Direct Interaction between the Catalytic Subunit of the Calmodulin-Sensitive Adenylate Cyclase from Bovine Brain with ^{125}I-Labeled Wheat Germ Agglutinin and ^{125}I-Labeled Calmodulin†

Arda-e-viraf M. Minocherhomjee, Sara Selfe, Nancy J. Flowers, and Daniel R. Storm*
Department of Pharmacology, SJ-30, University of Washington, Seattle, Washington 98195
Received January 14, 1987; Revised Manuscript Received March 12, 1987

ABSTRACT: A calmodulin-sensitive adenylate cyclase has been purified to apparent homogeneity from bovine cerebral cortex using calmodulin–Sepharose followed by forskolin–Sepharose and wheat germ agglutinin–Sepharose. The final product appeared as one major polypeptide of approximately 135 000 daltons on sodium dodecyl sulfate–polyacrylamide gels. This polypeptide was a major component of the protein purified through calmodulin–Sepharose. The catalytic subunit was stimulated 3–4-fold by calmodulin (CaM) with a turnover number greater than 1000 min^{-1} and was directly inhibited by adenosine. The catalytic subunit of the enzyme interacted directly with ^{125}I-CaM on a sodium dodecyl sulfate–polyacrylamide gel overlay system, and this interaction was Ca^{2+} concentration dependent. In addition, the catalytic subunit was shown to directly bind ^{125}I-labeled wheat germ agglutinin using a sodium dodecyl sulfate–polyacrylamide gel overlay technique, and *N*-acetylglucosamine inhibited binding of the lectin to the catalytic subunit. Calmodulin did not inhibit binding of wheat germ agglutinin to the catalytic subunit, and the binding of calmodulin was unaffected by wheat germ agglutinin. These data illustrate that the catalytic subunit of the calmodulin-sensitive adenylate cyclase is a glycoprotein which interacts directly with calmodulin and that adenosine can inhibit the enzyme without intervening receptors or G coupling proteins. It is concluded that the catalytic subunit of adenylate cyclase is a transmembrane protein with a domain accessible from the outer surface of the cell.

Stimulation of adenylate cyclase by calmodulin (CaM)[1] was first reported by Brostrom et al. (1975) and Cheung et al. (1975). Bovine cerebral cortex contains both CaM-sensitive and CaM-insensitive forms of adenylate cyclase which can be resolved by CaM–Sepharose affinity chromatography (Brostrom et al., 1977; Westcott et al., 1979). Yeager et al. (1985) first reported the purification of a CaM-sensitive form of the enzyme from bovine cerebral cortex using CaM–Sepharose and heptanediamine–Sepharose chromatography. The enzyme was purified as a complex of the catalytic subunit and the guanyl nucleotide regulatory complex, G_S. The specific activity of the enzyme prepared by this method is 2–3 μmol of cAMP min^{-1} mg^{-1}, when stimulated by forskolin. Subsequently, Smigel (1986) and Pfeuffer et al. (1985) purified adenylate cyclase catalytic subunits (C) devoid of G_S using forskolin–Sepharose and wheat germ agglutinin–Sepharose chromatography. Although both of these investigators used forskolin–Sepharose affinity chromatography, the enzyme isolated by Pfeuffer et al. showed little or no sensitivity to CaM whereas the enzyme purified by Smigel showed stimulation by CaM. Since bovine brain contains a mixture of CaM-sensitive and -insensitive adenylate cyclases, characterization of the CaM-sensitive form of the enzyme necessitates the removal of the CaM-insensitive forms of the enzyme. In this paper, we report the purification and characterization of the CaM-sensitive catalytic subunit using CaM–Sepharose, forskolin–Sepharose, and wheat germ agglutinin–Sepharose chromatography.

