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Purification of the Calmodulin-Sensitive Adenylate Cyclase from Bovine Cerebral Cortex†

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ABSTRACT: A calmodulin-sensitive adenylate cyclase was purified 3000-fold from bovine cerebral cortex using DEAE-Sephacel, calmodulin–Sepharose, and two heptanediamine–Sepharose column steps. The purified enzyme activity was stimulated by calmodulin, forskolin, 5′-guanylyl imidodiphosphate, and NaF. The molecular weight of the protein component was estimated as 328 000 with a smaller form of M_r 153 000 obtained in the presence of Mn^{2+}. The most highly purified preparations contained major polypeptides of 150 000, 47 000, and 35 000 daltons on sodium dodecyl sulfate (SDS) gels. Photoaffinity labeling of the preparation with azido[^{125}I]iodocalmodulin gave one product of 170 000 daltons on SDS gels. It is proposed that the catalytic subunit of the calmodulin-sensitive enzyme is 150 000 ± 10 000 daltons and that the enzyme exists as a complex of one catalytic subunit and the stimulatory guanyl nucleotide regulatory complex. These data are consistent with the previous report that the catalytic subunit of this enzyme has a molecular weight of 150 000 ± 10 000 [Andreasen, T. J., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1983) *Biochemistry 22*, 2757].

Adenylate cyclase sensitive to stimulation by calmodulin (CaM)[1] was first reported by Brostrom et al. in 1975, and independently in the same year by Cheung et al. (1975). In contrast to hormone-stimulated adenylate cyclase, which is present in almost every mammalian cell type (Ross & Gilman, 1980), CaM-sensitive adenylate cyclase has been demonstrated only in a limited number of tissues including brain, pancreatic islet cells, and adrenal medulla (Brostrom et al., 1975; LeDonne & Coffee, 1975; Valverde et al., 1979). Bovine cerebral cortex is believed to contain both CaM-sensitive and CaM-insensitive forms of adenylate cyclase (Brostrom et al., 1977; Westcott et al., 1979). It has been difficult to characterize the CaM-sensitive adenylate cyclases using membranes or unfractionated detergent-solubilized extracts because of the presence of calmodulin-insensitive forms of the enzyme and other CaM binding proteins. The ultimate characterization of CaM-sensitive adenylate cyclase and the elucidation of the regulatory mechanisms of the enzyme await the availability of a homogeneous preparation.

In this study, we describe a procedure for the partial purification of the CaM-sensitive adenylate cyclase which separates the enzyme from CaM-insensitive adenylate cyclase and several major CaM binding proteins. The subunit composition and molecular weight of the enzyme complex are reported.

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Materials and Methods

Materials

Bio-Gel A-5M was purchased from Bio-Rad. GppNHp was obtained from P-L Biochemicals. ATP, cAMP, and protein standards were from Sigma. [α-^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear and International Chemical Nuclear, respectively. 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

[1] Abbreviations: CaM, calmodulin; N_s, stimulatory guanyl nucleotide regulatory complex of adenylate cyclase; N_i, inhibitory guanyl nucleotide regulatory complex of adenylate cyclase; GppNHp, 5′-guanylyl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; IAP, islet activating protein from *Bordetella pertussis*; MABI, methyl azidobenzimidate; EDTA, (ethylenedinitrilo)tetraacetic acid; DMPC, dimyristoylphosphatidylcholine; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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standard errors of less than 5%. Sensitivity to GppNHp was determined by preincubation of the enzyme with 0.10 mM GppNHp for 30 min at 30 °C in the presence of 5 mM $MgCl_2$ prior to assay.

Protein concentrations were determined by the method of Peterson (1977).

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

Cholera Toxin Labeling. Cholera toxin was preactivated by incubation with 20 mM DTT for 20 min at 30 °C. Protein preparations were labeled with [^{32}P]NAD by incubation with 40 μg/mL cholera toxin, 250 μM [^{32}P]NAD (10^9 cpm/μmol), 30 μM ATP, 12 mM arginine, 10 mM thymidine, 100 μM GppNHp, 50 mM Tris, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT in 0.1% Lubrol PX for 30 min at 30 °C. The reaction was stopped by cooling to 0 °C and addition of unlabeled NAD at a final concentration of 5 mM. Labeled polypeptides were separated in 10% polyacrylamide slab gels run in SDS and identified by autoradiography of the dried gel.

Bordetella pertussis Toxin Labeling. Adenylate cyclase preparations were labeled with [^{32}P]NAD by using *B. pertussis* culture supernatant as a source of islet activating protein (IAP). Protein samples were incubated for 1 h at 30 °C in buffer containing 70 mM Tris, pH 7.4, 7 mM thymidine, 0.7 mM ATP, 3.5 mM $MgCl_2$, 2 mM DMPC, 0.7 mM DTT, 0.7 mM EDTA, 35 μM [^{32}P]NAD (10^9 cpm/μmol), and 27 μg/mL *B. pertussis* culture supernatant. The reaction was stopped by addition of unlabeled NAD at a concentration of 1 mM. Labeled polypeptides were then run on 7.5% polyacrylamide slab gels in SDS and identified by autoradiography of the dried gel.

Partial Purification of Calmodulin-Sensitive Adenylate Cyclase. Frozen bovine cerebral cortex (500 g), obtained from a local slaughterhouse, was fractured with a hammer and thawed in phosphate-buffered saline: 22.5 mM KH_2PO_4, 75 mM NaCl, and 12.8 mM NaOH, pH 7.2. Thawed cortex was drained and homogenized with a Waring blendor (30 s) in an equal volume of homogenization buffer: 20 mM glycylglycine, pH 7.2, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM DTT, and 1 mM PMSF. The homogenate was further disrupted with a Polytron homogenizer (30 s at the maximum setting). The resulting homogenate was centrifuged in a Sorvall RC-3B centrifuge for 30 min at 4500 rpm. The pelleted membranes were resuspened in an equal volume of homogenization buffer, and the Polytron homogenization, centrifugation, and resuspension steps were repeated 3 times.

