Preparation of azidocalmodulin: A photoaffinity label for calmodulin-binding proteins
(phosphodiesterase/myosin light chain kinase/membranes/Ca\(^{2+}\)/crosslinking)

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ABSTRACT A photoaffinity label for calmodulin-binding proteins was prepared from \(^{125}\)I-labeled calmodulin (\(^{125}\)I-calmodulin) and methyl-4-azidobenzimidate. Azidocalmodulin containing one azido group per calmodulin retained its ability to stimulate the Ca\(^{2+}\)-sensitive phosphodiesterase purified from bovine heart muscle. The concentrations of calmodulin and azidocalmodulin required for half-maximal stimulation of phosphodiesterase activity were 170 and 230 pM, respectively. Azido-\(^{125}\)I-calmodulin was used to photoaffinity label troponin I, myosin light chain kinase, and the Ca\(^{2+}\)-sensitive phosphodiesterase. Formation of crosslinked complexes required the presence of Ca\(^{2+}\) or Mn\(^{2+}\) and was inhibited by excess unmodified calmodulin. The calmodulin-binding subunits all formed 1:1 complexes with calmodulin, and the molecular weights of the crosslinked products obtained with troponin I, the phosphodiesterase, and myosin light chain kinase were 43,000, 79,000, and 116,000, respectively. Photolysis experiments using azido-\(^{125}\)I-calmodulin and bovine cerebral cortex membranes or detergent-solubilized membranes resulted in formation of a limited number of specifically labeled polypeptides. Azidocalmodulin appears to be an appropriate photoaffinity label for the identification and characterization of calmodulin-binding subunits.

Calmodulin (CaM) is an important regulatory protein that binds Ca\(^{2+}\) (1) and mediates Ca\(^{2+}\) stimulation of several enzyme activities, including the Ca\(^{2+}\)-sensitive phosphodiesterase (2-4), brain adenylate cyclase (5), human erythrocyte Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase (6, 7), myosin light chain kinase (8), and phosphorylase kinase (9). In addition, CaM also interacts with troponin I (10) and calcineurin (11, 12). It has been demonstrated by a variety of techniques that the affinity of CaM for these proteins is enhanced in the presence of Ca\(^{2+}\) (10-17). Interactions between Ca\(^{2+}\), CaM, and the catalytic subunit of the Ca\(^{2+}\)-sensitive phosphodiesterase are best represented by the following general model (17):

\[
4 \text{Ca}^{2+} \rightleftharpoons \text{CaM-Ca}_{4}^{2+}
\]
\[
2 \text{CaM-Ca}_{4}^{2+} + \text{[Phosphodiesterase]} \rightleftharpoons (\text{Phosphodiesterase})_{2}(\text{CaM-Ca}_{4}^{2+})_{2}
\]

Because CaM stimulation of these enzymes requires complex formation with CaM, there is considerable interest in the identification and characterization of specific CaM-binding subunits.

It would be advantageous to develop methods for the identification of specific CaM-binding subunits both with purified enzymes and impure preparations. Utilization of \(^{125}\)I-labeled CaM (\(^{125}\)I-CaM) with bifunctional crosslinking reagents has been used with some success (17); however, it is clear that a CaM derivative that covalently labels CaM-binding proteins would be a valuable tool for characterization of CaM-regulated systems. In the present study we describe the synthesis of azido-\(^{125}\)I-CaM and demonstrate that it can be used to photoaffinity label several different CaM-binding proteins.

MATERIALS AND METHODS

Materials. Radioisotopes were purchased from New England Nuclear. Bovine serum albumin, \(\beta\)-galactosidase, ovalbumin, aldolase, and lactate dehydrogenase were obtained from Sigma. Methyl-4-azidobenzimidate (MABI) was purchased from Pierce. All other reagents were the best available grade from commercial sources.

Protein Preparations. The Ca\(^{2+}\)-sensitive phosphodiesterase was purified from bovine heart muscle as described (17). CaM was purified from bovine brain (17). Troponin I was purified from rabbit skeletal muscle by the method of Wilkinson (18). Myosin light chain kinase was purified to apparent homogeneity from rabbit skeletal muscle. This preparation ran as one band on NaDodSO\(_4\) gels with a molecular weight of 95,000 to 97,000. Details of preparation will be presented in a subsequent publication. Membranes were prepared from bovine cerebral cortex and solubilized with Lubrol PX as described (16).

