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Development of a Novel Photoreactive Calmodulin Derivative: Cross-Linking of Purified Adenylate Cyclase from Bovine Brain†

Jeffrey K. Harrison,‡ Richard G. Lawton,§ and Margaret E. Gnegy*,‡
Departments of Pharmacology and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109
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ABSTRACT: A novel photoreactive calmodulin (CaM) derivative was developed and used to label the purified CaM-sensitive adenylate cyclase from bovine cortex. ^{125}I-CaM was conjugated with the heterobifunctional cross-linking agent *p*-nitrophenyl 3-diazopyruvate (DAPpNP). Spectral data indicated that diazopyruvoyl (DAP) groups were incorporated into the CaM molecule. Iodo-CaM–DAPs behaved like native CaM with respect to (1) Ca^{2+}-dependent enhanced mobility on sodium dodecyl sulfate–polyacrylamide gels and (2) Ca^{2+}-dependent stimulation of adenylate cyclase activity. ^{125}I-CaM–DAP photochemically cross-linked to CaM-binding proteins in a manner that was both Ca^{2+} dependent and CaM specific. Photolysis of forskolin–agarose-purified adenylate cyclase from bovine cortex with ^{125}I-CaM–DAP produced a single cross-linked product which migrates on sodium dodecyl sulfate–polyacrylamide gels with an apparent molecular weight of approximately 140 000.

Calmodulin (CaM) mediates a wide variety of calcium-dependent cellular processes, including cyclic nucleotide metabolism [reviewed by Stoclet et al. (1987)]. Its action as a regulator of brain cyclic AMP (cAMP) phosphodiesterase was discovered by Cheung (1970) and Kakiuchi et al. (1970), and it was subsequently found to regulate the adenylate cyclase activity from brain (Brostrom et al., 1975; Cheung et al., 1975). CaM appears to mediate the calcium-dependent stimulation of adenylate cyclase by interacting directly with the catalytic subunit of this enzyme (Smigel, 1986; Minocherhomjee et al., 1987). While GTP or GTP-binding protein subunits are not required for the stimulation of adenylate cyclase by CaM (Heideman et al., 1982; Seamon & Daly,

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* To whom correspondence should be addressed.
‡ Department of Pharmacology.
§ Department of Chemistry.

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1982), these agents can affect the way in which the enzyme activity is regulated by CaM (Mickevicius et al., 1986; Katada et al., 1987; Harrison et al., 1988).

Photoaffinity labeling of biological structures has been widely used. Photolabile derivatives of CaM have been synthesized and used to label a number of CaM-binding proteins in vitro (Hinds & Andreasen, 1981; Andreasen et al., 1983; Klevit & Vanaman, 1984). We have developed a novel photoaffinity-labeled CaM derivative to explore the interactions between CaM and the adenylate cyclase components of bovine brain. ^{125}I-CaM was conjugated with the heterobifunctional photoreactive probe *p*-nitrophenyl 3-diazopyruvate (DAPpNP). This probe, which is incorporated into free amino groups, has a number of features that distinguish it from currently available photoactivatable probes. The small size and dipolar nature of the diazopyruvoyl (DAP) group as well as the complete loss of UV absorption of the activatable function, the diazoketocarboxyl group, potentially allow highly efficient cross-linking. This report describes the development and use of ^{125}I-CaM–DAP as an alternative photoreactive cross-linking reagent to study CaM-regulated functions. Specifically, we investigated the utility of this probe to study the regulation of bovine brain adenylate cyclase by CaM. ^{125}I-CaM–DAP effectively substituted for native CaM in the stimulation of adenylate cyclase activity by calcium. Photolysis of ^{125}I-CaM–DAP with purified preparations of adenylate cyclase from bovine cortex yielded distinct cross-linked products.

Materials and Methods

ATP, cAMP, and protein standards were from Sigma. [α-^{32}P]ATP, [^{3}H]cAMP, and Na^{125}I were from Amersham. CaM–Sepharose was prepared according to the method of Westcott et al. (1979). Forskolin–agarose was synthesized from 7-*O*-hemisuccinyldeacetylforskolin (Calbiochem) and Affi-Gel 102 (Bio-Rad) according to the method of Smigel (1986). All other reagents were of the purest available grade from commercial sources.