The washed membrane pellet was detergent extracted by the addition of solubilization buffer: 20 mM Tris, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.5% Lubrol PX at a detergent to protein ratio of 2.5:1 (w/w). In some cases, membranes were pretreated with 0.10 mM GppNHp for 30 min at 30 °C in the presence of 5 mM $MgCl_2$ prior to solubilization. The mixture was stirred overnight and centrifuged 2 h at 4500 rpm in a Sorvall RC-3B centrifuge, and the supernatant fluid was decanted.

Two liters of DEAE-Sephacel equilibrated in 50 mM Tris, pH 7.4 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1% Lubrol PX (buffer A) was added to 5 L of detergent extract and stirred for 45 min. 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 activity was eluted. Under these conditions, calmodulin was not eluted from the column. The enzyme was pooled on the basis of adenylate cyclase activity, diluted with an equal volume of buffer A, and brought to 1.1 mM $CaCl_2$. This dilution lowered the KCl concentration in the pooled enzyme solution from approximately 110 mM to about 55 mM. Both the addition of Ca^{2+} and the dilution of the KCl in the pool were required for successful CaM–Sepharose chromatography.

The diluted enzyme solution was loaded onto a 2.5 × 26 cm CaM–Sepharose column equilibrated in buffer A adjusted to 1.1 mM $CaCl_2$. The column was washed with this same buffer until the effluent absorbance at 280 nm reached a steady value, at which time the column was eluted with buffer A.

Adenylate cyclase eluted from CaM–Sepharose was pooled on the basis of activity, loaded onto a heptanediamine–Sepharose column equilibrated in buffer A, and eluted with a concave 0–500 mM NaCl gradient in buffer A. Fractions were pooled on the basis of adenylate cyclase activity and diluted with 1.5 volumes of buffer A. Calmodulin and $CaCl_2$ were added to the diluted pool to final concentrations of 5 μM and 1.1 mM, respectively. This pool was loaded onto the same heptanediamine–Sepharose column equilibrated in buffer A containing 1.1 mM $CaCl_2$ (buffer B). The column was eluted with a linear 0–500 mM NaCl gradient in loading buffer. Fractions were assayed for adenylate cyclase activity, frozen in liquid nitrogen, and stored at −70 °C for future use. All operations described in the purification procedure were at 4 °C.

Determination of Stokes Radius. The Stokes radius of the partially purified enzyme was determined by Bio-Gel A-5M chromatography. Adenylate cyclase pooled from either the CaM–Sepharose column or the second heptanediamine–Sepharose column was concentrated by ultrafiltration and applied to a 12-mL Bio-Gel A-5M column equilibrated in buffer A. Eluted enzyme was assayed in either the absence or the presence of CaM.

Sucrose Density Gradient Centrifugation. Adenylate cyclase was subjected to centrifugation through a 5–20% sucrose gradient in H_2O or D_2O for 18 h at 35000 rpm in a Beckman SW40-Ti rotor. Protein standards included β-galactosidase, catalase, alkaline phosphatase, and cytochrome *c*. Gradients contained 0.5% Lubrol PX, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. In some experiments, 10 mM $MnCl_2$ was also present. Linearity of gradients was confirmed by refractometry.

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, and 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, and 100 mM NaCl in a flow dialysis cell for 2 days in the dark at 4 °C. The azidofication 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 nine-well Pyrex spot plate (Corning 7220, VWR Scientific). Samples (up to six at a time) were irra-

Table I: Purification of Calmodulin-Sensitive Adenylate Cyclase from GppNHp-Treated Membranes

purification step	total act.[a]	% yield act.	total protein	sp act. of CaM-sensitive adenylate cyclase[b]	x-fold purification
membranes[c]	3620	100	45 g	0.08	1.0
detergent extract	2880	80	16 g	0.18	2.3
DEAE-Sephacel	1940	54	3.8 g	0.51	6.4
calmodulin–Sepharose	1800	50	34 mg	53.2	665.0
heptanediamine–Sepharose I	1080	30	9 mg	120.0	1500.0
heptanediamine–Sepharose II	500	14	2 mg	240.0	3000.0

[a] Adenylate cyclase was assayed as described under Methods in the presence of 10 mM $MnCl_2$ and 5 μM CaM. Total activity is expressed as nanomoles of cAMP per minute; specific activity is nanomoles of cAMP per minute per milligrams. [b] The specific activity of CaM-insensitive adenylate cyclase plus CaM-sensitive adenylate cyclase in membranes was 0.44. [c] Membranes were pretreated with 0.1 mM GppNHp for 30 min at 30 °C as described under Methods.

Table II: Purification of CaM-Sensitive Adenylate Cyclase from Membranes Not Treated with GppNHp

purification step	total act.[a]	% yield act.	total protein	sp act. of CaM-sensitive adenylate cyclase	x-fold purification
membranes	4180	100	46 g	0.09	1.0
detergent extract	3440	82	15 g	0.23	2.6
DEAE-Sephacel	560	13	2.4 g	0.23	2.6
calmodulin–Sepharose	830	20	33 mg	25.0	277.0
heptanediamine–Sepharose	420	10	7 mg	60.0	667.0

[a] Adenylate cyclase was assayed as described under Methods in the presence of 10 mM $MnCl_2$ and 5 μM CaM. Total activity is expressed as nanomoles of cAMP per minute; specific activity is nanomoles of cAMP per minute per milligram.

diated 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 SDS slab gels (Laemmli, 1970) with Du Pont Cronex Lightning Plus intensifying screens and Kodak XR-5 X-ray film.