Phosphodiesterase Assay. Phosphodiesterase activity was determined as reported (17). Assay mixtures contained 0.05 mM cyclic [\(^{3}\)H]AMP (60,000 cpm), 10 mM 4-morpholinepropanesulfonic acid (Mops) at pH 7.2, 1 mM MgCl\(_2\), 150 mM KCl, 0.1 mM CaCl\(_2\), [\(^{14}\)C]AMP (6000 cpm), 0.1 mM phenylmethanesulfonyl fluoride, bovine serum albumin at 0.1 mg/ml, 2 mM dithiothreitol, and various amounts of CaM or azido-CaM in a total volume of 0.5 ml.

Preparation of Azido-\(^{125}\)I-CaM. CaM was iodinated by the method of LaPorte and Storm (15). The specific activity of \(^{125}\)I-CaM used in these preparations was \(8 \times 10^7\) cpn/nmol of CaM. \(^{125}\)I-CaM was desalted into 50 mM sodium borate (pH 9.8)/0.2 mM CaCl\(_2\)/100 mM NaCl, yielding a final concentration of 100 \(\mu\)g of \(^{125}\)I-CaM per ml. MABI was dissolved in the same buffer at a concentration of 1.3 mg/ml and 10 \(\mu\)l was added to 100 \(\mu\)l of the \(^{125}\)I-CaM solution. The mixture was incubated at 25°C for 2 hr in the dark and the product was desalted into 20 mM Mops (pH 7.2)/100 mM NaCl. Incorporation of azido groups into CaM was estimated spectrophotometrically (19). At MABI-to-CaM molar ratios of 3, 10, and 100, there were 0.45, 0.95, and 1.45 mol of azido groups per mol of CaM, respectively. The azido-\(^{125}\)I-CaM used in this study contained 1.0 mol of azido

Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(\text{N,N',N''}\)-tetraacetic acid; Mops, 4-morpholinopropanesulfonic acid; MABI, methyl-4-azidobenzimidate.

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groups per mol of CaM. More highly substituted derivatives were less effective as activators of the Ca^{2+}-sensitive phosphodiesterase.

**Photolysis and NaDodSO_{4} Gel Electrophoresis.** Azido-125I-CaM at concentrations between 20 and 200 nM was mixed with various concentrations of the protein to be labeled in 20 mM Mops (pH 7.2)/100 mM NaCl in a total volume of 80 μl. In some experiments ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), CaCl_{2}, MnCl_{2}, MgCl_{2}, or unmodified CaM was present as indicated. Samples were irradiated for 3-5 min at 4°C by using a Mineralight UVS-11 positioned 1 cm above the sample. The samples were then run on NaDodSO_{4} slab gels (20), and the gels were dried and autoradiographed at -80°C, using Du Pont Cronex Lightning Plus intensifying screen and Kodak XR-5 x-ray film.

**RESULTS**

Stimulation of Phosphodiesterase Activity by Azido-CaM. Walsh and Stevens have determined that treatment of CaM with O-methylisourea, which modifies ε-amino groups of lysine with retention of the positive charge, does not significantly alter CaM activity (21). Therefore, MABI was used to prepare a photoaffinity label, because this reagent reacts with protein amino groups to form amidines, which also retain the positive charge on the modified residues. Azido-CaM retained its ability to stimulate the Ca^{2+}-sensitive phosphodiesterase purified from bovine heart muscle (Fig. 1). The maximal level of stimulation was identical to that of unmodified CaM, with half-maximal stimulation occurring at 170 pM CaM and 230 pM azido-CaM. These data indicated that azido-CaM was able to interact with the enzyme and suggested that it might be an appropriate photoaffinity label for CaM-binding proteins. Because it had been previously shown that 125I-CaM retained its activity (15), azido-125I-CaM was prepared and evaluated as a photoaffinity label.

**Photoaffinity Labeling of the Phosphodiesterase.** Photolysis of a mixture of azido-125I-CaM and the CaM-sensitive phosphodiesterase in the presence of 0.1 mM CaCl_{2} resulted in one crosslinked product having a molecular weight of 79,000 on NaDodSO_{4} gels (Fig. 2). No other crosslinked products were detected even after prolonged periods of autoradiography. This crosslinked polypeptide was not produced when the photolysis was carried out in the presence of EGTA or a 150-fold molar excess of unmodified CaM. Azido-125I-CaM and the catalytic subunit of the phosphodiesterase had apparent molecular weights on NaDodSO_{4} gels of 20,000 and 58,000, respectively. These data are consistent with previous studies indicating formation of a one-to-one complex between CaM and the catalytic subunit of the phosphodiesterase (17). In addition, the data in Fig. 2 confirm that azido-125I-CaM forms a calcium-dependent complex with the phosphodiesterase and illustrate the utility of azido-125I-CaM as a photoaffinity reagent.

