Structure of Brain Adenylate Cyclase: Proteolysis-Dependent Modifications†

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ABSTRACT: The associations of the components of eucaryotic adenylate cyclase are still poorly characterized. Enzyme activity is, however, thought to depend upon subunit conformations and states of association. Estimates of adenylate cyclase sizes corresponding to given levels of activity may thus give clues as to how the enzyme functions. Studying the rat brain enzyme, we found that samples protected from proteolysis throughout the fractionation procedure yielded, upon Lubrol solubilization, a soluble protein complex of 9.1S sedimentation coefficient and 11.5-nm Stokes radius. These values are much larger than those previously reported. The soluble enzyme specific activity, *but not its size*, was dependent upon the various effectors preincubated with the membranes. Proteolysis is known to first activate and then decrease adenylate cyclase activity. Proteolysis of the brain samples, whether due to trypsin or to endogeneous proteases, decreased the adenylate cyclase *s* value, Stokes radius, and specific activity altogether. The magnitude of the shifts depended upon the nature of the enzyme effector preincubated with the membranes. We recently showed that some brain membrane proteins can be ADP-ribosylated by cholera toxin, concomitantly with adenylate cyclase activation [Berthillier, G., d'Alayer, J., & Monneron, A. (1982) *Biochem. Biophys. Res. Commun. 109*, 297–304]. Trypsin treatment of such samples led to a quick degradation of the labeled polypeptides and especially of the M_r 47000 protein. This Lubrol soluble protein is likely to be the brain G/F stimulatory subunit.

Eucaryotic plasma membranes all contain adenylate cyclase. This enzyme is known to be a multicomponent complex, of which only one set of regulatory proteins, the so called G/F subunits, is fairly well-known (Sternweis et al., 1981). Attempts to purify the catalytic subunit have yielded promising but very preliminary results (Pfeuffer & Metzger, 1982), while biochemical evidence for the existence of inhibitory subunits is just starting to appear (Hanski & Gilman, 1982; Katada & Ui, 1982; Smith & Limbird, 1982). Thus, the regulation of adenylate cyclase has been tentatively related to changes in protein conformation and/or state of association of the components (Rodbell, 1980; Ross & Gilman, 1980; Limbird, 1981). Indeed, much work has been devoted toward determining the size and composition of adenylate cyclase complexes in the presence of various enzyme effectors: hormones or ligands of the receptors, GTP or its nonhydrolyzable analogues such as Gpp(NH)p, NaF. The problem has been approached with both the membrane-bound and the detergent-solubilized enzyme. However, no clear picture of the enzyme's organization has of yet been obtained. Even within the same species, and for one given tissue, adenylate cyclase in one defined state of activity has been assigned different sizes by various laboratories by using different or even the same methods. Varying the experimental conditions chosen for tissue or cell disruption may indeed result in selective extraction of some adenylate cyclase components (Sahyoun et al., 1981a,b; Rasenick et al., 1981).

In this investigation, we tried to correlate the experimental conditions (sample preparation and preincubation with different enzyme effectors, solubilization, and fractionation) with the apparent sizes of the solubilized enzyme complexes. We chose to study the brain enzyme because it has a high specific activity and because it mediates at least some of the effects of various neurotransmitters (Daly, 1977). As a result we found that attempts to protect the enzyme from proteolytic degradation largely affected the properties of the solubilized enzyme. The enzyme obtained from samples carefully protected from proteolysis was larger than that expected from published data. Its size was independent from the level of activity of the membrane-bound enzyme, no matter which effector was present. Published data, on the contrary, referred to complexes of much smaller sizes, both the *s* value and specific activity of which depended on the effector present (Pfeuffer, 1979; Goldhammer et al., 1980; Neer et al., 1980; Guillon et al., 1981). We found similar results when we studied proteolyzed material. Since the very active brain enzyme species is almost twice as large as the lesser active forms, we believe that this large size results not only from the absence of proteolysis of the components existing in the smaller sized enzyme (G/F and catalytic subunits) but also from the association of additional subunits.