Results

Purification of Calmodulin-Sensitive Adenylate Cyclase. It was discovered during preliminary studies that the yield of adenylate cyclase activity and the levels of purification were greatly improved by pretreatment of the membranes with GppNHp. However, this treatment rendered the final preparation insensitive to GppNHp. Therefore, we report purifications starting with GppNHp-treated membranes (Table I) and untreated membranes (Table II). The major difference in the two purification schemes occurred during DEAE-Sephacel chromatography. The yields of adenylate cyclase activity at this stage were 50% and 13% for GppNHp-treated and untreated preparations, respectively. Although DEAE-Sephacel afforded some purification (Table I), the major function of this column was to remove endogenous CaM. As illustrated in Figure 1, CaM was well separated from adenylate cyclase when Ca^{2+} was chelated with EDTA. CaM is a relatively acidic protein and required much higher salt concentrations for elution. Since the DEAE-Sephacel elution was highly reproducible, the 50–600 mM KCl gradient shown in Figure 1 was routinely replaced with a 150 mM KCl step gradient. Attempts to remove endogenous CaM from the

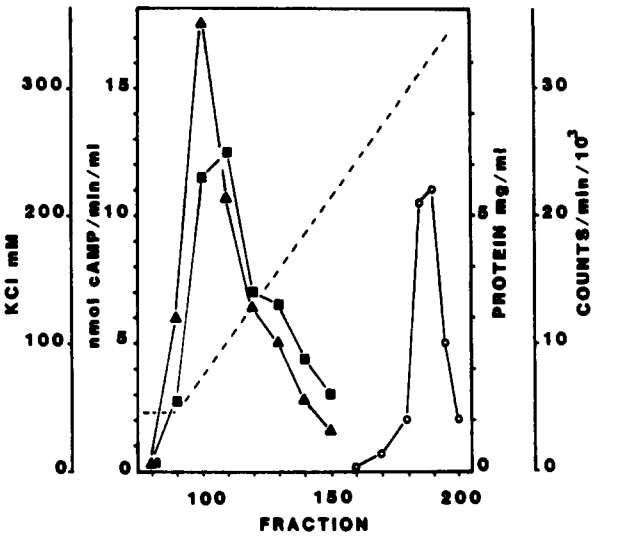

FIGURE 1: DEAE-Sephacel chromatography of Lubrol PX detergent extract. Detergent-solubilized adenylate cyclase was applied to DEAE-Sephacel as described under Methods. The column (9 × 30 cm) was washed with buffer A containing 50 mM KCl (4 L) and eluted with a 50–600 mM KCl gradient (---) in buffer A. Fractions (15 mL) were collected and assayed for adenylate cyclase activity (▲) and protein (■). Radioiodinated calmodulin (○) was added to monitor the elution of calmodulin.

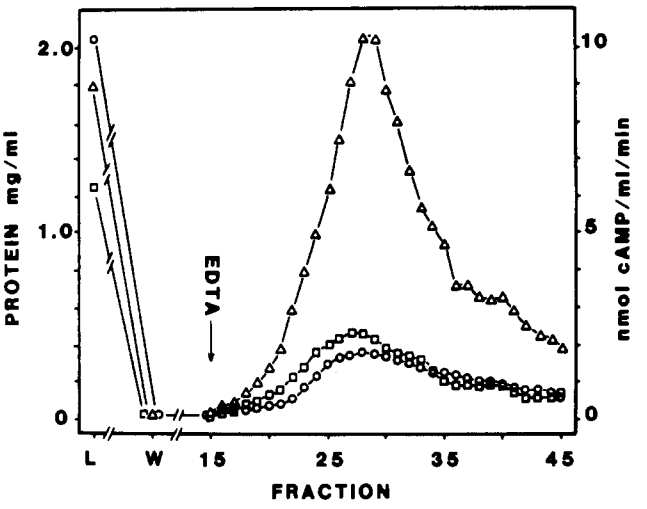

FIGURE 2: Calmodulin–Sepharose chromatography. Adenylate cyclase pooled from DEAE-Sephacel was applied to a 2.5 × 26 cm calmodulin–Sepharose column as described under Methods. The column was washed with calcium-containing buffer (buffer B) and eluted with buffer containing EDTA and no added calcium (buffer A). Loaded material flowing through the column and the material flowing through during the calcium wash were collected as single bulk fractions. Fractions (7 mL) were collected during the EDTA elution and assayed for adenylate cyclase activity in the presence (△) and absence (□) of calmodulin and for protein (○).

starting membranes with repeated washes of chelator-containing buffers were unsuccessful. This is apparently due to a protein present in the membranes which has significant affinity for CaM even in the presence of EDTA or EGTA (Andreasen et al., 1983a).

CaM–Sepharose chromatography provided significant purifications with excellent yields (Figure 2, Table I). When the adenylate cyclase pooled from CaM–Sepharose was reapplied to CaM–Sepharose, 95% of the applied activity was recovered by EDTA elution. Purifications by CaM–Sepharose were not improved by the use of chelator gradients or attempts to remove any nonspecifically absorbed proteins by washing the column with high salt or increased Lubrol PX concen-

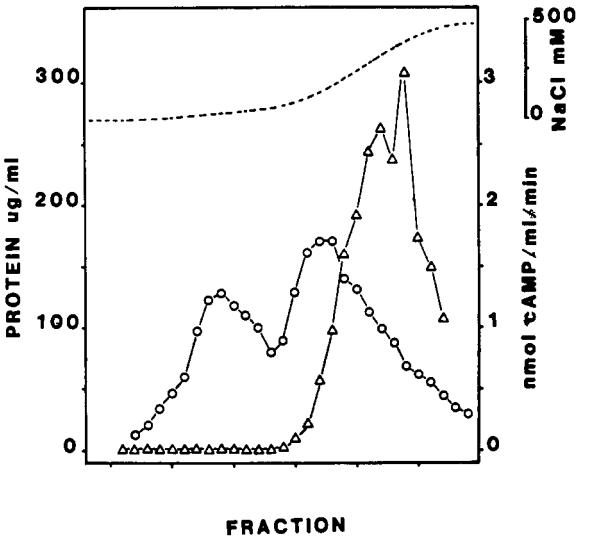

FIGURE 3: Heptanediamine–Sepharose chromatography I. Calmodulin-sensitive adenylate cyclase pooled from calmodulin–Sepharose (80 mL) was applied to a heptanediamine–Sepharose column (10 mL) equilibrated in buffer A as described under Methods. The column was eluted with a concave 0–500 mM NaCl gradient (---) in buffer A, and fractions were assayed for adenylate cyclase in the presence of calmodulin and Mn^{2+} (△) and for protein (○).

