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Resolution and Activity of Adenylate Cyclase Components in a Zwitterionic Cholate Derivative [3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate]†

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ABSTRACT: Bovine brain adenylate cyclase was solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium cholate, sodium deoxycholate, or these detergents plus $(NH_4)_2SO_4$. The specific activity of the extract obtained with 13 mM CHAPS alone was several times those of the other detergent extracts with or without $(NH_4)_2SO_4$. After solubilization with 13 mM CHAPS, gel filtration completely separated the catalytic unit (C) from the guanine nucleotide binding protein (G/F). C activity when assayed with 5 mM Mn^{2+} was 5 times that assayed with 10 mM Mg^{2+} and was unresponsive to GPP(NH)P. C activity was increased ~150% by GPP(NH)P in the presence of G/F extracted from human erythrocyte ghosts and ~100% by Ca^{2+} plus calmodulin in assays with Mg^{2+}. On gel filtration and/or density gradient centrifugation, the physical properties of C from brain or AC^- cells and G/F from bovine or pig erythrocytes in CHAPs were similar to those observed in other detergents. It appears that the use of CHAPS for solubilization of adenylate cyclase and separation of C and G/F may well prove advantageous in studies of the molecular interactions between the protein subunits and activators of the enzyme as well as for the initial purification of C.

Hormone-sensitive adenylate cyclase is believed to consist of at least three protein components: a hormone receptor, the catalytic unit (C),[1] and a guanyl nucleotide binding protein (G/F). To study the regulatory interactions of the proteins, it is desirable to be able to separate each component from the others and subsequently reassemble them in artificial membranes. Other workers have separated C and G/F by extraction of plasma membranes with sodium cholate and high concentrations of $(NH_4)_2SO_4$, followed by gel filtration of the extracts (Strittmatter & Neer, 1980; Ross, 1981). High concentrations of $(NH_4)_2SO_4$, however, change the properties of cholate and are also severely inhibitory to adenylate cyclase. It would be advantageous to separate C and G/F in the absence of the salt.

The zwitterionic detergent CHAPS (Hjelmeland, 1980) was recently introduced as an alternative to both ionic detergents, such as cholate (Hoffman, 1979; Strittmatter & Neer, 1980; Ross, 1981) and deoxycholate (Londos et al., 1979; Hebdon et al., 1981), and nonionic detergents, such as Lubrol (Haga et al., 1977; Neer et al., 1980) and Triton (Neer et al., 1980), all of which have been used for solubilization of adenylate cyclase. We compared the effectiveness of CHAPS, sodium cholate, and sodium deoxycholate or these detergents plus $(NH_4)_2SO_4$ for solubilization of bovine brain adenylate cyclase and found that CHAPS was significantly better than either of the bile salts for dispersing adenylate cyclase from brain particulate fractions. As reported here, the use of CHAPS for solubilization of adenylate cyclase from bovine brain allows the separation of C from G/F by gel filtration, and in CHAPS the hydrodynamic properties of both C and G/F correspond quite closely to values obtained with other detergents.

Experimental Procedures

Solubilization of Brain Adenylate Cyclase. Fresh bovine brain was stored at −70 °C. For each experiment, a small piece of cortex was removed, thawed in cold 50 mM glycine buffer, pH 8.0, and homogenized by hand in 5 volumes of the same buffer. The homogenate was centrifuged for 10 min at 15000*g*. The pellet was washed twice with 20 volumes of 50 mM glycine (pH 8.0) containing 0.5 mM EGTA and once with 10 volumes of 50 mM glycine, pH 8.0 (with centrifugation at 30000*g* after each wash), and dispersed in the same buffer (final protein concentration 2 mg/mL). Detergent was added as indicated, and after 30 min on ice the mixture was centrifuged at 100000*g* for 60 min; the supernatant contained the solubilized adenylate cyclase.