The observation by Pfeuffer et al. (1985) and Smigel (1986) that the catalytic subunit of adenylate cyclases binds to wheat germ agglutinin–Sepharose suggests that the catalytic subunit of adenylate cyclase may be a glycoprotein, which has important implications concerning the orientation and integration of the catalytic subunit in the membrane. However, association of the catalytic subunit with WGA–Sepharose could be due to indirect interactions with a glycolipid or a glycoprotein contaminant. In this report, we show direct interaction between the catalytic subunit of CaM-sensitive adenylate cyclase with ^{125}I-WGA and ^{125}I-CaM on SDS gel overlay systems.

Materials and Methods

GppNHp, wheat germ agglutinin–agarose, and adenosine were obtained from P-L Biochemicals. ATP, cAMP, and protein standards were from Sigma. [α-^{32}P]- and [^{3}H]cAMP were purchased from New England Nuclear and International Chemical Nuclear, respectively. All other reagents were of the purest available grade from commercial sources. Forskolin–Sepharose was synthesized by the method of Pfeuffer et al. (1985).

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salomon et al. (1979) using [α-^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. Assays contained the following 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, 0.1% bovine serum albumin, and 5 mM $MgCl_2$ or 10 mM $MnCl_2$ as indicated. All results are presented as the mean of triplicate assays with standard errors of less than 5%.

Purification of Bovine Brain Adenylate Cyclase. The CaM-sensitive enzyme was purified by using CaM–Sepharose

† This work was supported by NIH Grant NS 20498.

[1] Abbreviations: CaM, calmodulin; WGA, wheat germ agglutinin; GppNHp, guanylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

0006-2960/87/0426-4444$01.50/0

followed by forskolin–Sepharose and wheat germ agglutinin–Sepharose (WGA–Sepharose) chromatography. The membranes were prepared as described by Yeager et al. (1985). Frozen bovine cerebral cortex (500 g), obtained from a local slaughterhouse, was fractured with a hammer and thawed in phosphate-buffer 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 blender (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 resuspended in an equal volume of homogenization buffer, and the Polytron homogenization, centrifugation, and resuspension steps were repeated 3 times.

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

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 DTT, 1 mM PMSF, and 0.1% Tween-20 (buffer A) was added to 5 L of detergent extract and stirred for 45 min. The anion-exchange resin was washed on a sintered glass funnel with 4 L of buffer A containing 50 mM KCl and then transferred to a column. A single protein peak containing the adenylate cyclase activity was eluted with buffer A containing 150 mM KCl. Under these conditions, CaM was not eluted from the column. The enzyme was pooled on the basis of adenylate cyclase activity (DEAE pool), 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 for 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 CaM–Sepharose chromatography.

The diluted enzyme (DEAE pool) solution 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 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 (CaM pool) and prepared for loading onto the forskolin–Sepharose column.

The CaM pool was concentrated to approximately 30 mL. NaCl and CHAPS were added to the concentrated pool at final concentrations of 0.5 M and 6 mM, respectively. The NaCl/CHAPS-containing CaM pool was loaded at 30 mL/h on the forskolin–Sepharose column (15 mL), equilibrated in buffer A containing 0.5 M NaCl and 6 mM CHAPS (substituted for 0.1% Tween-20) (buffer B). The column was washed with 250 mL of buffer B at 60 mL/h (forskolin flow-through). The enzyme was eluted with 100 mL of buffer B containing 0.075 mM forskolin (added as an ethanolic solution). The enzyme was pooled on the basis of adenylate cyclase activity (forskolin pool).

The forskolin pool was concentrated to 5–7 mL on a PM-10 Amicon ultrafiltration membrane, diluted with 3 volumes of buffer A without PMSF and sucrose, and loaded at 30 mL/h onto the wheat germ agglutinin–agarose column (10 mL) equilibrated in buffer A without PMSF. The column was then washed with 40 mL of equilibration buffer (WGA flow-through). The enzyme was eluted with 50 mL of the equilibration buffer containing 0.1 M *N*-acetyl-D-glucosamine (WGA pool). Both the WGA flow-through and the pool were concentrated on an Amicon PM-10 ultrafilration membrane to approximately 5 mL.