Synthesis of CaM–DAP, I-CaM–DAP, and ^{125}I-CaM–DAP. CaM was prepared from bovine testes by the method of Dedman et al. (1977) using phenyl–Sepharose chromatography (Gopalakrishna & Anderson, 1982). CaM was iodinated essentially by the method of Graf et al. (1982). Iodo-CaMs were purified over phenyl-Sepharose (0.4 mL) in the presence of 50 mM imidazole, pH 7.2, with 1 mM $CaCl_2$ and eluted with 50 mM imidazole, pH 7.2, and 1 mM EGTA. Iodo-CaMs were pooled on the basis of protein and/or radioactivity and made 3 mM in $CaCl_2$. When ^{125}I-CaM was synthesized, a final specific activity of 2–4 μCi/μg of CaM was achieved with 1–1.4 iodines per CaM molecule. CaM and iodo-CaM were lyophilized overnight. CaMs were resuspended in an original volume of dry DMSO. To this solution was added a small aliquot of *p*-nitrophenyl 3-diazopyruvate (DAPpNP) in DMSO so as to achieve either 50:1 or 150:1 DAPpNP to CaM molar ratios. Typically, the concentration of CaM or ^{125}I-CaM was 10 μM, determined spectrally by using E_{276} = 0.18 or by specific radioactivity. Reaction of DAPpNP with CaM or ^{125}I-CaM proceeded for 30 min under low light conditions. At the end of this time, diazopyruvamide–CaM conjugates (CaM–DAPs) were adsorbed to phenyl-Sepharose (0.4 mL) in the presence of 50 mM Tris, pH 7.2, with 1 mM $CaCl_2$. The CaM–DAPs were eluted as a single peak with 50 mM Tris, pH 7.2, and 1 mM EGTA. The hydrolysis products of DAPpNP (*p*-nitrophenol and 3-diazopyruvate) were not adsorbed to phenyl-Sepharose under these conditions. Yields of CaM-DAPs varied between 40 and 60%. CaM–DAPs were stored at 4 °C until further use.

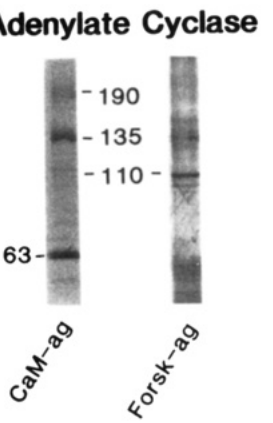

FIGURE 1: SDS–polyacrylamide gel electrophoresis of purified adenylate cyclase preparations. The CaM–agarose (CaM–ag)-purified (10 μg) preparation was subject to SDS–polyacrylamide gel electrophoresis on a 7.5% acrylamide gel with subsequent staining with Coomassie Brilliant Blue. The forskolin–agarose (Forsk–ag)-purified preparation was subject to SDS–polyacrylamide gel electrophoresis on an 8.75% acrylamide gel and subsequently silver stained.

Purification and Assay of CaM-Sensitive Adenylate Cyclase from Bovine Brain. CaM-sensitive adenylate cyclase from bovine cortex was purified through either CaM–Sepharose or forskolin–agarose according to Minocherhomjee et al. (1987). The SDS–polyacrylamide gel patterns of the various preparations are shown in Figure 1. CaM–Sepharose-purified proteins (10 μg) were run on 7.5% acrylamide gels (Laemmli, 1970) and stained with Coomassie Brilliant Blue while the forskolin–agarose-purified material was run on an 8.75% acrylamide gel and silver stained. All preparations were from fractions that had the highest adenylate cyclase activity as measured in the presence of Ca^{2+} and CaM. Molecular weights shown are $\times 10^{-3}$ and were calculated from a standard curve generated by simultaneously electrophoresing standard proteins of known molecular weight (myosin, 205K; β-galactosidase, 116K; phosphorylase, 97K; bovine serum albumin, 66K; ovalbumin 45K; carbonic anhydrase, 29K).

Adenylate cyclase activity was measured at 30 °C in an assay containing the following (final volume 100 μL): 20 mM NaHEPES, pH 7.4, 3 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM [α-^{32}P]ATP (1 μCi/tube), 2 mM cAMP, 4 mM phosphoenolpyruvate (PEP), 0.12 mM isobutylmethylxanthine (IBMX), and 20 μg of pyruvate kinase.