Preparation of AC^- Membranes and Solubilization of C. AC^- cells, kindly provided by Dr. Alfred G. Gilman, were grown at 37 °C in Dulbecco's modified Eagle's medium

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[1] Abbreviations: C, catalytic unit; G/F, guanyl nucleotide binding protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; APP(NH)P, 5′-adenylyl imidodiphosphate; GPP(NH)P, guanylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

containing 10% horse serum (Ross et al., 1977). Cells (~3 × 10^6/mL) were harvested by centrifugation at 5000*g*, washed twice in 150 mM NaCl/2 mM $MgCl_2$/1 mM EDTA/20 mM Hepes buffer, pH 8.0, and broken in a cell disruption bomb (Parr Instrument Co.) after equilibration under 500 psi of N_2 for 30 min. The suspension of broken cells was centrifuged (750*g*, 5 min), and the supernatant was centrifuged at 25000*g* for 10 min. The membrane pellet was washed twice in 2 mM $MgCl_2$/1 mM EDTA/20 mM Hepes buffer, pH 8.0, and suspended in the same buffer (protein concentration 15–20 mg/mL). The membranes were used to assay erythrocyte G/F (Figure 1) or were solubilized by adding CHAPS to a final concentration of 13 mM. After 30 min on ice, the mixture was centrifuged at 100000*g* for 60 min. The supernatant containing solubilized C was used for gel filtration or sucrose density gradient analysis (Figure 2).

Preparation of Erythrocyte Ghosts and Partial Purification of Solubilized G/F. Fresh human or pig blood anticoagulated with EDTA was centrifuged (5000*g*, 10 min). Plasma and buffy coat were discarded. The packed erythrocytes were washed twice with 150 mM NaCl/10 mM Tris, pH 7.5, and lysed in 20 volumes of 10 mM Tris, pH 7.5. The ghosts were washed extensively with the same buffer either by repeated centrifugation at 25000*g* or by the use of a Pellicon Cassette System (Millipore Corp.) equipped with a membrane filter (no. PTHK 000 05) and stored at −70 °C. G/F activity in these membranes was stable for at least 3 months. G/F was solubilized by adding CHAPS (final concentration 13 mM) to thawed ghosts. After 30 min on ice, the mixture was centrifuged at 100000*g* for 60 min. The supernatant (protein concentration ~2.5 mg/mL) contained G/F activity.

Pig erythrocyte G/F was partially purified by mixing the solubilized preparation with an equal volume of DE52 which had been previously equilibrated with 13 mM CHAPS/10 mM Tris, pH 7.5. The supernatant, which contained no G/F activity, was discarded, and the DE52 was washed 3 times with equal volumes of 13 mM CHAPS/10 mM Tris, pH 7.5. G/F was eluted with 200 mM NaCl in the same buffer. This partially purified G/F could be stored at 4 °C for 2–3 weeks without significant loss of activity.

Adenylate Cyclase Assay. Assays unless otherwise noted contained 0.1 mM ATP, (4–5) × 10^5 cpm of [α-^{32}P]ATP, 50 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM theophylline, 3 mM phospho(enol)pyruvate, 1 unit of pyruvate kinase, 0.1 mg/mL bovine serum albumin, 0.1 mM ascorbate, and enzyme as indicated in a total volume of 0.1 mL. After incubation for 20 min at 30 °C, 0.1 mL of a solution containing 40 mM ATP, 1.4 mM cAMP, 2% sodium dodecyl sulfate, 50 mM Tris (pH 7.5), and (5–6) × 10^3 cpm [^{3}H]cAMP was added, and cAMP was isolated for radioassay (Salomon et al., 1974).

So that solubilized C or G/F could be combined with erythrocyte ghosts or AC$^-$ membranes, respectively, the two components were mixed and kept on ice for 30 min before cyclase assays were initiated.

For addition of phospholipid to cyclase assays, phosphatidylcholine (in chloroform/methanol, 9:1) was taken to dryness under a stream of N_2. Lysophosphatidylcholine was added to the same tube, and both lipids were dispersed in water with a small glass rod and a Vortex mixer. Samples (5 μL) of this suspension were added to samples of adenylate cyclase in assay tubes, and after 20 min on ice assays were initiated. A mixture of phosphatidylcholine (5 mg/mL in the assay) and lysophosphatidylcholine (1 mg/mL in the assay) produced maximal enhancement of adenylate cyclase activity in assays containing ~2.5 mM CHAPS.