Materials and Methods

Preparation and Solubilization of Synaptosomes. Three-week-old Wistar rats were decapitated; their brains were chilled and homogenized in an ice-cold solution containing 50 mM Tris-HCl,[1] pH 7.5, 5 mM $MgCl_2$, 10% (w/v) sucrose, and protease inhibitors: 0.3 mM phenylmethanesulfonyl fluoride, 10 μM benzethonium chloride, 1 mg of pepstatin A, and 1 mg of kallikrein–trypsin inhibitor/g of brain tissue. Rat brain synaptosomes were prepared by flotation in Ficoll–sucrose gradients (Monneron & d'Alayer, 1980a). Synaptosomes were lysed by incubation with magnetic stirring for 0.5 h in a cold (4 °C) 1 mM triethanolamine buffer, pH 8.1, containing 2 mM dithiothreitol, 1 mM EDTA, and 0.2 mM ATP. Synaptosomal membranes were collected by centrifugation. Synaptosomes and synaptosomal membranes were solubilized

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; *r*, Stokes radius; Tg, thyroglobulin; β-Gal, β-galactosidase; Cat, catalase; ADH, alcohol dehydrogenase; MDH, malate dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Adenylate Cyclase Specific Activities (nmol of cAMP mg^{-1} min^{-1}) from One Typical Fractionation

	divalent ion concentration in the assay					
	10 mM $MgCl_2$		10 mM $MgCl_2$ + 2 mM $MnCl_2$		10 mM $MgCl_2$ + 2 mM $MnCl_2$ + 0.2 M $(NH_4)_2SO_4$	
	basal activity	Gpp(NH)p stimulation factor	basal activity	Gpp(NH)p stimulation factor	basal activity	Gpp(NH)p stimulation factor
synaptosomes	0.34	×1.6	0.42	×2	0.66	×2.7
synaptosomal membranes	0.47	×1.8	0.77	×1.7	1.3	×2.4
unfractionated Lubrol supernatant	1.1	×2.3	3.5	×1.5	6.6	×2
9S fraction from the gradient	2.75	×3.3	11	×1.2	14.6	×1.7
peak enzymatic fraction from the Bio-Gel column	4.1	×1.8	16	×2	22	×1.6

in Lubrol-PX by using a Potter glass–Teflon homogenizer (40 strokes, at 0 °C). Unless otherwise stated, the solubilization medium ("A" medium) contained 0.9% Lubrol [Lubrol/protein ratio (w/w) = 2], 2 mM dithiothreitol, 1 mM EDTA, and divalent ions as indicated in the text (usually 1 mM $MgCl_2$). Samples were centrifuged at 45000*g* for 10 min at 4 °C, so as to remove all insoluble membrane remnants. The supernatants were used as the source of the enzyme. When stored, the samples were frozen in liquid nitrogen.

Ultracentrifugation and Gel Filtration of Solubilized Membrane Proteins. The Lubrol supernatants were mixed with calibrating proteins: porcine thyroglobulin (s = 19.3 S; r = 9.8 nm); β-galactosidase (s = 15.9 S; r = 8.2 nm); catalase (s = 11.3; r = 5.2 nm); alcohol dehydrogenase (s = 7.4 S; r = 4.6 nm); malate dehydrogenase (s = 3.6 S; r = 3.3 nm). A sample 0.4–0.8 mL of supernatant was layered on 12.2-mL linear, 2–4.5% or 10–18% sucrose gradients containing, unless otherwise stated, 50 mM triethanolamine, pH 8.1, 2 mM dithiothreitol, 1 mM EDTA, 1 mM $MgCl_2$, and 0.05% Lubrol-PX. The gradients were centrifuged in a Beckman rotor SW 41 at 288000*g* at 4 °C for 12.5 h (2–4.5%) or 24 h (10–18%); 350-μL fractions were collected.

Gel filtration of the soluble extract was performed through a Bio-Gel A-5m column (1.9 × 85 cm) buffered with 50 mM triethanolamine, pH 8.1, containing 2.5% sucrose, 2 mM dithiothreitol, 1 mM EDTA, 1 mM $MgCl_2$, 0.05% Lubrol, and 0.02% N_3Na. Three milliliters of Lubrol extract containing calibrating proteins was layered on top of the column. The flow rate was 10 mL/h at 4 °C. Samples of 1.4 mL were collected. Stokes radii were calculated according to Bon et al. (1973).