Photolysis of ^{125}I-CaM–DAP and Purified Adenylate Cyclase Preparations. Adenylate cyclase preparations were incubated with ^{125}I-CaM–DAP in 1.5-mL microfuge tubes at 30 °C in the presence of low light under identical conditions as the adenylate cyclase activity measurements. At specified times (0.5–6 min), aliquots (50–100 μL) were placed on depressed-well glass slides and then irradiated with three flashes (occurring over 2 s) from a high-intensity studio flash unit (Balcar Monobloc). SDS–PAGE sample buffer (Laemmli, 1970) was then added, and cross-linked products were visualized by running the samples on SDS–polyacrylamide gels (7.5% acrylamide), drying the gels, and exposing them to KODAK X-OMAT AR film with fast tungstate intensifying screens.

Results

Determination of the Molar Incorporation of Diazopyruvate (DAP) into CaM. *p*-Nitrophenyl 3-diazopyruvate (DAPpNP) reacts with free primary amino groups of which CaM has seven. The absorbance spectra between 240 and 300 nm of

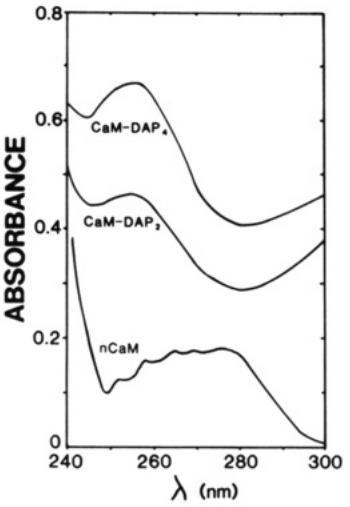

FIGURE 2: UV absorption spectra of CaM–DAPs. The absorption spectra between 240 and 300 nm of native CaM (nCaM) and CaM–DAPs isolated from conjugation reactions containing either 150:1 DAPpNP to CaM (CaM–DAP_4) or 50:1 DAPpNP to CaM (CaM–DAP_2) were measured in an SLM-Aminco DW-2C UV–vis spectrophotometer. The concentrations of the CaMs were 1 mg/mL nCaM, 0.33 mg/mL CaM–DAP_2, and 0.27 mg/mL CaM–DAP_4.

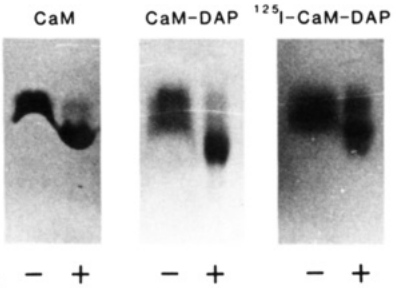

FIGURE 3: Effect of $CaCl_2$ on the mobility of CaM, CaM–DAP, and ^{125}I-CaM–DAP in SDS–polyacrylamide gels. CaM, CaM–DAP, and ^{125}I-CaM–DAP were made 10 mM in EGTA (−) or 10 mM in $CaCl_2$ (+) and then run on SDS gels containing 13% acrylamide. Gels containing CaM or CaM–DAP were stained with Coomassie Brilliant Blue while the gel containing the ^{125}I-CaM–DAP was dried and autoradiographed as described under Materials and Methods.

the CaM–DAPs synthesized were measured and are shown in Figure 2. CaM–DAP absorption spectra displayed maxima at 257 nm. Absorption maxima for the model compound sarcosine–DAP were also found to include one at 257 nm. From the measurement of the protein content using the Bradford protein assay (native CaM as the standard) and the major assumption that the extinction coefficient of the CaM–DAP at 257 nm is equal to the extinction coefficient of the sarcosine–DAP, it was determined that 4.4 mol of DAP was conjugated per mole of CaM when a 150:1 DAPpNP to CaM molar ratio was used during the conjugation reaction. Similarly, when a 50:1 DAPpNP to CaM ratio was used, 2.2 mol of DAP was incorporated per mole of CaM. Spectral data indicate that DAP moieties were incorporated and that the molar incorporation determined on the basis of the above-stated assumption is reasonable considering the number of available free amine groups on CaM. ^{125}I-CaM–DAP used in subsequent experiments was synthesized by using 150:1 DAPpNP to ^{125}I-CaM molar ratios in the conjugation reaction.