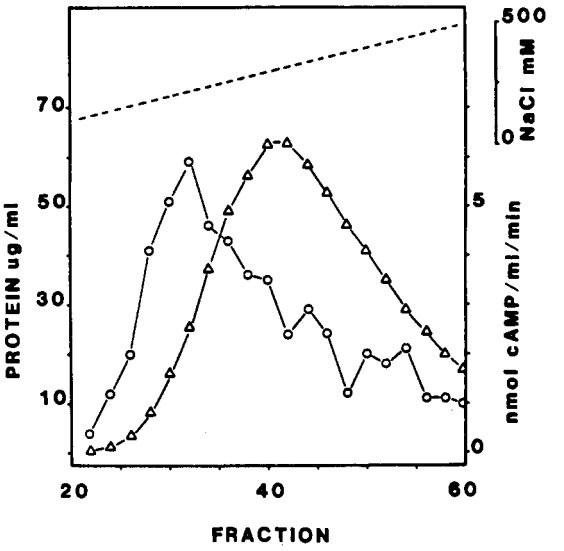

FIGURE 4: Heptanediamine–Sepharose chromatography II. Adenylate cyclase pooled from heptanediamine–Sepharose was diluted with buffer A, adjusted to 1.1 mM calcium and 5 μM calmodulin, and reloaded onto heptanediamine–Sepharose equilibrated in buffer B as described under Methods. The column was eluted with a linear 0–500 mM NaCl gradient in buffer B (---). Fractions (3.5 mL) were assayed for protein activity (○) and for adenylate cyclase activity in the presence of Mn^{2+} and calmodulin (△).

trations. The major contaminants of the adenylate cyclase preparation obtained by CaM–Sepharose are CaM binding proteins, several of which have been identified.

The adenylate cyclase preparation purified through CaM–Sepharose contained as its major contaminant a CaM-sensitive phosphodiesterase. This enzyme and several other contaminating proteins were removed by heptanediamine–Sepharose chromatography (Figure 3). The elution profile from this column consisted of two major protein peaks. The CaM-sensitive phosphodiesterase was present in the first protein peak; adenylate cyclase was associated with the second. The specific activity of pooled enzyme from this column (120 nmol min^{-1} mg^{-1}) was actually greater than individual fractions, suggesting that subunits of the enzyme complex may actually fractionate over the peak of enzyme activity. Further purification was achieved by addition of CaM and Ca^{2+} to the pooled enzyme and reapplication to heptanediamine–Sepharose. Complex formation between CaM and adenylate cyclase caused the enzyme to shift up the gradient and displace it from the major peak of protein (Figure 4). The final specific activity of the pooled preparation was 240 nmol min^{-1} mg^{-1} with a 14% yield. The stability of adenylate cyclase activity through CaM–Sepharose was excellent; however, the final preparation was considerably less stable at 4 °C. The most highly purified preparations were frozen at −80 °C without loss of activity for 12 months.

The CaM-sensitive phosphodiesterase was also separated from adenylate cyclase by Bio-Gel A-5M gel filtration (Figure 5). In 0.1% Lubrol PX, CaM-sensitive adenylate cyclase ran with an apparent Stokes radius of 75 Å. The elution position of the enzyme was unaffected by GppNHp treatment. A peak of CaM-sensitive phosphodiesterase activity eluted with an apparent Stokes radius of 52 Å.

Pooled samples from each stage of the purification summarized in Table I were submitted to SDS–polyacrylamide gel electrophoresis (Figure 6). The Coomassie Blue staining pattern indicates that some protein fractionation was achieved by detergent extraction and DEAE-Sephacel chromatography; however, the most obvious change in polypeptide composition followed CaM–Sepharose chromatography. The major CaM

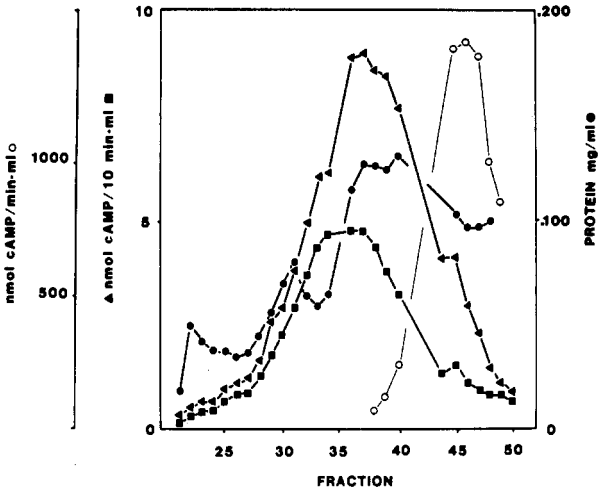

FIGURE 5: Bio-Gel elution profile. A 2.5 × 74 cm Bio-Gel A-5M column equilibrated in 50 mM Tris·HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1% Lubrol PX was loaded with 15 mL of adenylate cyclase activity pooled from CaM–Sepharose as described under Methods. Fractions (4.5 mL) were collected and assayed for adenylate cyclase activity in the presence of 10 mM $MnCl_2$ either in the presence of 4 μM CaM (▲) or in the absence of CaM (■). Protein (●) and phosphodiesterase activities in the presence of 4 μM CaM (○) are also reported.

binding protein eluting from CaM–Sepharose had an apparent molecular weight of 64 000. This protein, which comprised more than 90% of the total protein present in the CaM–Sepharose pool, was subsequently identified as a CaM-stimulated phosphodiesterase (data not shown). Another component of the CaM–Sepharose pool was a polypeptide having a molecular weight of 140 000. The CaM-sensitive ATPase purified from erythrocytes (Graf et al., 1982) or brain synaptic membranes (Papazian et al., 1984) has a molecular weight of 138 000–140 000. When pure samples of the ATPase were run on the same gel as our adenylate cyclase preparations, the ATPase ran parallel with the 140 000-dalton polypeptide. The 64 000- and 140 000-dalton polypeptides were almost completely removed by the two heptanediamine–Sepharose columns, al-