Iodocalmodulin Gel Overlay. Adenylate cyclase (WGA pool) was subjected to SDS–PAGE [7.5% acrylamide/0.17% bis(acrylamide)], fixed with 40% methanol/10% acetic acid, and washed with 10% ethanol overnight. The gel was washed with water and equilibrated with buffer C (50 mM imidazole hydrochloride, pH 7.5, 0.15 M NaCl, 1 mg/mL defatted bovine serum albumin, and 1 mM $CaCl_2$) for 30 min. The gel was incubated in buffer C containing ^{125}I-labeled CaM ± WGA (0.5 mg/mL) for 16 h. The gel was then washed at least 6 times with buffer C to remove nonspecifically bound ^{125}I-CaM. For control, 1 mM $CaCl_2$ was replaced by 1 mM EGTA. The gel was finally stained with Coomassie blue, dried, and autoradiographed.

Iodo-WGA Gel Overlay. The gel was treated as described for iodocalmodulin gel overlay; however, buffer C was replaced by buffer D (50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 1 mg/mL defatted BSA, and 1 mM $CaCl_2$). The gel was incubated with buffer D containing ^{125}I-WGA ± CaM (10 μM) for 16 h and washed, stained, dried, and autoradiographed as described above. For a control, 0.2 M *N*-acetyl-D-glucosamine was added to buffer D.

RESULTS

Purification of Calmodulin-Sensitive Adenylate Cyclase. We developed a new protocol for purification of the CaM-sensitive adenylate cyclase in order to isolate the catalytic subunit devoid of G_S with significant CaM sensitivity. This new purification scheme was necessary because the method of Yeager et al. (1985) yielded catalytic subunit associated with G_S. Furthermore, Smigel (1986) and Pfeuffer et al. (1985) both employed forskolin–Sepharose for purification of brain adenylate cyclase, but one preparation was CaM sensitive and the other showed little or no sensitivity to CaM. Since brain membrane probably contain mixtures of CaM-sensitive and CaM-insensitive adenylate cyclases, we felt it was important to resolve CaM-sensitive adenylate cyclase from CaM-insensitive forms of the enzyme to avoid characterizing mixtures of isozymes. The purification described in this study was similar to that published by Yeager et al. (1985) through CaM–Sepharose, except that Lubrol-PX used in chromatography buffers was replaced by Tween-20 and CaM–Sepharose was followed by forskolin–Sepharose and WGA–Sepharose. Tween-20 was used in place of Lubrol-PX in order to optimize chromatography on forskolin–Sepharose and WGA–Sepharose. As reported in Table I, only 20% of the enzyme bound to the CaM–Sepharose column, while the remaining activity flowed through CaM–Sepharose. The enzyme activity that did not bind to the CaM–Sepharose column was not stimulated by CaM/Ca^{2+} whereas the enzyme specifically eluted from the CaM–Sepharose column in the presence of EDTA was stimulated by CaM/Ca^{2+}. The adenylate cyclase preparation obtained after CaM–Sepharose chromatography provided significant purification of the enzyme with good yields (>10 mg) (Table I). The catalytic subunit of adenylate cyclase in the CaM–Sepharose pool was detectable as a major band of M_r 135 000 on SDS gels (Figure 1A). One of the major contaminants of the enzyme at this stage of the purification

Table I: Purification of Calmodulin-Sensitive Adenylate Cyclase from Bovine Brain Membrane[a]

purification step	total activity (nmol/min)	% total activity (membrane)	% total activity (CaM pool)	total protein (mg)	specific activity (nmol min^{-1} mg^{-1})	x-fold purification
membrane	1414	100		20350.0	0.694	1.0
Lubrol solubilized	1390	99		6858.0	2.03	2.92
DEAE-Sephadex	918	65		1170.0	7.85	11.3
CaM–Sepharose	178	13	100.0	11.25	158.0	227.0
forskolin (flow-through)	33		19.0	9.96	55.0	79.0
forskolin (pool)	25		14.0	0.08	3000.0	4403.0
WGA–Sepharose (flow-through)	13		7.0	0.046	4500.0	6476.0
WGA–Sepharose (pool)	6		3.5	0.032	1920.0	2763.0

[a] Membranes were pretreated with GppNHp (0.1 mM) for 30 min at 30 °C. Adenylate cyclase was assayed as described under Materials and Methods in the presence of 10 mM $MnCl_2$ and 5 μM CaM.

