Photoaffinity Labeling of Brain Adenylate Cyclase Preparations with Azido[^{125}I]iodocalmodulin†

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ABSTRACT: A partially purified calmodulin-sensitive adenylate cyclase from bovine cerebral cortex was photoaffinity labeled with azido[^{125}I]iodocalmodulin. Sodium dodecyl sulfate gel electrophoresis followed by autoradiography revealed several cross-linked polypeptides ranging in molecular weight from 37 000 to 200 000. The calmodulin-sensitive enzyme was submitted to a number of purification steps to determine if any of the calmodulin binding polypeptides copurified with adenylate cyclase activity. Fractionation procedures used included Bio-Gel A5M and Ultragel AcA 34 gel chromatography, isoelectric focusing, and native gradient gel electrophoresis. One cross-linked peptide having a molecular weight of 170 000 correlated with adenylate cyclase activity through all purification steps. Native gradient gel electrophoresis in the presence of 0.03% deoxycholate gave one peak of adenylate cyclase activity with a Stokes radius of 40 Å, consistent with a molecular weight of 140 000–150 000. It is proposed that the molecular weight of the adenylate cyclase catalytic subunit is 150 000 and that each catalytic subunit interacts with one calmodulin.

Cerebral cortex contains a calmodulin- (CaM)[1] sensitive adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975) that can be adsorbed to CaM–Sepharose in the presence of Ca^{2+} and eluted with buffers containing Ca^{2+} chelators (Westcott et al., 1979). These observations indicate that CaM interacts directly with one or more subunits of the brain adenylate cyclase complex. Recent data from both Neer's and Vaughan's laboratories show that the guanyl nucleotide regulatory complex (G/F) may not be an obligatory requirement for CaM stimulation of brain adenylate cyclase since preparations lacking GppNHp sensitivity were stimulated by CaM (Salter et al., 1981; Bitonti et al., 1982). In addition, GTP is not required for CaM stimulation of brain adenylate cyclase (Heideman et al., 1982; Seamon & Daly, 1982). Therefore, it seems likely that CaM may interact directly with the catalytic subunit of brain adenylate cyclase although additional interactions with regulatory subunits of the enzyme cannot be ruled out. This conclusion is consistent with the general observation that CaM binds directly to the catalytic subunits of enzymes regulated by Ca^{2+}_n·CaM (Andreasen et al., 1981).

We have developed a radioactive photoactivatable derivative of calmodulin, azido[^{125}I]iodoCaM, which has been used to photoaffinity label the CaM binding subunits of a number of proteins including the Ca^{2+}-sensitive phosphodiesterase, the (Ca^{2+} + Mg^{2+}) ATPase, troponin I, myosin light chain kinase, phosphorylase kinase (Andreasen et al., 1981), and CaM-binding proteins in coated vesicles (Linden, 1982). In the present study, partially purified CaM-sensitive adenylate cyclase preparations were photoaffinity labeled with azido-[^{125}I]iodoCaM in an attempt to affinity label the CaM-binding subunit of adenylate cyclase. We report the labeling of a single polypeptide that correlates with adenylate cyclase activity over a variety of separation techniques.

Experimental Procedures

Materials

Pharmalyte 5-8, DEAE-Sephacel, and cyanogen bromide activated Sepharose 4-B were purchased from Pharmacia. Enzymobeads and Bio-Gel A5M were obtained from Bio-Rad. ATP, cAMP, GppNHp, CHAPS, and protein standards were from Sigma. [α-^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear. [^{125}I]NaI was obtained from Amersham and Ultragel AcA 34 was from LKB. All other reagents were of the finest available grade from commercial sources.

Methods

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salomon et al. (1974) using [α-^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. Assays contained in a final volume of 250 μL 20 mM Tris-HCl, pH 7.5, 1 mM [α-^{32}P]ATP (20 cpm/pmol), 5 mM theophylline, and 0.1% bovine serum albumin. All results are presented as the mean of triplicate assays with standard errors of less than 5%. Protein concentrations were determined by the method of Peterson (1977).

Preparation of CaM. CaM was prepared from bovine brain by the procedure of Dedman et al. (1977a) as modified by LaPorte et al. (1979). CaM–Sepharose was prepared from purified CaM and cyanogen bromide activated Sepharose 4B according to the procedure of Westcott et al. (1979).