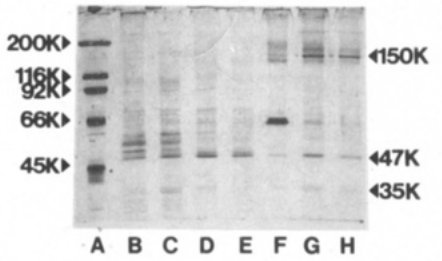

FIGURE 6: SDS–polyacrylamide gel electrophoresis of CaM-sensitive adenylate cyclase after each step in the purification scheme. Samples of adenylate cyclase (11 μg) from each stage of the purification scheme summarized in Table I were diluted to 0.4 mL in buffer A, brought to 1.0 mL with distilled water, and precipitated with 0.1 mL of 0.15% sodium deoxycholate and 72% trichloroacetic acid, respectively. The precipitates were pelleted by centrifugation for 30 min at 3500 rpm. The pellets were then washed with 1 mL of cold acetone and recentrifuged for 30 min. The acetone was removed by aspiration, and the residues were dissolved in 150 μL of Laemmli sample buffer. The samples were subjected to SDS–polyacrylamide gel electrophoresis on a 7.5%, 1.5′mm-thick slab gel. The gel was stained with Coomassie Brilliant Blue and photographed wet. (A) Bio-Rad high molecular weight standards; (B) bovine cerebral membranes; (C) Lubrol PX solubilized membranes; (D) DEAE-Sephacel pool; (E) flow-through from CaM–Sepharose Ca^{2+} wash; (F) CaM–Sepharose EDTA elution pool; (G) heptanediamine–Sepharose I pool; (H) heptanediamine–Sepharose II pool.

Table III: Sensitivity of Purified Adenylate Cyclase[c] to CaM, Forskolin, and GppNHp

addition[a]	adenylate cyclase act. (nmol of cAMP min^{-1} mL^{-1})	x-fold stimulation
Mg^{2+}[b]		
basal	0.20	1.0
NaF	0.26	1.3
GppNHp	0.72	3.6
forskolin	1.40	7.0
CaM	1.94	9.7
Mn^{2+}		
basal	0.99	1.0
NaF	1.88	1.9
GppNHp	1.74	1.8
CaM	2.94	3.0
forskolin	4.99	5.0

[a] When present, GppNHp, forskolin, NaF, and CaM were 100 μM, 10 μM, 15 mM, and 4 μM, respectively. Samples treated with GppNHp were incubated at 30 °C for 30 min prior to assay as described under Methods. The enzyme was purified as described in Table II. [b] The enzyme was assayed with either 5 mM $MgCl_2$ or 10 mM $MnCl_2$ as the supporting divalent cation. [c] Pooled adenylate cyclase from CaM–Sepharose was used.

though they still contaminated the final preparation. The most highly purified preparation contained polypeptides of molecular weight 240 000, 160 000, 150 000, 47 000, and 35 000. The 240 000-dalton polypeptide was removed in separate experiments by gel filtration and is apparently not the catalytic subunit of the enzyme.

Sensitivity to Mn^{2+}, NaF, GppNHp, and Forskolin. The enzyme purified by either purification scheme (Tables I and II) was stimulated 8–10-fold by 10 μM CaM and 7-fold by forskolin when Mg^{2+} was the supporting divalent cation. The enzyme prepared without pretreatment with GppNHp was also stimulated by NaF and GppNHp (Table III). The basal activity was approximately 5 times higher when Mn^{2+} was present as the divalent cation compared to Mg^{2+}. Mn^{2+} was also able to replace Ca^{2+} in supporting CaM stimulation of the enzyme; however, in contrast to Ca^{2+}, Mn^{2+} had no inhibitory effect at concentrations up to 20 mM. It has also been reported that Ca^{2+} but not Mn^{2+} inhibits turkey erythrocyte adenylate cyclase (Hanski et al., 1977). The sensitivity of the

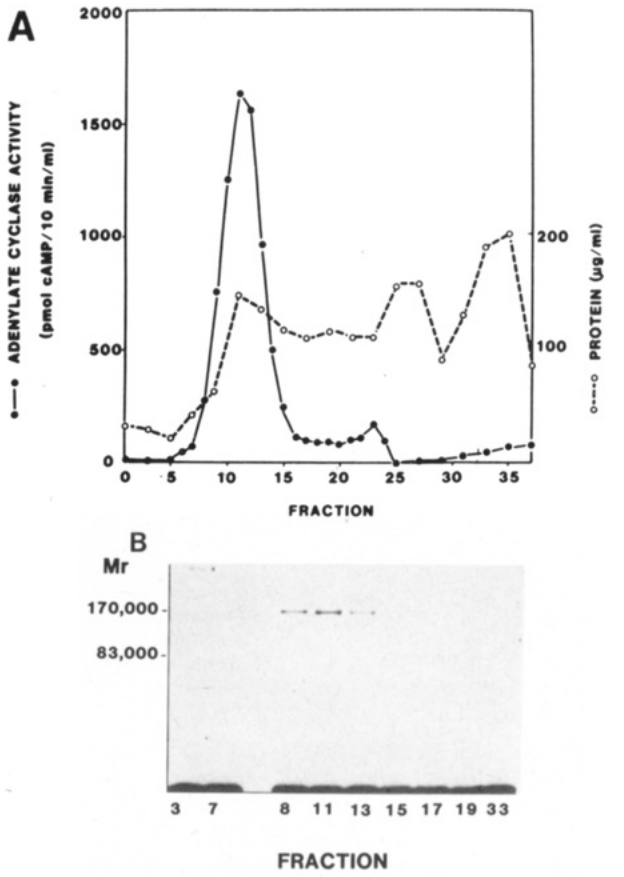

FIGURE 7: Photoaffinity labeling of adenylate cyclase with azido-[^{125}I]iodoCaM. (A) Adenylate cyclase purified through CaM–Sepharose (2 mg of protein) was treated with 13 mM CHAPS detergent and submitted to Ultrogel AcA 34 gel chromatography using buffer A containing 0.1% CHAPS for elution. (B) Fractions from the Ultrogel column were photoaffinity labeled with azido[^{125}I]iodoCaM, run on SDS gels, and autoradiographed as described under Methods. The CaM-sensitive phosphodiesterase was eluted in fractions 15–19 and formed a 83 000-dalton cross-linked product with azido[^{125}I]-iodoCaM.

partially purified enzyme to NaF and GppNHp indicates functional coupling of the stimulatory guanyl nucleotide regulatory complex with the CaM-sensitive adenylate cyclase.

