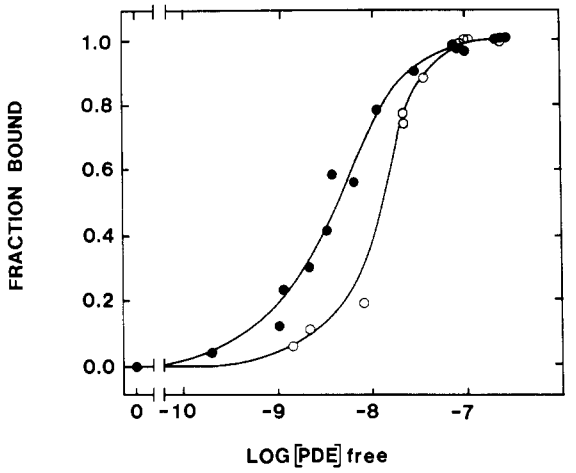

FIGURE 5: Titration of immunoaffinity-purified AEDANSCaM and nonpurified AEDANSCaM with bovine heart phosphodiesterase. The fraction of purified AEDANSCaM (●) and nonpurified AEDANSCaM (○) bound is plotted as a function of the log of the free molar phosphodiesterase concentration. AEDANSCaM (7.5 pmol) in 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM Mg Cl_2, 2 mM $CaCl_2$, and 2 mM dithiothreitol was titrated with PDE as described for TnI in the legend to Figure 4. The final sample volume was 0.515 mL, and the blank fluorescence intensity did not exceed 25%. The respective anisotropies of the free AEDANSCaM species and bound AEDANSCaM–PDE species (from the limits of the titration) were 0.0430 and 0.0822. The ratio of the fluorescence intensity of the bound species over that of the free species was 0.90. A titration curve similar to the curve for nonpurified AEDANSCaM was previously published by LaPorte et al. (1981) and is included for comparison.

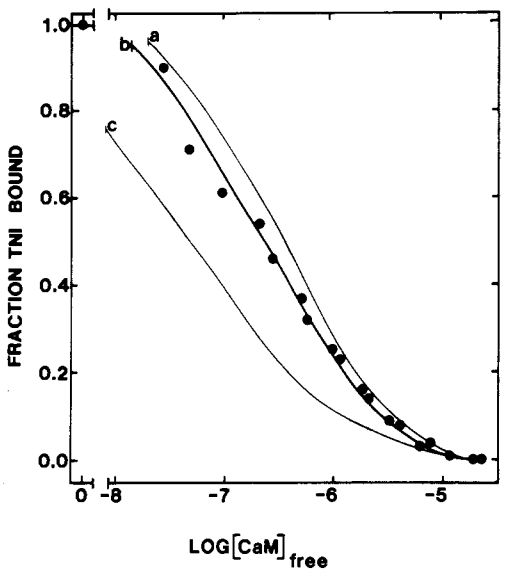

FIGURE 6: Determination of native dissociation constant (K_d) for interaction of bovine brain calmodulin with rabbit skeletal troponin I. Immunoaffinity-purified AEDANSCaM (490 pmol) and TnI (375 pmol) in 0.375 mL of 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2.0 mM DTT were titrated with CaM (●). The log of the free molar CaM concentration was plotted as a function of the fraction of troponin I bound to AEDANSCaM. Calculation of the free-CaM concentration is directly from the fluorescence anisotropy values as described under Materials and Methods. The solid line represents the expected values for free CaM concentration calculated from eq 1 (Materials and Methods) by assumption of a native K_d of 9 nM (a), 3 nM (b), and 1 nM (c). The Ca^{2+}-dependent dissociation constant (K_d') for the AEDANSCaM–TnI complex was 15 nM (Figure 5). The sample was titrated in parallel with a buffer blank, and the blank fluorescence intensity did not exceed 1%. The final sample volume was 0.500 mL.

stant for the $n$$Ca^{2+}$–AEDANSCaM–TnI complex. A similar titration curve for nonimmunoaffinity-purified AEDANSCaM with TnI is included for comparison. The respective dissociation constants obtained from the midpoints of the titration curves for immunoaffinity purified and nonpurified AEDANSCaM were 15 nM and 60 nM. The logarithmic free-concentration interval between 10% and 90% saturation is 1.908 for a single class of noninteracting sites. The logarithmic interval of free concentration can be used as an index of binding heterogeneity (Weber, 1975). The logarithmic interval of free TnI concentrations between 10% and 90% saturation for the immunoaffinity-purified and nonpurified AEDANSCaM derivatives was 1.90 and 2.10, respectively. The immunoaffinity-purified AEDANSCaM preparation shows no heterogeneity in binding rabbit skeletal muscle TnI. It is noteworthy that substantial quantitative differences result in the titration curve of AEDANSCaM binding to TnI on removal of residual CaM from the AEDANSCaM preparation.