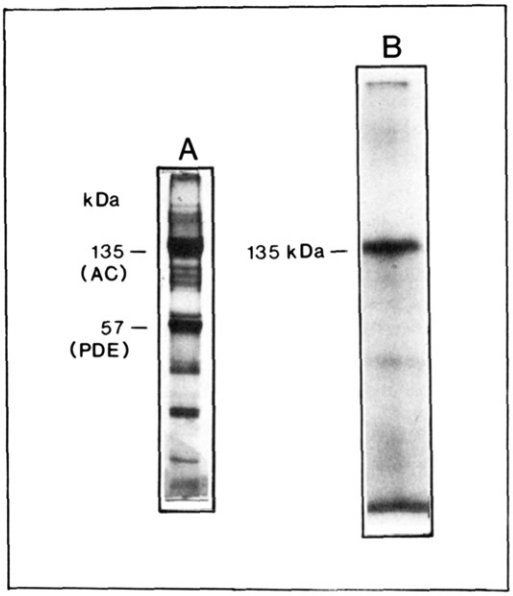

FIGURE 1: SDS–polyacrylamide gel of CaM-sensitive adenylate cyclase purified through CaM–Sepharose and WGA–Sepharose. (Left panel) Adenylate cyclase purified through CaM–Sepharose (10 μg) was electrophoresed on a 7.5% SDS–polyacrylamide gel and stained by Coomassie blue. (Right panel) SDS–polyacrylamide gel of adenylate cyclase purified through wheat germ agglutinin–Sepharose (Table I). The WGA pool (200 ng) was iodinated by using Chloroamine-T and electrophoresed on a 7.5% gel [7.5% acrylamide/0.17% bis-(acrylamide)]. The dried gel was exposed to film for 3 h. The molecular weight of adenylate cyclase is indicated.

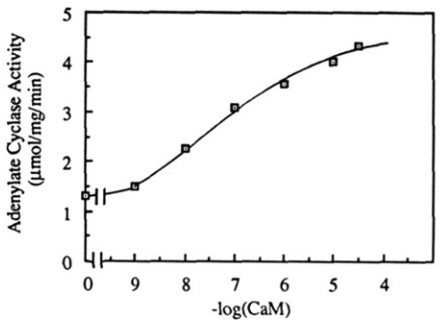

FIGURE 2: CaM stimulation of adenylate cyclase purified through WGA–Sepharose chromatography. Adenylate cyclase activity was measured at various concentrations of CaM in the presence of 5 mM $MgCl_2$. Free calcium was controlled by inclusion of 200 μM EGTA and 200 μM calcium in the assay buffer.

was a CaM-sensitive phosphodiesterase, M_r 57 000.