Partial Purification of CaM-Sensitive Adenylate Cyclase. One kilogram of frozen bovine cerebral cortex was fractured with a hammer and thawed in phosphate-buffered saline (22.5 mM KH_2PO_4, 75 mM NaCl, 12.8 mM NaOH, pH 7.2). Thawed cortex was drained and homogenized in an equal volume of homogenization buffer with a Waring blender for 30 s. Homogenization buffer contained 20 mM glycylglycine,

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[1] Abbreviations: CaM, calmodulin; G/F, the guanyl nucleotide regulatory complex of adenylate cyclase; GppNHp, 5′-guanylyl imidodiphosphate; $NaDodSO_4$, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; TEMED, tetramethylethylenediamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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pH 7.2, 1 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. The preparation was then further disrupted by three strokes of a dounce homogenizer with a loose-fitting pestle and centrifuged at 7000 rpm for 30 min in a Sorval GSA rotor. The pelleted membranes were resuspended in an equal volume of homogenization buffer, and the dounce homogenization, centrifugation, and resuspension were repeated 3 times. The final pellet was resuspended in 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol and centrifuged as before. The washed membrane pellet was detergent extracted by addition of 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, and 1% Lubrol PX to a detergent to protein ratio of 2.5:1 (w/w). The mixture was stirred at 4 °C for 60 min and centrifuged at 10000*g* for 2 h and the supernatant fluid was decanted. Two liters of DEAE-Sephacel equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Lubrol PX (buffer A) was incubated for 90 min with 5 L of detergent extract with stirring at 4 °C. The resin was washed on a sintered glass funnel with 4 L of buffer A containing 50 mM KCl, poured into a 9 × 30 cm column, and eluted with buffer A containing 150 mM KCl. A single protein peak containing the adenylate cyclase was eluted. CaM that did not elute from DEAE-Sephacel below 300 mM KCl was not eluted. The enzyme was pooled on the basis of activity and diluted with an equal volume of buffer A containing 2.2 mM $CaCl_2$. The dilution lowered the KCl concentration of the pooled enzyme solution from approximately 110 to 55 mM and provided Ca^{2+} in excess of EDTA. Both Ca^{2+} and the lower salt concentration were required for CaM–Sepharose affinity chromatography. The diluted pool was loaded onto a 2.5 × 26 cm CaM–Sepharose column equilibrated in buffer A containing 1.1 mM $CaCl_2$. The column was washed with the equilibration buffer until the absorbance at 280 nm reached a steady value and was eluted with buffer A. Adenylate cyclase eluted from the column was pooled and concentrated 8-fold by ultrafiltration in an Amicon XM 100A filter. This preparation is designated F-2. F-2 was then loaded onto a 2.5 × 74 cm Bio-Gel A5M column equilibrated in buffer A. Fractions were assayed for adenylate cyclase activity, pooled, frozen in liquid nitrogen, and stored at −70 °C for later use. When F-2 was submitted to isoelectric focusing or native gradient electrophoresis, it was first incubated at 30 °C for 1 h with 100 μM GppNHp for stabilization.

Native Polyacrylamide Gradient Gel Electrophoresis. Polyacrylamide gradient slab gels for native gel electrophoresis were poured from 3% and 20% acrylamide stocks [bis-(acrylamide) = 5% of total] into a gradient former. Stock solutions contained 90 mM Tris, 80 mM borate, pH 8.4, 0.07% w/v ammonium persulfate, 0.03% v/v TEMED, and 0.1% Lubrol PX. The 20% stock also contained 10% sucrose to aid in gradient stabilization during formation. Gradients were poured with a 10-well comb in place on the glass gel plates. Extra 3% solution was added to form the sides of the wells, and polymerization was carried out at 4 °C after enclosing the gel in a plastic bag filled with N_2. After a 1 h preelectrophoresis, 80–100 μL of F-2 (80–100 μg of protein) containing 15% glycerol and 1% RBY dye was loaded and electrophoresed at 150 V for 30 h at 4 °C. After termination of the run, the slab gel was placed over a glass plate to which had been attached a transparent Xerox copy of standard 10 × 10 (to the cm) graph paper. The plates were placed onto a bed of ice in a glass dish, and this assembly was set atop a light box. Precise and reproducible gel slices would then be obtained by simultaneously slicing several wells with a razor blade. For adenylate cyclase assays, gel slices were placed in small glass tubes and overlaid with 150 μL of assay cocktail. After a 4–6-h incubation at 4 °C, the assay was initiated by placing the samples in a 30 °C bath for 15 min. Assays were terminated by addition of 850 μL of ice-cold water. After a 4-h incubation on ice, the assay solutions were applied to Dowex columns (Salomon et al., 1974), while the gel slices were discarded. Protein standards used for estimation of Stokes radii were thyroglobulin (85 Å), apoferritin (61 Å), catalase (52 Å), lactate dehydrogenase (40 Å), aldolase (47 Å), myoglobin (21 Å), and β-galactosidase (69 Å). For second-dimension electrophoresis in $NaDodSO_4$, gel slices from native gradients were incubated in $NaDodSO_4$ sample buffer and run on $NaDodSO_4$ slab gels as described below.