Identification of Enzyme Subunits. Since the final preparation contained a limited number of polypeptides, we attempted to identify them by specific labeling using cholera toxin, *Bordetella pertussis* islet activating protein, and azido[^{125}I]iodoCaM. We have recently photoaffinity labeled partially purified CaM-sensitive adenylate cyclase preparations with azido[^{125}I]iodoCaM and discovered that only one cross-linked peptide of molecular weight 170 000 correlated with adenylate cyclase activity (Andreasen et al., 1983b). We proposed that the catalytic subunit of the enzyme is 150 000 daltons. Figure 7 shows that the 170 000-dalton polypeptide is the only azido[^{125}I]iodoCaM cross-linked polypeptide that is obtained with highly purified adenylate cyclase. The 83 000-dalton cross-linked polypeptide shown in Figure 7 is formed from the CaM-sensitive phosphodiesterase which is well separated from adenylate cyclase activity on this column.

The stimulatory guanine nucleotide regulatory complex of adenylate cyclase (N_S) mediates stimulation by guanine nucleotides and fluoride (Northup et al., 1982; Sternweis & Gilman, 1982). It is a dimer of an α subunit (45 000 ± 2000 daltons) and a β subunit (35 000 daltons). Cholera toxin catalyzes the ADP-ribosylation of the α subunit. Labeling of pooled adenylate cyclase fractions through the purification scheme with cholera toxin and [^{32}P]NAD showed a number

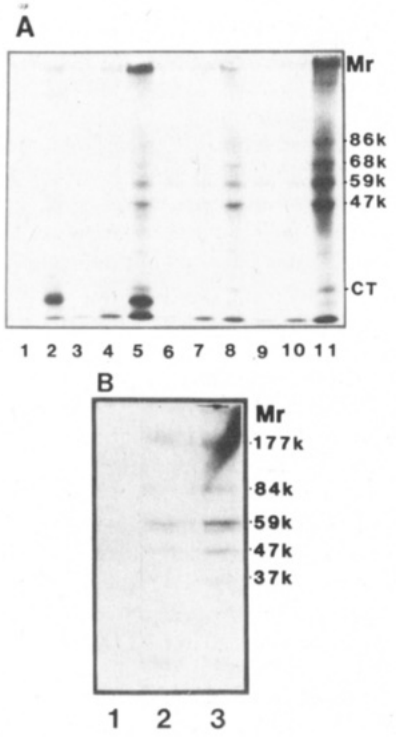

FIGURE 8: Cholera toxin labeling of adenylate cyclase preparations. Enzyme preparations were labeled with cholera toxin and [^{32}P]NAD as described under Methods. Some samples contained 10 μg of rat liver cytosol as indicated. (A) 80-μg samples were labeled, subjected to SDS gel electrophoresis, and analyzed by autoradiography: (1) membranes; (2) membranes + cholera toxin; (3) detergent extract; (4) detergent extract + liver cytosol; (5) detergent extract + liver cytosol + cholera toxin; (6) DEAE-Sephacel pool; (7) DEAE-Sephacel pool + liver cytosol; (8) DEAE-Sephacel pool + cytosol + cholera toxin; (9) CaM–Sepharose flow-through; (10) CaM-Sepharose flow-through + cytosol; (11) CaM–Sepharose flow-through + liver cytosol + cholera toxin. (B) An adenylate cyclase preparation (100 μg) purified through CaM–Sepharose was incubated with 50 μg of DEAE-Sephacel pool and labeled with cholera toxin and [^{32}P]NAD, and then the sample was purified on CaM–Sepharose. It was then run on an SDS gel and autoradiographed. (1) No cholera toxin; (2) cholera toxin; (3) cholera toxin + liver cytosol.

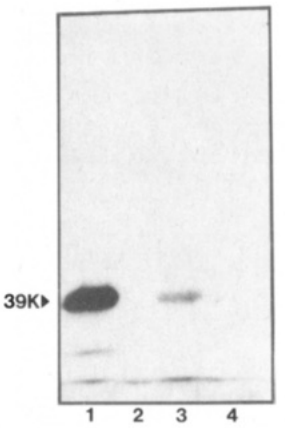

FIGURE 9: IAP labeling of CaM-sensitive adenylate cyclase. Adenylate cyclase purified by the method summarized in Table I (0.79 mL) was combined with 0.6 mL of IAP labeling buffer [6 mM sonicated DMPC, 200 mM Tris·HCl (pH 7.4), 2 mM ATP, 2 mM DTT, 2 mM EDTA, 100 μM NAD, 20 mM thymidine, and 10 mM $MgCl_2$], 0.015 mL of [^{32}P]NAD (2 mCi/mL), and either 0.35 mL of concentrated *B. pertussis* culture supernatant (136 μg of protein/mL) (lanes 1 and 3) or 0.35 mL of buffer A (lanes 2 and 4). The labeling mixtures were incubated for 60 min at 30 °C and then made 1 mM in NAD and precipitated with 0.17 mL of sodium deoxycholate and 0.17 mL of 72% trichloroacetic acid. The precipitates were pelleted by centrifugation for 30 min at 3500 rpm on a Sorvall RC3B centrifuge; the pellets were then washed with 1 mL of cold acetone and recentrifuged for 30 min. The acetone was removed by aspiration, and the residues were taken up in 150 μL of Laemmli sample buffer. The samples were subjected to SDS–polyacrylamide gel electrophoresis on a 7.5% slab gel. The gel was silver stained, vacuum dried, and autoradiographed. Lanes 1 and 2 represent adenylate cyclase (60 μg per lane) purified through the CaM–Sepharose chromatography step as described under Methods. Lanes 3 and 4 represent the enzyme (400 μg per lane) purified through the first heptanediamine–Sepharose step as described under Methods.