The CaM-sensitive adenylate cyclase was further purified by forskolin–Sepharose and wheat germ agglutinin–Sepharose chromatography. The final product appeared as one major band of approximately M_r 135 000 on SDS–PAGE (Figure 1B), although the molecular weight of this polypeptide varied considerably with the gel composition (135 000 ± 10 000). The only contaminating protein was the CaM-sensitive phosphodiesterase of M_r 57 000, and there was no detectable G_S α subunit present in the preparation. The absence of G_S α subunit from this preparation is consistent with the work of Smigel (1986) and Pfeuffer et al. (1985) which demonstrated that forskolin–Sepharose and wheat germ agglutinin–Sepharose can be used to prepare catalytic subunit free of G_S. The turnover number of the enzyme used in this study was greater than 1000 min^{-1}. It is noteworthy that the final yield was only a few micrograms of protein compared to milligram quantities obtained after CaM–Sepharose chromatography. Furthermore, the 19-fold increase in specific activity obtained after forskolin–Sepharose chromatography was due in part to stimulation of the enzyme by forskolin which was used to elute the enzyme from the affinity column. We estimate that the forskolin–Sepharose affinity column gave approximately 3-fold purification, with the addition of forskolin stimulating the enzyme about 6-fold. The advantage of this preparation over that reported by Yeager et al. (1985) is that the catalytic subunit was obtained unassociated with G_S.

Interaction of the Catalytic Subunit with ^{125}I-Calmodulin. The enzyme isolated by the procedure described above was stimulated 3–4-fold by CaM (Figure 2). Half-maximal stimulation occurred at approximately 10^{-7} M CaM; however, the log concentration–response curve for CaM stimulation of the catalytic subunit extended over more than 3 log units, suggesting heterogeneity of CaM binding sites or negative cooperativity between multiple binding sites. The CaM dose–response curve for this enzyme is comparable to that for the catalytic subunit associated with G_S (Keller et al., 1980).

Direct interaction of the catalytic subunit with CaM was shown by the ^{125}I-CaM gel overlay technique described under Materials and Methods. The catalytic subunit of CaM-sensitive adenylate cyclase interacted directly and specifically with CaM in the presence of Ca^{2+} but not in its absence (Figure 3). The contaminating CaM binding protein at M_r 57 000 which interacted with ^{125}I-CaM is a CaM-sensitive cAMP phosphodiesterase. The addition of unlabeled WGA (0.5 mg/mL) during the ^{125}I-CaM gel overlay had no effect on the ability of the catalytic subunit to bind ^{125}I-CaM. Furthermore, WGA had no effect on CaM stimulation of the enzyme. These results suggest that the CaM binding domain is not coincident with or overlapping with the lectin binding domain of the catalytic subunit.

It is interesting that Andreasen et al. (1983) had previously reported that ^{125}I-azido-CaM interacts directly with the catalytic subunit of adenylate cyclase in the presence of free Ca^{2+} but not in the absence of Ca^{2+}. These data are consistent with the observations that CaM stimulation of adenylate cyclase can occur in the absence of GTP (Heideman et al., 1982;

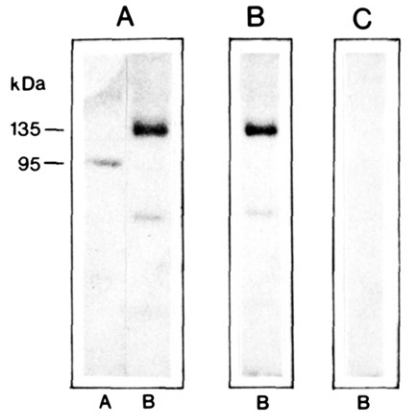

FIGURE 3: ^{125}I-CaM binding to the catalytic subunit of adenylate cyclase demonstrated by ^{125}I-CaM gel overlay. Adenylate cyclase (WGA pool) was subjected to SDS–PAGE [7.5% acrylamide/0.17% bis(acrylamide)], fixed with 40% methanol/10% acetic acid, and washed with 10% ethanol overnight. The gel was washed with water and equilibrated with buffer C (50 mM imidazole hydrochloride, pH 7.5, 0.15 M NaCl, 1 mg/mL defatted bovine serum albumin, and 1 mM $CaCl_2$) for 30 min. The gel was incubated in buffer C containing ^{125}I-CaM for 16 h. The gel was then washed 6 time with buffer C to remove nonspecifically bound ^{125}I-CaM. For control, 1 mM $CaCl_2$ was replaced by 1 mM EGTA. The gel was finally stained with Coomassie blue, dried, and autoradiographed. (Panel A) Lane A, molecular weight markers (myosin, 200 000; β-galactosidase, 116 250; phosphorylase *b*, 95 000; bovine serum albumin, 66 000; ovalbumin, 45 000); lane B, buffer C with Ca^{2+}, purified catalytic subunit with Ca^{2+} present. (Panel B) Purified catalytic subunit with Ca^{2+} and WGA (0.5 mg/mL) present. (Panel C) Purified catalytic subunit with EGTA substituted for Ca^{2+}.