Table I: Solubilization of Brain Adenylate Cyclase with with Detergents[a]

detergent, concn	additions to assay: PL[b]	GPP-(NH)P[c]	CaM[d]	adenylate cyclase [pmol min^{-1} (mg of protein)$^{-1}$]: undiluted	diluted
CHAPS, 13 mM	0	0	0	200 ± 2.9	100 ± 6.2[e]
	+	0	0	490 ± 51	460 ± 49
	0	+	0	220 ± 37	96 ± 20
	+	+	0	583 ± 34	580 ± 87
	0	0	+	390 ± 11	
	+	0	+	630 ± 31	
deoxycholate, 12 mM	0	0	0	0.13 ± 0.086	9.1 ± 1.4
	+	0	0	21 ± 2.9	140 ± 5.7
	0	+	0	1.6 ± 0.37	18 ± 13
	+	+	0	170 ± 20	420 ± 31
	0	0	+	0.26 ± 0.15	
	+	0	+	60 ± 4.1	
cholate, 15 mM	0	0	0	2.9 ± 1.0	6 ± 1.6
	+	0	0	20 ± 1.1	23 ± 2.0
	0	+	0	6.4 ± 1.3	2.3 ± 0.5
	+	+	0	33 ± 3.1	27 ± 2.5
	0	0	+	11 ± 1.3	
	+	0	+	49 ± 0.95	

[a] Adenylate cyclase was solubilized with the indicated detergent. Samples (20 μL) of undiluted supernatant or of supernatant diluted with 2 volumes of 50 mM glycine buffer, pH 8.0, were assayed. Supernatant protein concentrations were the following: CHAPS, 2.3 mg/mL; deoxycholate, 2.8 mg/mL; and cholate, 1.4 mg/mL. The data with each detergent were obtained in experiments run at the same time on the same sample of brain. [b] Phosphatidylcholine, 5 mg/mL, plus lysophosphatidylcholine, 1 mg/mL. [c] 100 μM GPP(NH)P. [d] Calmodulin, 1 μg/assay plus 50 μM Ca^{2+}. [e] Standard deviation based on triplicate assays.

Materials. Phosphatidylcholine (egg yolk), lysophosphatidylcholine (egg yolk), and sodium deoxycholate were purchased from Sigma; sodium cholate and CHAPS were from Calbiochem; GPP(NH)P, APP(NH)P, and [α-^{32}P]APP(NH)P were from ICN; [α-^{32}P]ATP and [^{3}H]cAMP were from New England Nuclear. Calmodulin was the gift of Dr. Randall L. Kincaid, who prepared it. Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Solubilization of Brain Adenylate Cyclase. Solubilization of brain adenylate cyclase was optimal with concentrations of CHAPS between 6.5 and 13 mM (particulate protein concentration ~2 mg/mL). Activities in these extracts were optimal when assays contained 2.6 mM CHAPS; less activity was observed when the CHAPS concentration was <1 mM or >4 mM.

Specific activities of brain adenylate cyclase solubilized with 13 mM CHAPS were much higher than those in extracts prepared with 12 mM deoxycholate or 15 mM cholate. Dilution of extracts before assay increased specific activity of cholate or deoxycholate extracts but decreased that in CHAPS extracts as the concentration of CHAPS in the assay was decreased from 2.6 to 0.87 mM (Table I). Inclusion of phosphatidylcholine and lysophosphatidylcholine in assays increased activity of all preparations. In assays with phospholipids, the activity of all extracts was enhanced by GPP(NH)P or by calmodulin plus calcium. The relative magnitude of these effects was greater with deoxycholate extracts than with the other two detergents. Under all assay conditions, however, the specific activity of the CHAPS extracts was highest (Table I). Deoxycholate added to assays inhibited CHAPS-solubilized C (data not shown).