of ADP-ribosylated polypeptides. Major labeled polypeptides had molecular weights of 47 000, 59 000, 68 000, and 86 000 (Figure 8A). However, more highly purified preparations obtained subsequent to CaM–Sepharose yielded no ADP-ribosylated polypeptides. Since a protein factor is required for cholera toxin labeling of purified N_S from liver (Northup et al., 1980; Schleifer et al., 1982), we suspected that CaM–Sepharose chromatography may have removed the protein required for cholera toxin labeling. Therefore, a sample of enzyme purified through CaM–Sepharose was mixed with a small amount of the DEAE-Sephacel pool, labeled with cholera toxin and [^{32}P]NAD, and purified on CaM–Sepharose. Figure 8B shows the autoradiogram of this preparation following SDS gel electrophoresis. The two most prominent labeled bands had molecular weights of 47 000 and 59 000 with less prominent bands at 177 000, 84 000, 56 000, and 37 000 daltons. These data are consistent with those reported by Berthillier et al. (1983), who reported major cholera toxin substrates of 47 000 and 59 000 daltons from brain synaptosomal membranes. The only major polypeptide present in the most highly purified adenylate cyclase preparations that was a cholera toxin substrate was the 47 000-dalton band. Therefore, we suspect that this is the α subunit of N_s and that the 35 000-dalton polypeptide is the β subunit. SDS gels of the most highly purified enzyme preparation did not show equal staining of the 47K and 35K bands (Figure 6). However, GppNHp treatment may cause dissociation of the 35K subunit and result in nonstoichiometric levels of this polypeptide in the most highly purified preparation.

The most highly purified adenylate cyclase preparation was readily labeled with islet activating protein and [^{32}P]NAD (Figure 9). The only labeled polypeptide had a molecular weight of 39 000 on SDS gels which did not correspond to any of the polypeptides stained by Coomassie Blue or silver staining. Therefore, we feel that in contrast to the cholera toxin substrate, the IAP substrate is present only as a minor contaminant.

Determination of the Molecular Weight of the Enzyme Complex. The purified adenylate cyclase was obtained in 0.1% Lubrol PX, and the complex would be expected to contain a mixture of detergent and protein. Therefore, the general method of Clark (1975) for the determination of the molecular weights of the detergent-solubilized membrane proteins was used. It was discovered that the inclusion of Mn^{2+} in buffers used either for gel chromatography or for sucrose gradients converted the enzyme to a much smaller form than that observed without Mn^{2+}. Although it is not clear why Mn^{2+} caused the appearance of the smaller form of adenylate cyclase, it is noteworthy that Mn^{2+} does cause functional dissociation of N_s from the catalytic subunit in frog erythrocyte membranes (Limbird et al., 1979). The size of both forms of the enzyme was estimated. The Stokes radii determined by Bio-Gel A-5M chromatography were 75.4 and 52.5 Å for the large and small forms, respectively (Figure 10). Sucrose density gradient centrifugation of the purified adenylate cyclase was performed in both H_2O and D_2O. In the absence of Mn^{2+}, a $s_{20,w}$ value of 10.3 S was obtained with a partial specific volume ($\bar{V}$) of 0.75. In the presence of 10 mM $MnCl_2$, the $s_{20,w}$ was reduced

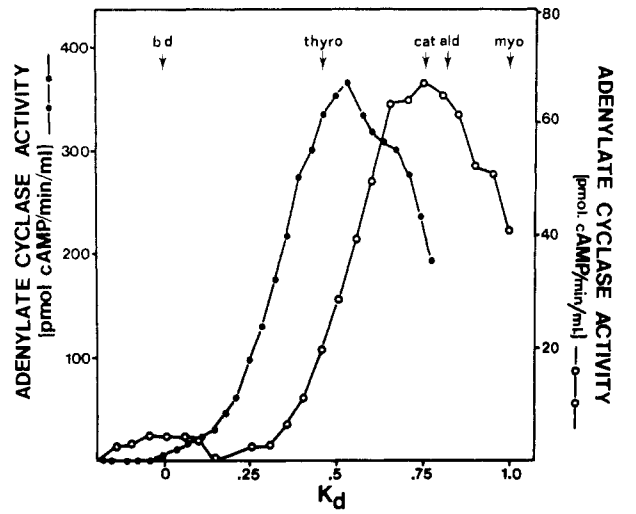

FIGURE 10: Determination of the Stokes radius of CaM-sensitive adenylate cyclase by gel filtration. The purified enzyme obtained from GppNHp-treated membranes was submitted to Bio-Gel A-5M chromatography in buffer A in the presence or absence of 10 mM $MnCl_2$. The larger form (Stokes radius 75.4 Å) was obtained without Mn^{2+} present; the smaller form (52.5 Å) was obtained with Mn^{2+} present. The void volume (bd) was determined by using blue dextran. The column was calibrated with thyroglobulin (r = 85 Å), catalase (r = 52 Å), aldolase (r = 47.4 Å), and myoglobin (r = 18.9 Å). This figure summarizes data from five separate experiments. (●) No Mn^{2+}; (○) 10 mM $MnCl_2$ present throughout column buffers.

Table IV: Hydrodynamic Properties of Calmodulin-Sensitive Adenylate Cyclase

	large form	small form[a]
$s_{20,w}$ (S)	10.3	7.4
partial specific volume	0.75	0.80
% Lubrol PX binding	7.0	31.0
Stokes radius[b] (Å)	75.4	52.5
M_r[c] (complex)	353000	220000
M_r[d] (adenylate cyclase)	328000	153000

[a] Determined in the presence of 10 mM Mn^{2+}. [b] Determined as described in the legend to Figure 10. [c] Molecular weight of the detergent–enzyme complex was determined by the method of Clarke (1975). [d] Molecular weight of the protein component was determined by assuming $\bar{V}$ = 0.73.

to 7.4 S and $\bar{V}$ was 0.80 (Table IV).