Titration of Immunoaffinity-Purified AEDANSCaM and Nonpurified AEDANSCaM with Bovine Heart Phosphodiesterase. The binding curves for the titrations of purified and nonpurified AEDANSCaM with PDE were monitored by fluorescence anisotropy (Figure 5). Bovine heart phosphodiesterase is a dimer of identical 57 000-dalton monomers that bind one molecule of CaM for each phosphodiesterase monomer (LaPorte et al., 1979). The titration curves in Figure 5 illustrate the large differences for the Ca^{2+}-dependent interactions of immunoaffinity-purified AEDANSCaM and nonpurified AEDANSCaM with PDE. The dissociation constant for immunoaffinity-purified $n$$Ca^{2+}$–AEDANSCaM–PDE complex was 3.0 nM, and for the nonpurified $n$$Ca^{2+}$–AEDANSCaM–PDE complex the K_d was 10 nM. The titration curve for the Ca^{2+}-dependent binding of nonpurified $n$$Ca^{2+}$–AEDANSCaM [prepared as described by LaPorte al. (1981)] to PDE displays marked apparent positive cooperativity. The log-interval free concentration of PDE between 10% and 90% saturation for the nonpurified AEDANSCaM titration curve was 1.20. In addition, the dissociation constant for nonpurified AEDANSCaM binding to PDE was not reproducible for different preparations of AEDANSCaM. These discrepancies were accounted for by the presence of varying amounts of residual CaM remaining in the fluorescent AEDANSCaM preparation. Removal of residual CaM by an antibody specific for the AEDANS dye resulted in a 4.0-fold decrease in the dissociation constant for interaction of AEDANSCaM with PDE. The immunoaffinity-purified AEDANSCaM titration curve (Figure 5) yielded a log-interval free concentration of PDE from 10% to 90% saturation equal to 1.90. The extremely low dissociation constants encountered in CaM interactions with CaM-binding proteins require removal of residual CaM from the AEDANSCaM preparations for accurate determinations of the dissociation constants.

Determination of Native K_d for CaM Interaction with TnI. Native CaM was titrated to compete for TnI complexed to purifed AEDANSCaM for determination of the native dissociation constant in the presence of Ca^{2+}. The fraction of TnI bound to $n$$Ca^{2+}$–AEDANSCaM (Figure 6) is plotted as a function of the log of the free molar CaM concentration. The fraction bound and free molar 4Ca^{2+}–CaM concentration are calculated directly from the fluorescence anisotropy values as described under Materials and Methods. The solid lines in Figure 6 are plots of the fraction of $n$$Ca^{2+}$–AEDANSCaM concentrations calculated from eq 1 with different dissociation constants. A native K_d of 3 nM was used to plot the center solid line in Figure 6. The upper and lower lines were calculated for dissociation constants of 9 nM and I nM, respectively. The experimental data are consistent with the calculated curve displaying a native Ca^{2+}-dependent dissoci-

ation constant equal to 3 nM (Figure 6). The dissociation constant for the interaction of native CaM and TnI in the presence of Ca^{2+} is 5-fold lower than the K_d of 15 nM for the interaction of immunoaffinity-purified AEDANSCaM and TnI in the presence of Ca^{2+}. A K_d of 60 nM was previously reported for the Ca^{2+}-dependent interaction of nonpurified AEDANSCaM with TnI (LaPorte et al., 1981). The K_d of 60 nM is 20-fold greater than the native K_d of 3 nM. It is noteworthy that the presence of 30–50% residual nonlabeled CaM causes a 20-fold increase in the apparent K_d for Ca^{2+}-dependent interaction of AEDANSCaM with TnI when compared with the interaction between the native $4Ca^{2+}$–CaM and TnI.

Discussion

We have developed a general technique for separation of fluorescent-labeled CaM from native CaM by antisera directed specifically against the AEDANS-labeled bovine serum albumin immobilized on Affi-Gel 10 (Bio-Rad Laboratories). This technique appears generally applicable for removal of native CaM from fluorescent or otherwise chemically modified CaM. Immunoaffinity-purified AEDANSCaM was used to determine the dissociation constant (K_d) of the nCa^{2+}–AEDANSCaM–TnI and $4Ca^{2+}$–AEDANSCaM–PDE complexes. A competitive titration was developed to enable determination of the native dissociation constant of the $4Ca^{2+}$–CaM–TnI complex.