Determination of the Functionality of CaM–DAPs. The ability of the modified CaMs to behave like native CaM was assessed by two methods. First, the ability of $CaCl_2$ to enhance the mobility of CaM on SDS–polyacrylamide gels was tested. In the presence of $CaCl_2$, CaM migrated at a faster rate or lower apparent molecular weight on SDS–polyacrylamide gels.

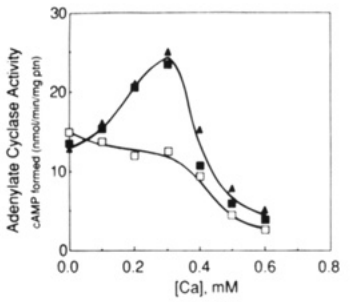

FIGURE 4: Effect of $CaCl_2$ concentration on the stimulation of adenylate cyclase by CaM and iodo-CaM–DAP. The effect of $CaCl_2$ concentration on the activity of a CaM–Sepharose-purified adenylate cyclase was measured in the absence (□) or presence of 300 nM CaM (■) or 300 nM iodo-CaM–DAP (▲). The concentration of EGTA present was 0.5 mM.

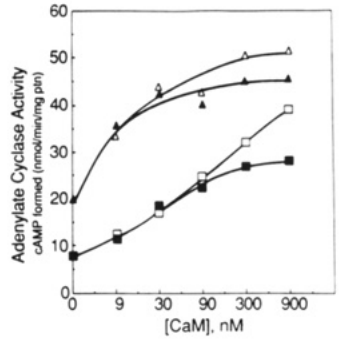

FIGURE 5: Concentration-dependent activation of adenylate cyclase activity by CaM and iodo-CaM–DAP. The concentration-dependent activation of CaM–Sepharose-purified adenylate cyclase activity by CaM (■, ▲) and iodo-CaM–DAP (□, △) was measured in the presence of 5 mM $MnCl_2$ (▲, △) or 0.3 mM $CaCl_2$ with 0.5 mM EGTA (■, □).

The ability of 10 mM $CaCl_2$ to affect the mobility of CaM–DAP and ^{125}I-CaM–DAP on 13% polyacrylamide gels was assessed and is shown in Figure 3. Like native CaM, CaM–DAP and ^{125}I-CaM–DAP displayed Ca^{2+}-dependent enhanced mobility on SDS–polyacrylamide gels.

Unlabeled iodinated CaM–DAP (I-CaM–DAP) was synthesized and tested for its ability to stimulate a partially purified CaM-sensitive adenylate cyclase. Figure 4 shows the calcium sensitivity of the stimulation of the adenylate cyclase by native CaM and I-CaM–DAP. In the absence of added CaM, increasing concentrations of Ca^{2+} steadily inhibited enzyme activity. In the presence of either 300 nM native CaM or 300 nM I-CaM–DAP, calcium-dependent stimulation was observed. With 0.5 mM EGTA present in the assay, a concentration of 0.3 mM added $CaCl_2$ was needed to achieve maximal calcium- and calmodulin-stimulated adenylate cyclase activity. Both native CaM and I-CaM–DAP displayed the same calcium sensitivity. Figure 5 shows the dose-dependent activation of adenylate cyclase activity by native CaM and I-CaM–DAP in the presence of either 5 mM $MnCl_2$ or 0.3 mM $CaCl_2$ (with 0.5 mM EGTA). Activity in the presence of $MnCl_2$ was greater than that obtained in the presence of $CaCl_2$. I-CaM–DAP effectively substituted for native CaM in the stimulation of adenylate cyclase activity when measured in the presence of either Mn^{2+} or Ca^{2+}.

Photochemical Cross-Linking of ^{125}I-CaM–DAP with Proteins Purified from Bovine Brain. Figure 6 shows the time and concentration dependence of cross-linking of ^{125}I-CaM–DAP with bovine brain proteins purified from a CaM–Sepharose column. Photolysis was carried out after incubating the reaction mixture under identical conditions as the activity determinations (assay cocktail and temperature). Major