**Photoaffinity Labeling of Troponin I.** Troponin I is known to bind CaM in the presence of Ca^{2+} (10), and fluorescence studies have suggested that the two proteins form a one-to-one complex (22). On NaDodSO_{4} gels, troponin I and CaM ran with apparent molecular weights of 23,000 and 20,000. The single photolysis product obtained from a mixture of troponin I and azido-125I-CaM had a molecular weight of 43,000 (Fig. 3). This
same product was obtained when 5 mM MnCl₂ was substituted for CaCl₂ but not when 5 mM MgCl₂ was used. The 43,000-dalton polypeptide was not produced in the presence of excess EGTA or unmodified CaM. These data indicated that troponin I binds one CaM in a Ca²⁺-dependent manner.

**Photoaffinity Labeling of Myosin Light Chain Kinase.** Interactions between azido-¹²⁵I-CaM and myosin light chain kinase were also examined in order to test the general utility of the reagent as a photoaffinity label. Photolysis of a mixture of myosin light chain kinase and azido-¹²⁵I-CaM in the presence of Ca²⁺ gave one crosslinked product having a molecular weight of 116,000 (Fig. 4). There were no other higher molecular weight peptides formed, even when azido-¹²⁵I-CaM was in molar excess relative to the kinase. In the presence of 2 mM EGTA, 5 mM MgCl₂, or excess unmodified CaM, there was no crosslinking between the enzyme and azido-¹²⁵I-CaM. However, the 116,000-dalton peptide was formed when MnCl₂ was substituted for CaCl₂. The purified myosin light chain kinase ran with an apparent molecular weight of approximately 95,000 on NaDodSO₄ gels, which compares favorably with preparations described by other investigators (23). It appears that the catalytic subunit of myosin light chain kinase can bind one and only one molecule of CaM. This stoichiometry is consistent with recent kinetic studies, which also indicated that the catalytic subunit of the kinase binds only one CaM (23).

**Photoaffinity Labeling of Bovine Cerebral Cortex Membrane and Detergent-Solubilized Membranes.** The preceding photolysis experiments were conducted with pure proteins, and it was of some interest to carry out analogous experiments with membrane preparations. Because the membranes contained endogenous CaM, the first set of experiments was conducted with membrane preparations that had been washed with EDTA to remove endogenous CaM (Fig. 5). Azido-¹²⁵I-CaM labeled five membrane proteins in the presence of Ca²⁺, and no crosslinking occurred in the presence of EGTA or excess unmodified CaM. The apparent molecular weights of the crosslinked products were 77,000, 95,000, 107,000, 165,000, and 175,000. Because only a limited number of membrane proteins were specifically labeled with azido-¹²⁵I-CaM, this photoaffinity label may be a valuable tool for detecting CaM-binding proteins in impure preparations. An identical experiment was carried out with membranes that had not been washed with EDTA to remove endogenous CaM. A similar labeling pattern was obtained, although the amounts of crosslinked material formed were clearly lower. This experiment indicates that it is possible to exchange azido-¹²⁵I-CaM for endogenous CaM, although the extent of labeling is improved by prior removal of CaM.

Lubrol PX-solubilized membranes were also photoaffinity labeled with azido-¹²⁵I-CaM (Fig. 6). Four crosslinked products having molecular weights of 64,000, 77,000, 114,000, and 175,000 were formed. In contrast to other crosslinked peptides,

**FIG. 4.** Crosslinking of azido-¹²⁵I-CaM to myosin light chain kinase. Azido-¹²⁵I-CaM and myosin light chain kinase were photolyzed, run on 7.5% NaDodSO₄/polyacrylamide slab gels, and autoradiographed. Sample A, 27 nM myosin light chain kinase and 50 nM azido-¹²⁵I-CaM were incubated in the presence of 3 mM CaCl₂ and 1 mM EDTA prior to photolysis; sample B, same as A except no CaCl₂ was added; sample C, same as A except 20 μg of unmodified CaM was present; sample D, same as A except that 5 mM MgCl₂ replaced CaCl₂; sample E, same as A except that 5 mM MnCl₂ replaced CaCl₂.