Isoelectric Focusing. Isoelectric focusing was carried out at 4 °C in 4% polyacrylamide tube gels containing 2% Pharmalyte 5-8, 0.1% Lubrol PX, and 1 mM $MnCl_2$. The catholyte (upper reservoir) consisted of 10 mM ethanolamine–100 μM ATP, while the anolyte (lower reservoir) contained 10 mM glutamic acid–1 mM $MnCl_2$. Gels were prefocused for 30 min at 12 mA (constant power) and samples applied. Sample solutions contained 10% glycerol, 2 mM DTT, 1 mM ATP, and 2% Pharmolyte in addition to F-2. After application, samples were overlaid with 80 μL of the identical solution (at 5% glycerol) without F-2. Focusing was carried out for 20 h at 900 V. Gels were sliced and assayed for adenylate cyclase activity or prepared for $NaDodSO_4$ slab gel electrophoresis as described above for native gradient gel slices. The pH gradient was determined on slices from gels that contained no F-2 in the sample application solution.

Ultragel AcA 34 Chromatography in CHAPS Detergent. CHAPS was added to F-2 to a final concentration of 13 mM. After incubating for 1 h at 4 °C, 5 mL of this sample (2 mg of protein) was applied to a 0.9 × 45 cm column of Ultragel AcA 34 that had been washed with 4 column volumes of 0.1% CHAPS, 100 mM NaCl, 0.02% NaN_3, 250 mM sucrose, and 50 mM glycine, pH 8.0. The enzyme was eluted from the column with the same buffer at a flow rate of 2 mL/h, and 1-mL fractions were assayed for adenylate cyclase activity.

Preparation and Use of Azido[^{125}I]iodoCaM. CaM was iodinated to a specific activity of (0.5–1) × 10^8 cpm/nmol by the Enzymobead method (Bio-Rad). [^{125}I]IodoCaM was desalted into and dialyzed against 50 mM borate, pH 9.8–100 mM NaCl. MABI (Pierce) was added to a 20-fold excess over [^{125}I]iodoCaM, and the mixture was incubated for 2 h in the dark at room temperature. The reaction products were dialyzed vs. 20 mM Mops, pH 7.2–100 mM NaCl in a flow dialysis cell for 2 days in the dark at 4 °C. The azidification and both dialysis steps were carried out in the dialysis cell. This afforded convenient handling and a minimum of sample transference and allowed up to 0.5 mg of azido[^{125}I]iodoCaM to be synthesized at a time.

Photolysis experiments were carried out as follows: ingredients were mixed in 1.5-mL microfuge tubes under low light and transferred to a 9-well Pyrex spot plate (Corning 7220, VWR Scientific). Samples (up to six at a time) were irradiated for 2 min on ice under a Mineralight UVS-11 placed directly onto the spot plate. Cross-linked products were visualized as autoradiography bands from dried $NaDodSO_4$ slab gels (Laemmli, 1970) with Du Pont Cronex Lightning Plus intensifying screens and Kodak XR-5 X-ray film.

Mn^{2+} was used in place of Ca^{2+} to promote interactions between CaM and adenylate cyclase for activity assays and cross-linking experiments. This cation was used because of

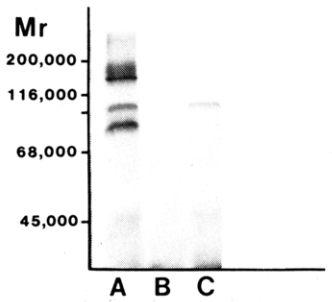

FIGURE 1: Photoaffinity labeling of F-2 adenylate cyclase preparation with azido[^{125}I]iodoCaM. (A) F-2 (40 μg of protein) and 1 μM azido[^{125}I]iodoCaM (2.5×10^4 cpm/pmol) were incubated in a final volume of 110 μL containing 1 mM EDTA and 3 mM $MnCl_2$. The sample was photolyzed, run on 7.5% $NaDodSO_4$–polyacrylamide gel electrophoresis, and autoradiographed as described under Methods. (B) Same as (A) except that 1 mM EGTA was substituted for $MnCl_2$. (C) Same as (A) except that F-2 was preincubated with 3 μM unmodified CaM for 5 min prior to the addition of azido[^{125}I]iodoCaM.

the following reasons: (a) like Ca^{2+}, Mn^{2+} activates CaM-regulated processes in vitro; (b) unlike Ca^{2+}, higher concentrations of Mn^{2+} (>0.1 mM free cation) do not cause inhibition of adenylate cyclase activity; (c) possible contaminants in our adenylate cyclase preparations include Ca^{2+}-activated proteases. These proteases are poorly activated by Mn^{2+}.