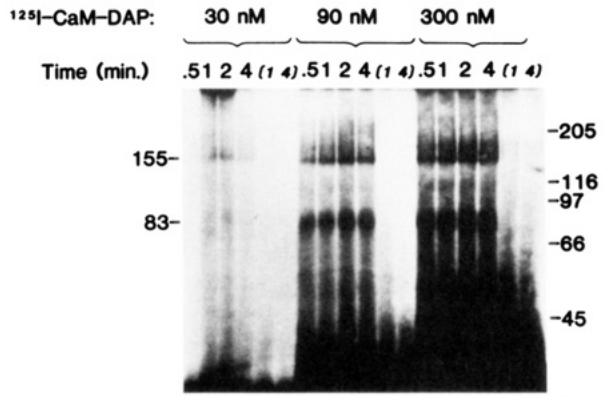

FIGURE 6: Time- and concentration-dependent cross-linking of ^{125}I-CaM-DAP with CaM-binding proteins from bovine cortex. ^{125}I-CaM-DAP at concentrations of 30, 90, and 300 nM was photolyzed with CaM-Sepharose-purified protein (2 μg/mL) at 0.5–4 min subsequent to the start of the incubation. The samples were run on SDS-polyacrylamide gels and autoradiographed as described under Materials and Methods. All photolysis reactions were carried out in the presence of 0.5 mM EGTA and 0.3 mM $CaCl_2$. Nonspecific labeling was determined at the 1- and 4-min time points (1 4) by incubating in the presence of 3 μM native CaM.

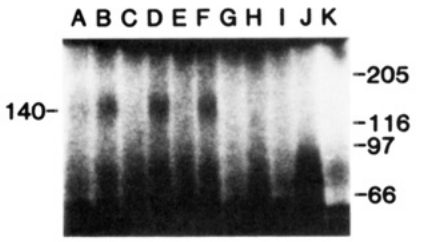

FIGURE 7: Cross-linking of ^{125}I-CaM-DAP and forskolin-agarose-purified adenylate cyclase from bovine cortex. CaM-Sepharose-purified proteins were subjected to chromatography over forskolin-agarose. The peak of CaM-sensitive adenylate cyclase activity eluted by forskolin was concentrated 10-fold by a Centricon-30 (Amicon) filter in the presence of 10 μg/mL bovine serum albumin (BSA). The concentrate was then photolyzed with 100 nM ^{125}I-CaM-DAP in the presence of EGTA alone (A) or with 0.3 mM $CaCl_2$ (B, C), 0.4 mM $CaCl_2$ (D, E, H–K), or 0.5 mM $CaCl_2$ (F, G). An equivalent amount of BSA (H, I) and 10 times the amount of BSA (J, K) (present in the adenylate cyclase photolyzed reactions) were photolyzed in the absence of the adenylate cyclase preparation. Nonspecific labeling was determined in the presence of 1 μM native CaM (lanes C, E, G, I, K). Samples were run on SDS-polyacrylamide gels and autoradiographed as described under Materials and Methods.

cross-linked adducts of apparent molecular weight 155000 and 83000 formed in an ^{125}I-CaM-DAP concentration-dependent manner. Adduct formation in the presence of 90 nM ^{125}I-CaM-DAP also appeared to be time dependent, with the amount of cross-linked products at the half-minute time point being less than that observed at subsequent times. Nonspecific cross-linking was also determined at 1 and 4 min by incubating in the presence of 3 μM native CaM. No major cross-linking was observed in the presence of excess cold CaM.

When the CaM-Sepharose preparation was passed over forskolin-agarose, a predominant silvered-stained band having an apparent molecular weight of 110000 was specifically eluted from the column in the presence of forskolin (Figure 1). The intensity of this band correlated with the level of CaM-sensitive adenylate cyclase activity in the eluted fractions. The enzyme was concentrated 10-fold in the presence of bovine serum albumin (BSA) and then subject to photolysis in the presence of 100 nM ^{125}I-CaM-DAP. Figure 7 shows that the predominant cross-linked product had an apparent molecular weight of 140000. Cross-linking was both calcium dependent (occurring in the presence of 0.3, 0.4, and 0.5 mM $CaCl_2$) and CaM specific. The M_r 140000 product was not observed when either an equivalent amount or 10 times the amount of BSA alone was photolyzed in the presence of ^{125}I-CaM-DAP.