The location and homogeneity of incorporation of AEDANS on CaM were determined by amino acid analysis and sequence analysis of tryptic peptides derived from native and AEDANS-labeled CaM. The labeling is at least 70–80% homogeneous for the C-terminal tryptic peptide (residues 127–145); however, the label may be present on one or both adjacent methionine residues. Isolation of immunoaffinity-purified AEDANSCaM yielded up to 50% residual nonlabeled CaM, suggesting the published extinction coefficient of 6000 M^{-1} cm^{-1} at 337 nm (Hudson & Weber, 1973) changes when covalently bound to CaM.

Immunoaffinity purification of AEDANSCaM resulted in an increase in the K_a for CaM-sensitive PDE activation of 2.3-fold to 650 nM. Immunoaffinity-purified AEDANSCaM yielded respective K_d values of 15 nM and 3 nM for Ca^{2+}-dependent interaction of AEDANSCaM with TnI and the CaM-sensitive phosphodiesterase. The competition assay using immunoaffinity-purified AEDANSCaM yielded a K_d of 3 nM for the Ca^{2+}-dependent interaction of native CaM with TnI, a value 20-fold lower than the K_d reported for the Ca^{2+}-dependent interaction of nonpurified AEDANSCaM with TnI.

The low K_a for CaM activation of PDE (120 pM) and other CaM-dependent enzymes requires careful interpretation of the activity of chemically modified CaM that contains residual nonlabeled CaM. Absolute demonstration for Ca^{2+}-dependent complex formation of chemically modified CaM with CaM-dependent enzymes requires methods independent of enzyme-activity determinations for detection of these complexes.

The nonpurified AEDANSCaM interaction with PDE appeared positively cooperative and was not reproducible for different preparations of AEDANSCaM. Immunoaffinity-purified AEDANSCaM binding to PDE was consistent with binding of AEDANSCaM to a single class of noninteracting sites. The large discrepancies for the binding of purified and nonpurified AEDANSCaM could be accounted for by the presence of residual nonlabeled CaM remaining in the AEDANSCaM preparation. The use of fluorescent-labeled or otherwise modified CaM containing residual unreacted CaM for quantitative determination of CaM or Ca^{2+}-binding interactions with CaM-binding proteins must be interpreted with extreme caution. The removal of residual native CaM from AEDANS-labeled CaM preparations resulted in large differences for the activation and binding constants for AEDANSCaM interactions with CaM-binding proteins.

Fluorescent AEDANSCaM purified by immunoaffinity chromatography is a well-characterized fluorescent-labeled reagent that will be useful for the study of CaM interaction with CaM-binding proteins. We have described a general method for the purification of chemically labeled CaM from residual nonlabeled CaM remaining after chemical modification. This method was useful for quantitative determination of CaM interactions with CaM-binding proteins.

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References

Blumenthal, D. K., & Stull, J. T. (1980) *Biochemistry 19*, 5608.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Cheung, W. Y. (1980) *Science (Washington, D.C.) 207*, 19.
Cox, J. A., Malnoe, A., & Stein, E. A. (1981) *J. Biol. Chem. 256*, 3218.
Crouch, T. H., Holroyde, M. J., Collins, J. H., Solaro, R. J., & Potter, J. D. (1981) *Biochemistry 20*, 6318.
Head, J. F., Mader, S., & Kaminer, B. K. (1979) *J. Cell Biol. 80*, 211.
Huang, C. Y., Chan, V., Chock, P. B., Wang, J. H., & Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 871.
Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154.
Hudson, L., & Fay, F. C. (1976) *Practical Immunology*, p 3, Blackwell, Oxford.
Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489.
Lambooy, D. K., Steiner, R. F., & Sternberg, H. (1982) *Arch. Biochem. Biophys. 217*, 517.
LaPorte, D. C., & Storm, D. R. (1978) *J. Biol. Chem. 253*, 3374.
LaPorte, D. C., Toscano, W. A., & Storm, D. R. (1979) *Biochemistry 18*, 2820.
LaPorte, D. C., Keller, C. H., Olwin, B. B., & Storm, D. R. (1981) *Biochemistry 20*, 3965.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.
Means, A. R., & Dedman, J. R. (1980) *Nature (London) 285*, 73.
Olwin, B. B., Keller, C. H., & Storm, D. R. (1982) *Biochemistry 21*, 5669.
Sasagawa, T., Ericsson, L. H., Walsh, K. A., Shreiber, W. E., Fischer, E. H., & Titani, K. (1982) *Biochemistry 21*, 2565.
Vanaman, T. C., Sharief, F., & Watterson, D. M. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) p 107, Elsevier, New York.
Wang, J. H., & Waisman, D. M. (1979) *Curr. Top. Cell. Regul. 15*, 47.
Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962.
Weber, G. (1975) *Adv. Protein Chem. 29*, 2.
Wilkinson, J. M. (1974) *Biochim. Biophys. Acta 359*, 379.