Results

Cross-Linking of Azido[^{125}I]iodoCaM to F-2. Preliminary estimations of the amount of adenylate cyclase present in bovine cerebral cortex membranes suggested that it might not be possible to detect cross-linked products from adenylate cyclase with crude membrane preparations. Therefore, the CaM-sensitive enzyme was partially purified by the procedure described under Methods. The pooled activities from CaM–Sepharose (F-2) and Bio-Gel A5M were purified 100- and 350-fold, respectively. The specific activities of these two preparations were 2.3×10^5 and 8.0×10^5 pmol of cAMP/(10 min·mg). Both preparations were stimulated 17-fold by $CaM{\cdot}Ca^{2+}_4$.

Photoaffinity labeling of F-2 with azido[^{125}I]iodoCaM yielded several products (Figure 1). Specificity of interactions between azido[^{125}I]iodoCaM and polypeptides was evaluated by control experiments in which cross-linking was carried out in the presence of either a large excess of EGTA substituted for $MnCl_2$ or 3 μM unmodified CaM (lanes B and C). On the basis of these criteria, cross-linked products having molecular weights of 83 000, 103 000, and 170 000 and a diffuse band between 150 000 and 200 000 were of interest. Occasionally, a pair of lower molecular weight bands in the 35 000–45 000 range could be seen. Since there were several CaM-binding polypeptides present in the preparation, F-2 was submitted to a variety of fractionation techniques to determine if one or more of the CaM-binding subunits copurified with adenylate cyclase activity.

Bio-Gel A5M Gel Filtration. When F-2 was applied to a Bio-Gel A5M column, CaM-sensitive adenylate cyclase eluted as a broad peak coincident with the elution volume of thyroglobulin, which has a Stokes radius of 85 Å (Figure 2A). The apparent size of the enzyme on Bio-Gel A5M was unchanged by the inclusion of CaM in the running buffers. A CaM-sensitive phosphodiesterase eluted with an elution volume coincident with catalase, which has a Stokes radius of 52 Å. Cross-linking of azido[^{125}I]iodoCaM with fractions containing adenylate cyclase or phosphodiesterase activity yielded the products reported in Figure 2B. The peak of adenylate cyclase activity yielded cross-linked products at 170 000 daltons, a

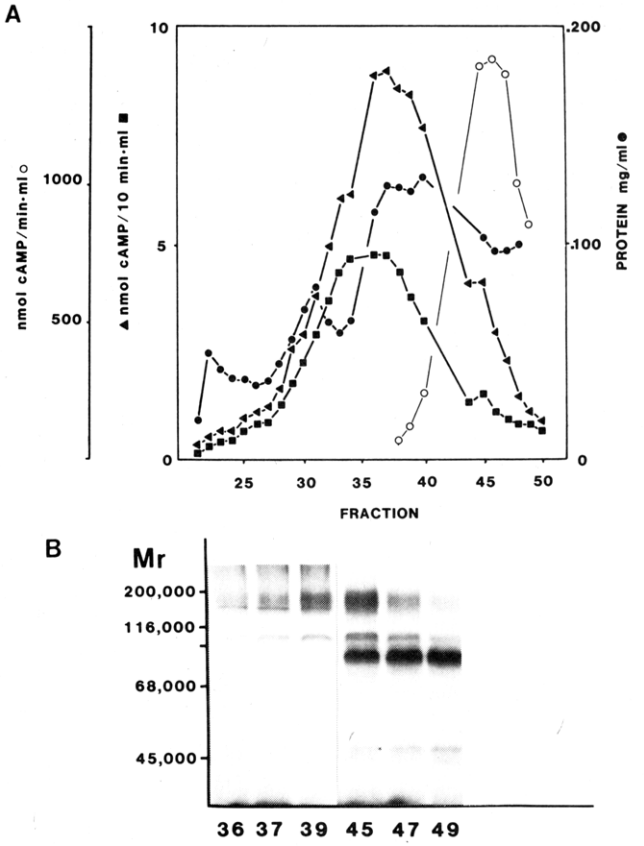

FIGURE 2: Fractionation of F-2 by Bio-Gel A5M chromatography and labeling with azido[^{125}I]iodoCaM. (A) F-2 adenylate cyclase was applied to a Bio-Gel A5M column as described under Methods. Fractions were assayed for adenylate cyclase activity in the presence (▲) or absence (■) of 4 μM CaM, phosphodiesterase activity in the presence of 4 μM CaM (○), and protein (●). (B) Aliquots from the peak of adenylate cyclase activity (fractions 36–39) and phosphodiesterase activity (45–49) were photoaffinity labeled with azido-[^{125}I]iodoCaM, run on 7.5% $NaDodSO_4$–polyacrylamide gel electrophoresis, and autoradiographed as described under Methods.

diffuse band between 170 000 and 200 000 daltons, and a band at 103 000 daltons. The phosphodiesterase fractions gave cross-linked products at 83 000 and 103 000 daltons, as well as the diffuse product from 170 000 to 200 000 daltons. The 83 000-dalton cross-linked peptide was greatly enriched in the peak of phosphodiesterase activity. This CaM-sensitive phosphodiesterase has been purified to apparent homogeneity and found to have a subunit molecular weight of 63 000 on $NaDodSO_4$ gels and 83 000 when cross-linked to azido-[^{125}I]iodoCaM (unpublished observations).