Discussion

This study characterizes the development and use of a novel photoreactive CaM derivative. ^{125}I-CaM was conjugated with the heterobifunctional photoreactive probe *p*-nitrophenyl 3-diazopyruvate (DAPpNP). DAPpNP has been synthesized, and its reactivity with amine nucleophiles has been established. This new photolabile derivative of CaM is introduced as an alternative to the preexisting azidocalmodulin probes previously used to cross-link CaM-binding proteins (Hinds & Andreasen, 1981; Andreasen et al., 1983; Klevit & Vanaman, 1984). The small size and dipolar nature of the incorporated diazopyruvoyl (DAP) group, as well as the complete loss of UV absorption of the activatable function, afford this CaM-DAP derivative with great potential for highly efficient cross-linking of CaM with its binding sites.

On the basis of the absorption spectrum of the CAMs isolated from the CaM/DAPpNP reaction mixtures, the DAP group was incorporated into the CaM molecule. We estimated that approximately 2 and 4 mol of DAP were incorporated per mole of CaM when 50:1 and 150:1 DAPpNP to CaM molar ratios were used during the conjugation reaction, respectively. This is a reasonable estimate considering that CaM has seven free amino groups. Iodinated CaM-DAPs behaved like native CaM on the basis of two criteria. First, ^{125}I-CaM-DAP displayed Ca^{2+}-dependent enhanced mobility on SDS-polyacrylamide gels. Second, I-CaM-DAP effectively substituted for native CaM in the calcium-dependent stimulation of partially purified adenylate cyclase activity from bovine brain.

Photolysis of ^{125}I-CaM-DAP with purified preparations of CaM-binding proteins that included adenylate cyclase activity generated cross-linked adducts of various molecular weights. Photolysis reactions were conducted under essentially identical conditions (assay contents and temperature) as the adenylate cyclase activity measurements. Cross-linked products of M_r 83000 and 155000 were consistently observed from preparations purified through CaM-Sepharose. Cross-linking was calcium dependent and CaM specific. These molecular weights correlated with the addition of one CaM molecule to each of the major Coomassie-stained proteins in those preparations (M_r 63000 and 135000). These probably represent cross-linked adducts between the ^{125}I-CaM-DAP and the CaM-sensitive phosphodiesterase and the CaM-sensitive ATPase, respectively.

Purification of the CaM-sensitive adenylate cyclase through forskolin-agarose yielded a preparation containing one major silver-stained protein having an apparent molecular weight of 110000 on SDS-polyacrylamide gels. This molecular weight is consistent with previously reported molecular weights of purified adenylate cyclase. Estimates of the molecular weight of adenylate cyclase from bovine brain have ranged from 115000 to 135000 (Smigel, 1986; Minocherhomjee et al., 1987; Mollner & Pfeuffer, 1988). Andreasen et al. (1983) have previously reported a cross-linked adduct of apparent molecular weight 170000 between ^{125}I-azido-CaM and preparations of bovine brain adenylate cyclase purified through CaM-Sepharose affinity and Bio-Gel A5M chromatography. They proposed that the apparent molecular weight of the adenylate cyclase was 150000 and demonstrated that adenylate cyclase activity correlated with a cross-linked adduct of apparent molecular weight 170000. Photolysis of our forskolin-agarose-purified preparation with ^{125}I-CaM-DAP

yielded a single high molecular weight adduct of 140000 whose formation was both calcium dependent and CaM specific. The difference could be due to the stage or method of purification, the detergent used, or the fact that our enzyme was purified from bovine striatum instead of bovine cerebral cortex. While the molecular weights of the silver-stained protein and the CaM do not exactly add in our study, this may be due to an anomaly in the electrophoretic mobility of the CaM–adenylate cyclase adduct. Prior to photolysis, the adenylate cyclase purified through the forskolin–agarose was concentrated so as to enhance the likelihood of detecting the cross-linked product. The concentration step not only concentrated the protein but also concentrated the Tween that was present in the purification buffer. The presence of excess Tween may compete for the SDS in the PAGE sample buffer which could then give rise to a higher apparent molecular weight of the cross-linked product. Alternatively, the M_r 110000 protein could be binding two CaM molecules, or the M_r 140000 adduct could be a complex of ^{125}I-CaM and a protein that is present in quantities lower than the sensitivity of the silver staining procedure. Despite this anomaly, ^{125}I-CaM–DAP cross-linked to proteins in a calcium-dependent and calmodulin-specific manner and will be useful in probing interactions between CaM and the systems which it regulates.

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Registry No. DAPpNP, 111337-51-0; adenylate cyclase, 9012-42-4.

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