Table II: Solubilization of Brain Adenylate Cyclase with Detergents and Ammonium Sulfate[a]

detergent, concn	AMS (0.5 M)	extract protein (mg mL^{-1})	PL in assay[b]	adenylate cyclase [pmol min^{-1} (mg of protein)$^{-1}$] undiluted	diluted
CHAPS,	0	1.8	0	280 ± 35[c]	125 ± 12
13 mM	0		+	490 ± 31	740 ± 163
	+	2.7	0	25 ± 4.6	81 ± 10
	+		+	63 ± 17	190 ± 21
deoxy-	0	3.0	0	0.44 ± 0.10	7.8 ± 0.44
cholate,	0		+	25 ± 2	89 ± 4.6
11 mM	+	1.9	0	2.8 ± 0.75	19 ± 1.4
	+		+	15 ± 2.2	36 ± 6.5
cholate,	0	0.9	0	6.4 ± 1.0	4.3 ± 0.80
15 mM	0		+	33 ± 5.4	30 ± 5.1
	+	3.1	0	5.6 ± 0.06	26 ± 3.3
	+		+	31 ± 3.7	110 ± 4.1

[a] Adenylate cyclase was solubilized with detergent with or without 0.5 M $(NH_4)_2SO_4$ (AMS) as indicated. Samples of supernatant (extract) were assayed with or without dilution as described in Table I. [b] Phosphatidylcholine, 5 mg/mL, plus lysophosphatidylcholine, 1 mg/mL. [c] Standard deviation of triplicate assays.

Table III: Activity of Solubilized C from Brain after Gel Filtration[a]

additions to assay G/F	GPP(NH)P	CaM	adenylate cyclase activity [pmol min^{-1} (mg of protein)$^{-1}$] 10 mM $MgCl_2$	5 mM $MnCl_2$
0	0	0	200 ± 15[b]	950 ± 123
0	+	0	240 ± 18	690 ± 36
0	0	+	390 ± 44	1300 ± 59
+	0	0	230 ± 21	1000 ± 100
+	+	0	500 ± 56	950 ± 102
+	+	+	690 ± 30	1040 ± 75

[a] Brain adenylate cyclase solubilized with 13 mM CHAPS was chromatographed on a column (0.9 × 45 cm) of Ultrogel AcA 34 as described in Figure 1. Fractions containing C (corresponding to 32-36 in Figure 1) were pooled, and samples (30 μL, approximately 3 μg of protein) were taken for assay with $MgCl_2$ or $MnCl_2$ and other additions as indicated. All assays contained phospholipids as described in Table I. Concentrations of GPP(NH)P and CaM (calmodulin plus calcium) were as described in Table I. For assays with G/F, samples of C were combined with solubilized human erythrocyte ghosts (50 μg of protein). [b] Standard deviation based on triplicate assays.

The presence of 0.5 M $(NH_4)_2SO_4$ during extraction with CHAPS solubilized somewhat more protein, but specific activities (assayed with or without phospholipids) were lower than those of extracts without $(NH_4)_2SO_4$ (Table II). Although all activities were lower with deoxycholate as the detergent, similar effects of $(NH_4)_2SO_4$ were noted when samples were assayed with phospholipid; in assays without phospholipid, extracts with $(NH_4)_2SO_4$ had higher activity than those without. With cholate, on the other hand, when assayed after dilution, with or without phospholipid, specific activities of extracts containing $(NH_4)_2SO_4$ were higher than those without. The latter contained only about one-third as much protein as those prepared with cholate and ammonium sulfate (Table II).

Gel filtration of CHAPS-solubilized brain adenylate cyclase on Ultrogel AcA 34 separated C from G/F (Figure 1). C activity (in the presence of phospholipids), when assayed with Mg^{2+}, was increased by ~100% by calmodulin plus calcium and ~150% by GPP(NH)P plus G/F but not by GPP(NH)P alone. Addition of calmodulin, calcium, GPP(NH)P, and G/F increased activity ~250% (Table III). In assays with Mn^{2+}, activities were higher than they were with Mg^{2+}, but effects of additions were relatively small (Table III).