Native Polyacrylamide Gradient Gel Electrophoresis. Since CaM did not affect the apparent size of F-2 adenylate cyclase on Bio-Gel A5M or sucrose gradients (unpublished observations), we anticipated that CaM bound to adenylate cyclase would have little effect on the mobility of the enzyme on native gradient gels. Therefore, parallel gel lanes were run containing either unmodified F-2 or F-2 cross-linked to azido[^{125}I]iodo-CaM. After termination of the run, gel lanes were either stained for protein and autoradiographed intact, sliced for determination of adenylate cyclase activity, or sliced and electrophoresed on a second dimension in $NaDodSO_4$.

Native gradient gels showed two peaks of adenylate cyclase activity at apparent Stokes radii of 85 and 54 Å (Figure 3A). Autoradiography of the intact gel lane loaded with F-2 cross-linked to azido[^{125}I]iodoCaM revealed a broad zone of radioactivity between 350 000 and 700 000 daltons and a sharp band at a native molecular weight of 170 000 (Figure 3A, inset).

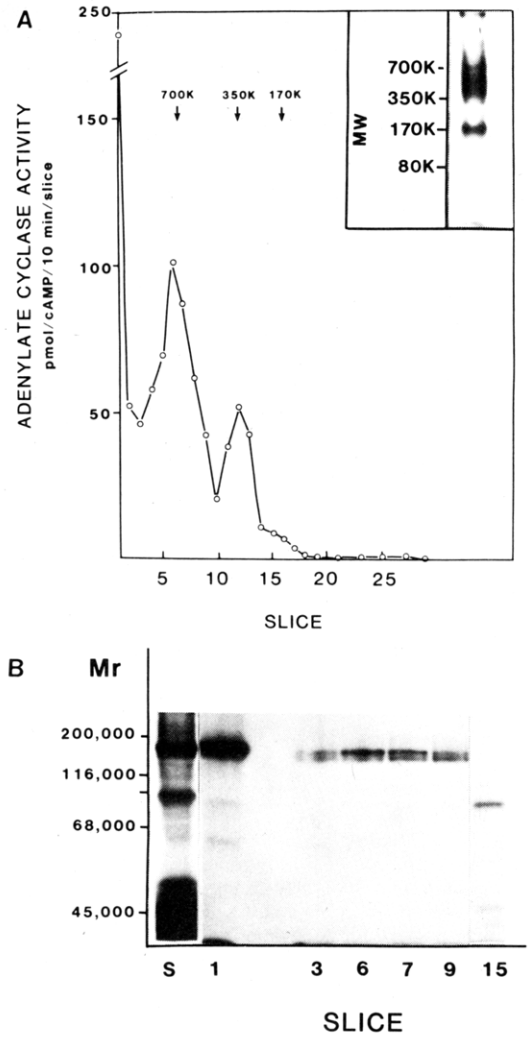

FIGURE 3: Fractionation of F-2 by native gradient gel electrophoresis and labeling with azido[^{125}I]iodoCaM. (A) 100 μg of F-2 was run on 3–20% linear polyacrylamide slab gel and assayed for adenylate cyclase activity as described under Methods. The inset shows an autoradiograph of a parallel gel slice to which F-2 cross-linked to azido[^{125}I]iodoCaM was applied. (B) Selected native gel slices containing both adenylate cyclase activity and ^{125}I counts were incubated in $NaDodSO_4$ sample buffer and run in a second dimension on $NaDodSO_4$ slab gels and autoradiographed. The starting material, F2, was run in the lane marked "S".

Selected native gel slices containing both adenylate cyclase activity and ^{125}I counts were soaked in $NaDodSO_4$ sample buffer and run on $NaDodSO_4$ slab gels (Figure 3B). Autoradiography of the $NaDodSO_4$ gel revealed cross-linked products of 160 000–170 000 daltons from the major peak of adenylate cyclase activity taken from the native gel. In addition, a significant amount of adenylate cyclase activity ran in slice 1 of the native gel (Figure 3A), and this fraction gave one major cross-linked product of 170 000 daltons. The azido[^{125}I]iodoCaM cross-linked peptides of M_r 83 000 and 103 000 present in F-2 were completely separated from adenylate cyclase activity on native gels.