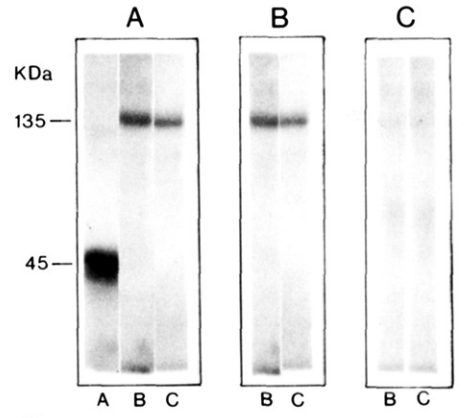

FIGURE 4: ^{125}I-WGA binding to the catalytic subunit of adenylate cyclase demonstrated by ^{125}I-WGA overlay. The gel was treated as described in Figure 3; however, buffer C was replaced by buffer D (50 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 1 mg/mL defatted BSA, and 1 mM $CaCl_2$). The gel was incubated with buffer D containing ^{125}I-WGA for 16 h and washed, stained, dried, and autoradiographed as described previously. For control, 0.2 M *N*-acetyl-D-glucosamine was added to buffer D. (Panel A) Lane A, molecular weight markers (same as in Figure 3); lane B, adenylate cyclase purified through forskolin–Sepharose; lane C, adenylate cyclase purified through WGA–Sepharose. (Panel B) Lane B, adenylate cyclase purified through forskolin–Sepharose with 10 mM CaM present; lane C, adenylate cyclase purified through WGA–Sepharose with 10 μM CaM present. (Panel C) Lane B, adenylate cyclase purified through forskolin–Sepharose with 0.2 M *N*-acetyl-D-glucosamine present; lane C, adenylate cyclase purified through WGA–Sepharose with 0.2 M *N*-acetyl-D-glucosamine present.

Seamon et al., 1982) and that impure preparations of brain adenylate cyclase resolved from G_S are CaM sensitive (Salter et al., 1981; Bitonti, 1982).

Interaction of the Catalytic Subunit with ^{125}I-Labeled Wheat Germ Agglutinin. Smigel (1986) and Pfeuffer et al. (1985) both observed that a catalytic subunit of adenylate cyclase bound to a wheat germ agglutinin–Sepharose column, suggesting that it may be a glycoprotein. Absorption to wheat germ agglutinin–Sepharose could, however, be due to association of the catalytic subunit with a glycolipid or a highly glycosylated protein contaminant. Furthermore, we found that wheat germ agglutinin had no effect on the activity of the enzyme or its sensitivity to CaM. Therefore, direct interaction between wheat germ agglutinin and the catalytic subunit of the CaM-sensitive adenylate cyclase was probed using ^{125}I-WGA and the SDS gel overlay technique described under Materials and Methods (Figure 4). The pure catalytic subunit of adenylate cyclase activity purified through CaM–Sepharose, forskolin–Sepharose, and WGA–Sepharose columns did interact with ^{125}I-WGA on an SDS gel overlay system. This interaction of the lectin with the catalytic subunit was specific. In addition, the presence of unlabeled CaM during the ^{125}I-WGA gel overlay had no effect on the ability of the catalytic subunit to bind ^{125}I-WGA (Figure 4B). These data are consistent with the results discussed above which suggested that the CaM binding domain (presumed to be on the inner membrane surface) does not overlap the lectin binding domain (presumed to be on the outer membrane surface). It appears that the catalytic subunit of the CaM-sensitive adenylate cyclase is a glycoprotein and that it therefore may be a transmembrane polypeptide.