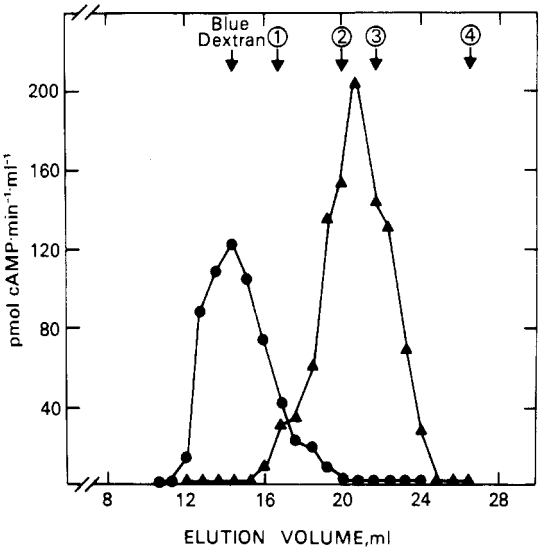

FIGURE 1: Separation of C and G/F solubilized from brain. A sample (0.6 mL, 1.5 mg of protein) of solubilized brain particulate (13 mM CHAPS) containing calibrating enzymes was applied to a column (0.9 × 45 cm) of Ultrogel AcA 34 previously equilibrated and eluted with 13 mM CHAPS/100 mM NaCl/0.02% NaN_3/50 mM glycine buffer, pH 8.0. Samples (30 μL) of fractions (0.4 mL) were assayed for C activity in the standard adenylate cyclase assay for 5 mM $MnCl_2$ and phosphatidylcholine, 5 mg/mL, plus lysophosphatidylcholine, 1 mg/mL (●). Other samples (30 μL) were combined with AC⁻ membranes (320 μg of protein) and assayed with 10 mM $MgCl_2$ and 100 μM GPP(NH)P (▲) to determine G/F activity. The fractions were also assayed with 10 mM $MgCl_2$ and 100 μM GPP(NH)P in the absence of AC⁻ membranes, and these values were subtracted from the values obtained in the presence of AC⁻ membranes. Calibrating enzymes (Stokes radius in parentheses) included (1) β-galactosidase (6.84 nm), (2) lactate dehydrogenase (4.75 nm), (3) malate dehydrogenase (3.69 nm), and (4) cytochrome *c* (1.87 nm).

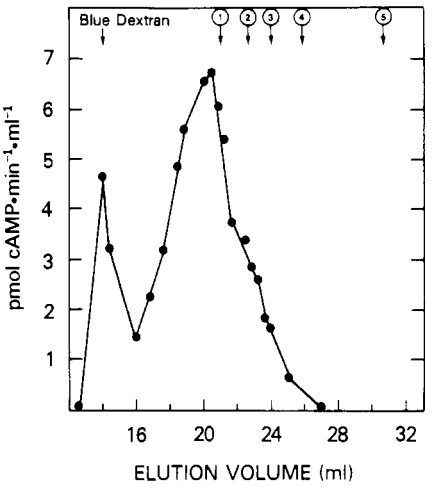

FIGURE 2: Gel filtration of C solubilized from AC⁻ membranes with CHAPS. A 1-mL sample of membranes (solubilized containing calibrating enzymes) was applied to a column (0.9 × 47 cm) of Sepharose 6B (Pharmacia) previously equilibrated with 13 mM CHAPS/2 mM $MgCl_2$/1 mM EDTA/20 mM NaCl/20 mM Hepes, pH 8.0. During elution with the same buffer (8 mL/h), 0.2-mL fractions were collected. Samples (40 μL) were assayed after combination with solubilized human erythrocyte ghosts (105 μg of protein). Calibrating enzymes (Stokes radius in parentheses), (1) β-galactosidase (6.84 nm), (2) catalase (5.21 nm), (3) lactate dehydrogenase (4.75 nm), (4) malate dehydrogenase (3.69 nm), and (5) cytochrome *c* (1.87 nm), were assayed by standard methods (Haga et al., 1977).