Isoelectric Focusing. While association of CaM with adenylate cyclase did not significantly change the mobility of the enzyme on sucrose gradients, Bio-Gel A5M, or native gradient gels, binding of CaM to the enzyme could affect the isoelectric point of the adenylate cyclase complex. CaM is a relatively acidic protein with a p*I* of 3.9 (Dedman et al., 1977b), and brain adenylate cyclase has been reported to have a p*I* of 6.1–6.3 (Franks & Malamud, 1976; Wahrmann et al., 1980). We would expect the p*I* of the CaM–adenylate cyclase complex to lie somewhere in between that of the individual proteins.

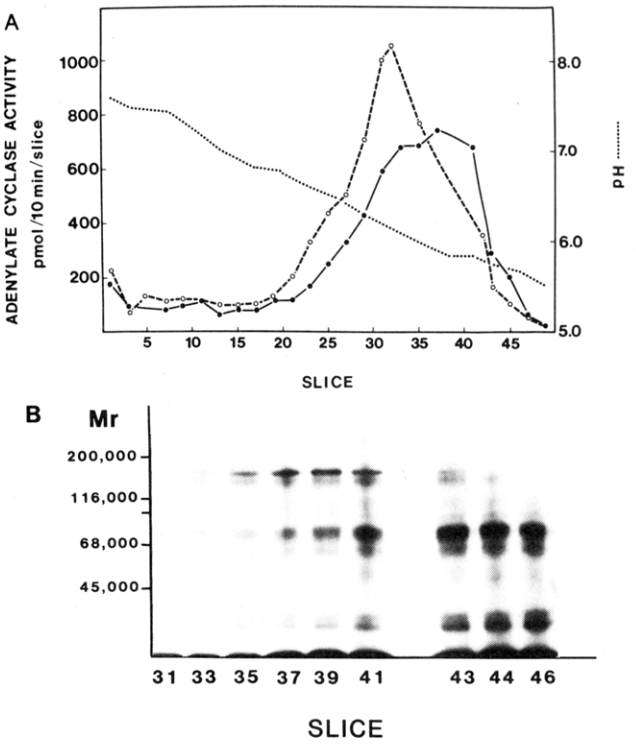

FIGURE 4: Fractionation of F-2 by isoelectric focusing and labeling with azido[^{125}I]iodoCaM. (A) F-2 (60 μg of protein) was isoelectric focused in polyacrylamide gel in the absence (○) or presence of 4 μM CaM (●) and assayed for adenylate cyclase as described under Methods. (B) Corresponding gel slices from a third gel, loaded with F-2 cross-linked to azido[^{125}I]iodoCaM, were incubated in $NaDodSO_4$ sample buffer, run on 7.5% $NaDodSO_4$–polyacrylamide gel electrophoresis, and autoradiographed as described under Methods.

Samples of F-2 adenylate cyclase were loaded into polyacrylamide tube gels containing a pre-formed pH 5–8 gradient as described under Methods. Sample A contained unmodified F-2, sample B consisted of F_2 plus CaM, and sample C was F-2 cross-linked to azido[^{125}I]iodoCaM. The gradients and the lower reservoir (anolyte) contained 1 mM Mn^{2+} in order to stabilize CaM–adenylate cyclase interactions. Over the course of a 20-h run, we would expect some fraction of the CaM–adenylate cyclase complexes of sample B to dissociate, giving peaks of activity corresponding to free adenylate cyclase and the enzyme–CaM complex. In the absence of CaM, adenylate cyclase ran as a single peak of activity with an apparent p*I* of approximately 6.2. Although separate peaks of activity were not resolved in the presence of CaM, there was a shoulder of activity corresponding to free adenylate cyclase and a peak at a p*I* of approximately 5.7. It seems likely that the latter may have been due to the CaM–adenylate cyclase complex since it ran at a p*I* more acidic than that of adenylate cyclase itself.

Gel slices from the sample that had been cross-linked with azido[^{125}I]iodoCaM were incubated in $NaDodSO_4$ sample buffer and run on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4B). The 170 000-dalton cross-linked product appeared in gel slices 35–41 (p*I* 5.7–5.6) and was absent from gel slice 31 (p*I* 6.2). The 40 000- and 83 000-dalton products were most conspicuous in more acidic fractions.