From these data and an assumption that $\bar{V}$ of the protein is typical ($\bar{V}$ = 0.73), one can calculate the molecular weight of the enzyme–detergent complex and the enzyme without detergent (Table IV). The apparent molecular weight of the large form was 328 000 and the small form 153 000. The major uncertainty in these determinations is the assumed $\bar{V}$ = 0.73. Since $\bar{V}$ can generally range from 0.70 to 0.75, the large form has a molecular weight of 328 000 ± 24 000 and the small form 153 000 ± 19 000. Neer & Salter (1981) have reported a Stokes radius of 54 Å and an apparent molecular weight of 130 000 for a small form of bovine brain adenylate cyclase obtained with impure Lubrol PX solubilized enzyme. This smaller form was obtained in the presence of ammonium sulfate. The preparations used in these two studies were significantly different; however, their molecular weights are within the experimental error inherent with these determinations.

Discussion

Although a highly purified preparation of the CaM-sensitive adenylate cyclase was obtained, the calculated turnover number was only about 80 min^{-1}. This indicates that either the preparation was not purified to homogeneity, that it is lacking an important component, or that the enzyme was damaged during purification. For example, the low turnover number may be due to the microenvironment of the detergent which only approximates the membrane lipid phase. SDS gels of the most highly purified preparation showed major bands at 35 000, 47 000, and 150 000 daltons. It is estimated that these polypeptides account for greater than 80% of the total protein present in the most highly purified preparations. Therefore, we feel that the protein has been purified extensively but that full activity is not expressed because of a missing component or inappropriate microenvironment for assay.

We have previously shown, using azido[^{125}I]iodoCaM, that the catalytic subunit of adenylate cyclase has a molecular weight of 150 000 (Andreasen et al., 1983b). In addition, we have been able to generate a small form of the enzyme under native conditions with an apparent molecular weight of 153 000. In preliminary studies, the 150 000-dalton polypeptide was isolated by SDS gel electrophoresis and used as an antigen to generate polyclonal anti-adenylate cyclase antibodies (G. B. Rosenberg et al., unpublished results). Therefore, we feel that the 150 000-dalton polypeptide present in our most highly purified preparations is very likely the catalytic subunit and that it binds one CaM per mole. It seems likely that the 47 000- and 35 000-dalton subunits are the α and β subunits of N_s since the purified enzyme was GppNHp sensitive (when prepared as summarized in Table II) and the 47 000-dalton subunit was a cholera toxin substrate. However, it is quite clear that the identity of none of these polypeptides has been unambiguously determined and stoichiometry between the stained bands on SDS gels has not been established.

The native molecular weight of the protein complex was 328 000 with a smaller form of 153 000 daltons produced in the presence of Mn^{2+}. The 328 000-dalton particle may be either a dimer of two catalytic subunits or a complex between the catalytic subunit and one or more N_s complexes. N_s has been reported to have a native molecular weight of 150 000 in Lubrol PX or 130 000 when the weight of bound detergent is substracted (Howlett & Gilman, 1980) or 70 000 in cholate (Sternweis et al., 1981). Our data do not distinguish between these two possibilities, but a physical association of N_s with the catalytic subunit seems more likely than a fortuitous co-purification of the two components. It is interesting in this respect that Levitzki's laboratory has recently shown that the turkey erythrocyte catalytic subunit is tightly associated with N_s through a substantial purification (Arad et al., 1984).

Acknowledgments

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Registry No. NaF, 7681-49-4; GppNHp, 34273-04-6; forskolin, 66575-29-9; adenylate cyclase, 9012-42-4.

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Epidermal Growth Factor from the Mouse. Physical Evidence for a Tiered β-Sheet Domain: Two-Dimensional NMR Correlated Spectroscopy and Nuclear Overhauser Experiments on Backbone Amide Protons†

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ABSTRACT: When H_2O-exchanged, lyophilized mouse epidermal growth factor (mEGF) is dissolved in deuterium oxide at low pH (i.e., below ~6.0), 13 well-resolved, amide proton resonances are observed in the downfield region of an NMR spectrum (500 MHz). Under the conditions of these experiments, the lifetimes of these amide protons in exchange for deuterons of the deuterium oxide solvent suggest that these amide protons are hydrogen-bonded, backbone amide protons. Several of these amide proton resonances show splittings (i.e., $J_{NH\alpha\text{-}CH}$) of approximately 8–10 Hz, indicating that their associated amide protons are in some type of β-structure. Selective nuclear Overhauser effect (NOE) experiments performed on all amide proton resonances strongly suggest that all 13 of these backbone amide protons are part of a single-tiered β-sheet structural domain in mEGF. Correlation of 2D NMR correlated spectroscopy data, identifying scaler coupled protons, with NOE data, identifying protons close to the irradiated amide protons, allows tentative assignment of some resonances in the NOE difference spectra to specific amino acid residues. These data allow a partial structural model of the tiered β-sheet domain in mEGF to be postulated.

The protein hormone, mouse epidermal growth factor (mEGF),[1] stimulates the growth and differentiation of various epidermal and epithelial tissues (Cohen, 1962, 1965; Cohen & Elliott, 1963; Turkington, 1969; Savage & Cohen, 1973). On a cellular level, mEGF works directly on skin cells by binding to a transmembrane receptor molecule and becoming internalized in the cell by endocytosis (McKanna et al., 1979; Carpenter & Cohen, 1976). Binding of mEGF to the receptor molecule in the presence of ATP also generates an enhanced phosphorylation of a number of membrane proteins; the formation of this mEGF–receptor complex (Hollenberg & Cuatrecasas, 1973; Carpenter et al., 1975) most likely activates a protein kinase that is one part of the mEGF receptor polypeptide chain (Buhrow et al., 1982, 1983). Physiological

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[1] Abbreviations: mEGF, mouse epidermal growth factor; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; NOE, nuclear Overhauser effect; FID, free-induction decay; 2D NMR COSY, two-dimensional NMR correlated spectroscopy; CIDNP, chemically induced dynamic nuclear polarization.

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