Inhibition of Adenylate Cyclase by Adenosine. There is indirect evidence that "P"-type adenosine inhibition of adenylate cyclase may be due to interactions of adenosine with the catalytic subunit (Londos et al., 1979; Yeager et al., 1986), although this has not been directly demonstrated using pure

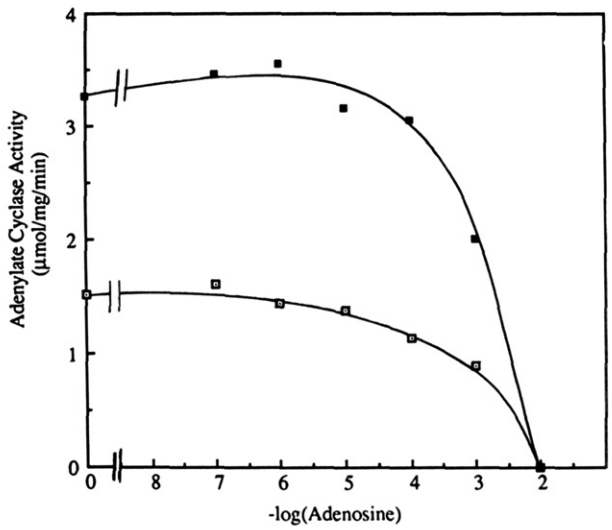

FIGURE 5: Dose–response curves for adenosine inhibition of the CaM-sensitive catalytic subunit of adenylate cyclase. Adenylate cyclase activity in the WGA flow-through (μ) and the WGA pool (μ) was measured in the presence of various concentrations of adenosine. The adenylate cyclase assay was performed in the presence of 10 mM $MnCl_2$.

catalytic subunit. The availability of pure catalytic subunit, without associated G_S, allowed direct examination of this question. As illustrated in Figure 5, adenosine directly inhibited the activity of the enzyme with an apparent K_d of about 1 mM. This K_d is somewhat higher than that observed for adenosine inhibition of the enzyme associated with G_S (Yeager et al., 1986) and suggests that G_S may have indirect affects on interactions of adenosine with the catalytic subunit. Wheat germ agglutinin (0.5 mg/mL) did not prevent adenosine inhibition of the purified enzyme. Thus, there are at least three molecules which can modify the activity of the CaM-sensitive adenylate cyclase by interacting directly with the catalytic subunit: CaM, forskolin, and adenosine.

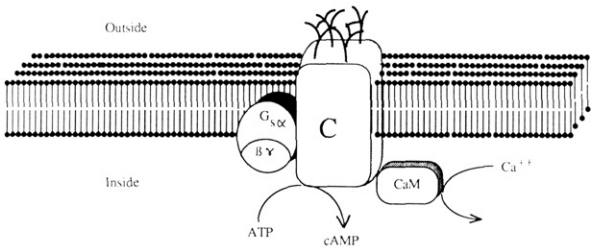

FIGURE 6: Model for the orientation of the CaM-sensitive adenylate cyclase in the membranes. It is proposed that the catalytic subunit of the calmodulin-sensitive adenylate cyclase from bovine brain is a transmembrane glycoprotein that interacts directly with CaM.

Discussion

In this study, we have used three affinity columns, CaM–Sepharose, forskolin–Sepharose, and WGA–Sepharose, to purify the catalytic subunit of the CaM adenylate cyclase in a CaM-sensitive form without associated components of G_S. It was our objective to determine if the catalytic subunit of this enzyme interacted directly with CaM, WGA, or adenosine. The enzyme was purified to a single polypeptide of M_r 135 000 on SDS gels with a specific activity of 2 μmol of cAMP min^{-1} mg^{-1}, and it was sensitive to CaM and adenosine. Although the yields of activity and total protein were inferior to the preparation described by Yeager et al. (1985), it was free of G_S and therefore more appropriate to examine direct interaction between the catalytic subunit and CaM or WGA. Since the yield of enzyme purified by forskolin–Sepharose and WGA–Sepharose is very low (Smigel, 1986; Pfeuffer et al., 1985), we explored interactions with the catalytic subunit and ^{125}I-CaM or ^{125}I-WGA on SDS gel overlay systems.