Ultragel AcA 34 Chromatography. F-2 adenylate cyclase was submitted to Ultragel gel filtration in the presence of CHAPS as described under Methods (Figure 5A). Although adenylate cyclase was separated from a significant amount of the applied protein, there was not an increase in specific ac-

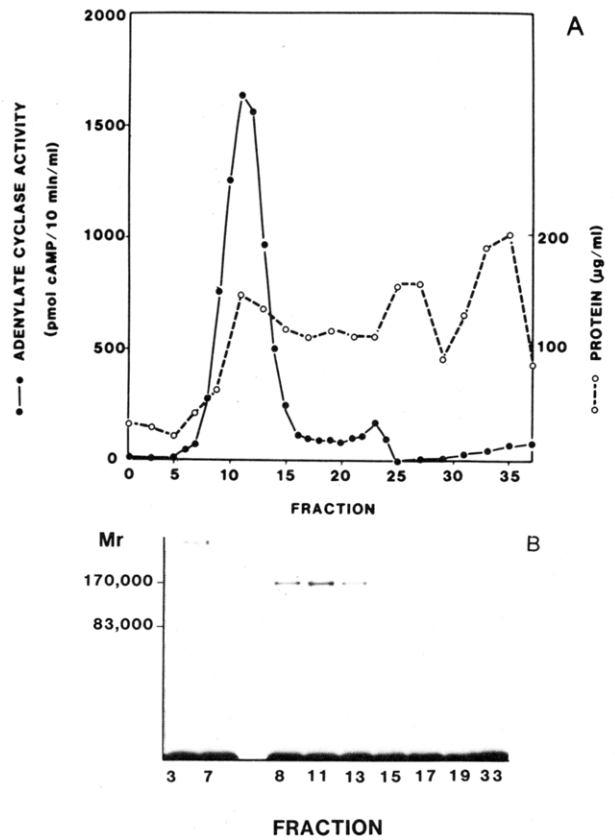

FIGURE 5: Fractionation of F-2 by Ultragel AcA 34 and labeling with azido[^{125}I]iodoCaM. (A) F-2 adenylate cyclase (2 mg of protein) was treated with 13 mM CHAPS detergent and submitted to Ultragel AcA 34 gel chromatography in the presence of 0.1% CHAPS as described under Methods. (B) Fractions from the Ultragel column were photoaffinity labeled with azido[^{125}I]iodoCaM, run on Na-DodSO$_4$–polyacrylamide gel electrophoresis, and autoradiographed as described under Methods.

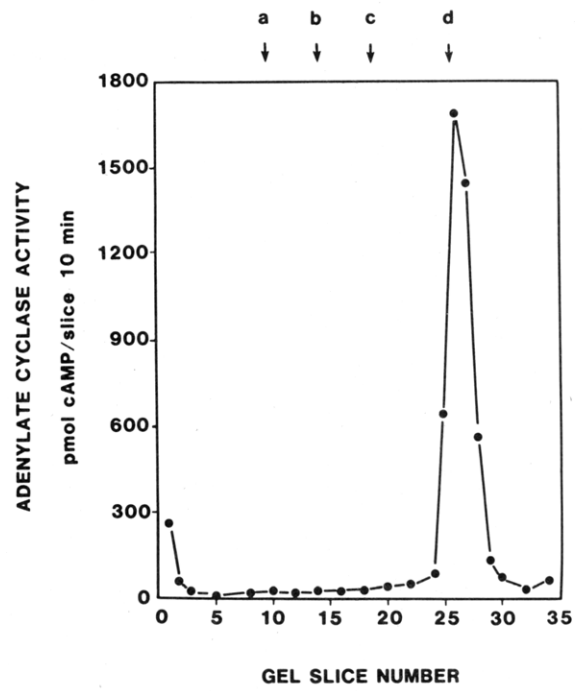

FIGURE 6: Polyacrylamide gradient gel electrophoresis of F-2 adenylate cyclase in the presence of 0.1% Lubrol PX and 0.03% deoxycholate. F-2 adenylate cyclase (80 μg) was treated for 1 h at 30 °C with 100 μM GppNHp–10 mM dithiothreitol in 0.03% deoxycholate and run on a 3–20% linear polyacrylamide gradient slab gel. The upper reservoir contained 20 mM Mops, pH 7.2, 100 μM ATP, 10 μM GppNHp, 0.1% Lubrol PX, and 0.03% deoxycholate. Gel lanes were sliced and assayed for adenylate cyclase as described under Methods. Molecular weight standards were (a) thyroglobulin, (b) ferritin, (c) catalase, and (d) lactate dehydrogenase.

tivity since approximately 90% of total enzyme activity was lost during this step. However, azido[^{125}I]iodoCaM was cross-linked to fractions containing adenylate cyclase since the protein elution profile indicated considerable protein fractionation. The peak of adenylate cyclase activity yielded only one cross-linked polypeptide of M_r 170 000 on NaDodSO$_4$ gels (Figure 5B). The CaM-sensitive phosphodiesterase appeared in fractions 15–20. Photoaffinity labeling of these fractions with azido[^{125}I]iodoCaM gave one cross-linked product of M_r 83 000.