Solubilization of C from AC⁻ Membranes. C, solubilized from AC⁻ membranes with 13 mM CHAPS and subjected to gel filtration on Sepharose 6B (Figure 2), had a Stokes radius

of 7.4 nm, a value in reasonable agreement with those reported for C in Lubrol 12A9 extracts of AC^- cells (Ross et al., 1978) or brain (Neer et al., 1980). On sucrose density gradient centrifugation, the sedimentation coefficient ($s_{20,w}$) was 8.8 S, similar to those observed by others (Ross et al., 1978; Neer et al., 1980).

Solubilization of G/F from Erythrocyte Membranes. G/F, solubilized from pig erythrocyte membranes with 13 mM CHAPS and chromatographed on Ultrogel AcA 34, had a Stokes radius of 4.2 nm corresponding to a molecular weight of ~135 000 as reported by Northup et al. (1980). Increasing the concentration of CHAPS above 13 mM did not decrease the apparent molecular size of G/F (or of C from AC^- membranes), whereas with lower concentrations of CHAPS these activities were associated with aggregates of larger size.

Discussion

The separation of C and G/F by gel filtration after solubilization with the zwitterionic detergent CHAPS is a significant improvement over existing procedures. Both C and G/F are obtained in active form and can be assayed more easily and accurately in CHAPS than in the presence of bile salts and high concentrations of $(NH_4)_2SO_4$. To show that brain C obtained by gel filtration on Ultrogel AcA34 is free of G/F, we have used essentially the same criteria as Ross (1981). C activity assayed with Mn^{2+} was 4–5 times that observed with Mg^{2+}. Activity was not stimulated by GPP(NH)P alone, but, in assays with Mg^{2+}, the addition of GPP(NH)P plus exogenous G/F from solubilized human erythrocyte ghosts increased activity ~150%. Calmodulin plus Ca^{2+} enhanced C activity ~100% in assays with Mg^{2+}, albeit only 35% in assays with Mn^{2+}. These findings are consistent with the possibility that calmodulin interacts directly with C, although other workers (Toscano et al., 1979) have concluded that G/F is probably necessary for the activation of adenylate cyclase by calmodulin.

The C obtained by the procedure described here from brain and AC^- cells and the G/F from brain and pig erythrocytes are very similar in hydrodynamic properties to preparations obtained with other detergents (Ross et al., 1978; Neer et al., 1980). CHAPS-solubilized adenylate cyclase also appears to be functionally similar to adenylate cyclase extracted with cholate (Strittmatter & Neer, 1980) or deoxycholate (Hebdon et al., 1981). Although the specific activity of adenylate cyclase in CHAPS extracts is considerably greater than that in other detergents tested, the enzymes respond similarly to GPP(NH)P, calmodulin, and phospholipids.

The reason that greater adenylate cyclase activity is observed with CHAPS than with cholate or deoxycholate is still obscure but may be related to the differences between the polar groups of CHAPS and the bile salt anions (cholate and deoxycholate). The bile salts have the potential for interaction with cationic groups on proteins, whereas CHAPS has no net charge at the pH used in these studies. Ionic interaction between the bile salts and adenylate cyclase may explain the inhibitory effects on these detergents. This may also explain why $(NH_4)_2SO_4$ increases the adenylate cyclase activity measured with cholate but not with CHAPS. The interaction of cationic groups in protein molecules and the negatively charged detergent is dependent on ionic strength. Increasing ionic strength would reduce interactions between the ionic groups and thereby permit expression of more adenylate cyclase activity. Increasing ionic strength would not be expected to improve CHAPS-solubilized adenylate cyclase activity, and in fact, $(NH_4)_2SO_4$ proved to be inhibitory. It appears that the use of CHAPS for solubilization of adenylate cyclase and separation of C and G/F may well prove advantageous in studies of the molecular interactions between the protein subunits and activators of the enzyme as well as for the initial purification of C.

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