**FIG. 5.** Crosslinking of azido-¹²⁵I-CaM to bovine cerebral cortex membranes. Membranes were prepared and diluted 1:20 into 20 mM Mops (pH 7.2)/2 mM EDTA/100 mM NaCl. After 4 min, membranes were centrifuged and resuspended in 20 mM Mops (pH 7.2)/0.2 mM CaCl₂/100 mM NaCl at 1 mg of protein per ml. Sample A, 50 μl of membranes was incubated with 1 μg of azido-¹²⁵I-CaM in the presence of 0.5 mM CaCl₂. After 5 min the membranes were centrifuged, resuspended in the same buffer, and photolyzed. Sample B, same as A except that 1 mM EGTA was present; sample C, same as A except that, prior to addition of azido-¹²⁵I-CaM, the membranes were incubated with 20 μg of unmodified CaM; sample D, same as A except that membranes were incubated with 20 μg of CaM after incubation with azido-¹²⁵I-CaM. Samples E–G, membranes were diluted into 20 mM Mops (pH 7.2)/0.2 mM CaCl₂/100 mM NaCl without a prior wash with an EDTA-containing buffer. Sample E, same as A; sample F, same as B; and sample G, same as C.

**FIG. 6.** Crosslinking of azido-¹²⁵I-CaM to detergent-solubilized membranes from bovine cerebral cortex. Sample A, 84 nM azido-¹²⁵I-CaM and solubilized membranes at 0.5 mg/ml were preincubated for 5 min in the presence of 1 mM EDTA and 2 mM CaCl₂. The sample was photolyzed and run on 7.5% NaDodSO₄ slab gels. Sample B, same as A except that 1 mM EDTA replaced CaCl₂. Sample C, same as A except that the solubilized extract was preincubated with unmodified CaM for 10 min prior to addition of azido-¹²⁵I-CaM.
the amount of the 77,000-dalton product formed actually increased in the presence of excess EGTA. This suggests that there is a 57,000-dalton polypeptide that has a higher affinity for CaM in the absence of Ca\(^{2+}\). However, there were detectable amounts of this crosslinked product formed even in the presence of a large molar excess of unmodified calmodulin, indicating that this interaction may be nonspecific. In addition, labeling of this polypeptide in intact membranes was not enhanced by chelation of Ca\(^{2+}\).

**Efficiency of Crosslinking.** The efficiency of crosslinking between azido-125I-CaM and the purified proteins was estimated by measuring radioactivities of gel slices corresponding to the photolysis product and untreated azido-125I-CaM in a gamma counter. For these determinations, the CaM-binding proteins were present in 4-fold molar excess in order to complex all azido-125I-CaM present. It was determined that 37% of the troponin I-azido-125I-CaM complexes formed covalent bonds subsequent to photolysis. The crosslinking efficiencies with the phosphodiesterase and myosin light chain kinase were 24% and 26%, respectively.

**DISCUSSION**

Since the discovery of CaM by Cheung (2), there has been a continuing search for CaM-regulated enzymes and considerable progress in characterizing CaM-regulated systems (1). CaM is the only protein known to function as a regulatory subunit for several different enzymes, and one might expect that these enzymes share a common CaM-binding domain. Biochemical studies are currently directed toward identification of CaM-regulated systems and characterization of CaM-protein interactions. A specific photoaffinity label for CaM-binding subunits would clearly be useful for these general goals.

We have determined that azido-125I-CaM is an effective photoaffinity agent for CaM-binding proteins. Although it was possible to incorporate up to two azido groups per CaM, the more highly substituted derivatives were less effective as activators of the Ca\(^{2+}\)-sensitive phosphodiesterase. The derivative used in this study contained one azido group per CaM, and dose–response curves for stimulation of the phosphodiesterase were comparable to those for underivatized CaM. Azido-125I-CaM formed crosslinked products with troponin I, myosin light chain kinase, and the Ca\(^{2+}\)-sensitive phosphodiesterase all formed crosslinked products with azido-125I-CaM with comparable efficiencies is consistent with the hypothesis that these proteins share a similar CaM-binding domain. It is also interesting that the CaM-binding subunits examined in this study all formed one-to-one complexes with CaM. There was no indication that any of these protein subunits could bind more than one CaM, providing further evidence for the specificity of this reagent.

Azido-125I-CaM, or related derivatives, may ultimately be useful for identifying CaM-binding subunits with enzymes containing nonidentical subunits such as phosphorylase kinase or brain adenylyl cyclase. In addition, data reported in this study indicate that it is possible to photoaffinity label CaM-binding proteins in crude unpurified preparations. For example, azido-125I-CaM has been successfully used to specifically label the CaM-sensitive ATPase of human erythrocytes (unpublished data).

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