Smallest Detectable Size of Adenylate Cyclase. The data reported thus far indicate that a single CaM-binding polypeptide correlates with adenylate cyclase activity through a number of purification steps. If it is assumed that this polypeptide only binds one CaM, like other CaM-binding subunits, then one can tentatively conclude that the CaM-binding subunit of adenylate cyclase has a molecular weight of approximately 150 000. Consequently, it was of some interest to determine the minimum size of adenylate cyclase. The data in Figure 3A suggested that the CaM-sensitive adenylate cyclase system may exist in different states of aggregation or that other proteins are nonspecifically associated with the enzyme. Therefore, native gradient gel electrophoresis was carried out in the presence of 0.03% deoxycholate to determine if smaller forms of adenylate cyclase activity were obtainable (Newby et al., 1978). In the presence of 0.1% Lubrol PX and 0.03% deoxycholate, the enzyme gave one peak of activity with a Stokes radius of approximately 40 Å (Figure 6). This size is consistent with a M_r of 140 000–150 000. We suspect that the 170 000-dalton cross-linked product obtained with azido-[^{125}I]iodoCaM and adenylate cyclase preparations is a covalent complex between CaM and the catalytic subunit of the enzyme.

Discussion

When a partially purified CaM-sensitive adenylate cyclase preparation was photoaffinity labeled with azido[^{125}I]iodo-CaM, a limited number of cross-linked products were formed. The general objective of this study was to determine if any of these CaM-binding polypeptides copurified with adenylate cyclase or could be separated from the enzyme by further purification. Implicit in this experimental design is the assumption that there would be enough of the cross-linked product between adenylate cyclase and azido[^{125}I]iodoCaM to detect by autoradiography of gels. Important parameters include the percentage of adenylate cyclase present in the preparations, the cross-linking efficiency achieved during photolysis, the specific radioactivity of the azido[^{125}I]iodoCaM, and the detection limits of autoradiography.

The F-2 adenylate cyclase preparation had a specific activity of 2.3 × 10^4 pmol of cAMP/(min·mg), and pooled activity from Bio-Gel A5M was 8.0 × 10^4 units/mg. Levitzki et al. (1975) have estimated that fully stimulated adenylate cyclase has a turnover number of 1000–1500 min^{-1}. On this basis, we would conservatively estimate that adenylate cyclase comprised a minimum of 0.5–1% of the F-2 protein. Thus, in a typical labeling experiment, 40 μg of protein applied to each gel lane would contain approximately 1 pmol of adenylate cyclase. The cross-linking efficiency of azido[^{125}I]iodoCaM with CaM-binding proteins is typically 10–30%, and the specific radioactivity of azido[^{125}I]iodoCaM used was 2.5 × 10^4 cpm/pmol. We would anticipate a minimum of

2000–3000 cpm of cross-linked product in a typical experiment such as that reported in Figure 1. This quantity can readily be detected by a few hours of autoradiographic exposure. Thus, it was reasonable to anticipate a detectable amount of cross-linked product between azido[^{125}I]iodoCaM and adenylate cyclase. Upon slicing and counting of the dried gel from Figure 1, it was determined that the product at M_r 170 000 contained approximately 4000 cpm.

The methods used in this study fractionated the proteins of F-2 on the basis of their size, molecular weight, or charge. In all experiments, pooled adenylate cyclase activity yielded a 170 000-dalton cross-linked peptide from azido[^{125}I]iodoCaM, and no other product correlated with adenylate cyclase activity. In fact, pooled adenylate cyclase activity from Bio-Gel AcA 34 chromatography gave one and only one cross-linked polypeptide having an M_r of 170 000. Extensive autoradiography of this film, 72 h of exposure, did not reveal any other labeled peptides. Therefore, we conclude that either this is the CaM-binding subunit of adenylate cyclase or we have greatly overestimated the amount of adenylate cyclase present in our preparations and the cross-linked product between the enzyme and azido[^{125}I]iodoCaM was simply not detectable.

Photoaffinity labeling of a variety of pure CaM-binding proteins including myosin light chain kinase, phosphorylase kinase, troponin I, (Ca^{2+} + Mg^{2+}) ATPase and phosphodiesterase has indicated that covalent attachment of azido-[^{125}I]iodoCaM leads to a molecular weight increase of approximately 20 000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. We suggest that the 170 000-dalton cross-linked polypeptide detected in this study is formed from azido-[^{125}I]iodoCaM and a 150 000-dalton peptide. It is interesting that the minimum functional size of this adenylate cyclase on native gradient gels was consistent with a protein having a molecular weight of 140 000–150 000, although the molecular weight of this activity was not rigorously determined. Therefore, we tentatively conclude that the catalytic subunit of the CaM-sensitive adenylate cyclase has a molecular weight of 150 000 and that each subunit binds one CaM. More direct proof of these conclusions will require either further purification of the enzyme or isolation of antibodies against the partially purified polypeptides.

Registry No. Adenylate cyclase, 9012-42-4.

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