The purified catalytic subunit of the enzyme interacted directly with ^{125}I-CaM in the presence of free Ca^{2+}, and inclusion of Ca^{2+} chelators inhibited this interaction. The catalytic subunit bound directly to ^{125}I-WGA, and this interaction was blocked by *N*-acetyl-D-glucosamine. Binding of CaM was unaffected by WGA, and the binding of WGA was insensitive to CaM. Although SDS gels of the purified enzyme showed the absence of G_S, contamination of the catalytic subunit with G_S subunits would not affect the major conclusions of this paper since interactions between catalytic subunit and CaM or between catalytic subunit and WGA were directly detected by an SDS gel overlay system in which the catalytic subunit was resolved from any contaminating polypeptides. These data illustrate that the catalytic subunit of the CaM-sensitive adenylate cyclase is indeed a glycoprotein which binds CaM and adenosine without intervening regulatory subunits. We propose that the catalytic subunit of this enzyme is a transmembrane protein that extends across the membrane as depicted in Figure 6. This orientation of the enzyme exposes a domain of the catalytic subunit to the extracellular media and opens the potential for direct regulation of the enzyme by extracellular effectors without the involvement of G coupling proteins or specific receptors. In this respect, it is interesting that the catalytic subunit can be directly regulated by adenosine. The properties of the adenylate cyclase described in this study may be unique to a single isozyme of adenylate cyclase and should not necessarily be generalized to all adenylate cyclases. During the course of purifying this enzyme from brain, we have observed adenylate cyclase activities that exhibited no affinity for CaM–Sepharose or WGA–Sepharose.

References

Andreasen, T. J., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1983) *Biochemistry 22*, 2757–2762.

Bitonti, A. J., Moss, J., Hjelmland, L., & Vaughan, M. (1982) *Biochemistry 21*, 3650–3653.

Brostrom, C. O., Huang, Y. C., Breckenridge, B. M., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 64–68.

Brostrom, C. O., Brostrom, M. A., & Wolff, D. J. (1977) *J. Biol. Chem. 252*, 5677–5685.

Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun. 66*, 1055–1062.

Heideman, W., Wierman, B. M., & Storm, D. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1462–1465.

Keller, C. H., LaPorte, D. C., Toscano, W. A., Storm, D. R., & Westcott, K. R. (1980) *Ann. N.Y. Acad. Sci. 356*, 205–219.

Londos, C., Wolff, J., & Cooper, D. M. F. (1979) in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Baer, H. P., & Drummond, G. I., Eds.) pp 271–281, Raven Press, New York.

Pfeuffer, E., Moller, S., & Pfeuffer, T. (1985) *EMBO J. 4*, 3675–3679.

Salomon, Y., Londos, C., & Rodbell, M. (1979) *Anal. Biochem. 58*, 541–548.

Salter, R. S., Krink, M. H., Klee, C. B., & Neer, E. J. (1981) *J. Biol. Chem. 256*, 9830–9833.

Seamon, K. B., & Daly, J. W. (1982) *Life Sci. 30*, 1457–1464.

Smigel, M. D. (1986) *J. Biol. Chem. 261*, 1976–1982.

Westcott, K. R., LaPorte, D. C., & Storm, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 204–208.

Yeager, R. E., Heideman, W., Rosenberg, G. B., & Storm, D. R. (1985) *Biochemistry 24*, 3776–3783.

Yeager, R. E., Nelson, R., & Storm, D. R. (1986) *J. Neurochem. 47*, 139–144.