Trypsin Digestion. Trypsin dissolved in triethanolamine buffer was added to membranes or to Lubrol supernatants equilibrated at 30 °C, at a final concentration of 18 μg/mL (10 μg of trypsin for 1 mg of membrane protein). After 1–4 min at 30 °C, proteolysis was stopped by addition of soybean trypsin inhibitor, 3 μg/μg of trypsin. Control samples were treated with a mixture of preincubated trypsin and trypsin inhibitor.

Other Assays. ADP-ribosylation of membranes by cholera toxin was performed according to Johnson et al. (1978), with modifications (Berthillier et al., 1982). For gel electrophoresis, 10% or gradient 7.5–12% acrylamide slab gels containing 0.1% $NaDodSO_4$ were used (Laemmli, 1970). They were either stained with the ultrasensitive silver method (Oakley et al., 1980) or processed for autoradiography: stained with Coomassie blue, dried, and exposed to Kodak X-Omat AR5 films for 2–10 days at −20 °C. Adenylate cyclase activity was measured at 30 °C (Berthillier et al., 1982) by using the assay procedure of Salomon et al. (1974). Proteins were determined according to Schaffner & Weissmann (1973).

Results

Properties of the Enzyme Solubilized from Synaptosomes Protected from Proteolysis. Since glial, vascular, and neuronal cells all contribute to total brain adenylate cyclase, we first prepared synaptosomes in order to selectively study the neuronal enzyme. We tried to avoid proteolysis of our material by using fresh tissue, by including several protease inhibitors throughout the subcellular fractionation, and by preparing samples devoid of lysosomes. The specific activity of adenylate cyclase in synaptosomes and synaptosomal membranes assayed in diverse conditions is given in Table I. When synaptosomes or membranes were solubilized by Lubrol, the 45000*g* Lubrol supernatant contained 60 ± 5% of the total protein and 95 ± 5% of the adenylate cyclase activity (Monneron & d'Alayer, 1980a). Such a high enzyme recovery in the supernatant ensured that the complex subjected to analysis was representative of the bulk of enzyme activity. The specific activity of the supernatants is given in Table I. As shown by Neer & Salter (1981), the inclusion of 0.2 M ammonium sulfate enhanced the activity (Table I) and abolished the lag in Gpp(NH)p stimulation.

The supernatant was analyzed on sucrose gradients. However, we made no attempts to correlate the apparent s value of adenylate cyclase with molecular weight, since the fractions probably contained micelles of different detergent/protein ratios (Tanford & Reynolds, 1976). Adenylate cyclase activity peaked at 9.1 ± 0.25 S (n = 35) (Figure 1, ●—●). The reported s values for solubilized brain enzyme are smaller: 4 and 7 (Sano & Drummond, 1981) and 7.3–7.6 S [as measured on the graphs (Neer et al., 1980)]. The 9.1S value we found did not change when a number of experimental parameters were varied: incubating synaptosomes at 30 (up to 20 min) or at 4 °C (up to several hours) with various stimulatory effectors [0.2 mM Gpp(NH)p, or 10 mM NaF] and enzyme inhibitors [4 mM Ca^{2+}, 1 mM 2′-deoxyadenosine, 1 mM $Pb(NO_3)_2$, and 0.4 mM *N*-ethylmaleimide]. The same value of 9.1 S for the enzyme was obtained whether adenylate cyclase was solubilized from whole brain homogenates or from freshly prepared synaptosomes or membranes. Changes in the solubilization medium (A) and/or in the gradients have been reported to shift the enzyme's apparent s value (Guillon et al., 1981). In our experiments, the enzyme's s value remained constant with the following changes: Lubrol concentration (0.4–2% in the A medium, Lubrol to protein ratio 0.8 to 7), divalent ion concentrations (0–10 mM $MgCl_2$, 0–10 mM $MnCl_2$, in both A medium and gradients), dithiothreitol concentrations (0–5 mM in both A medium and gradients), and temperature of solubilization (0 and 36 °C). (Omission

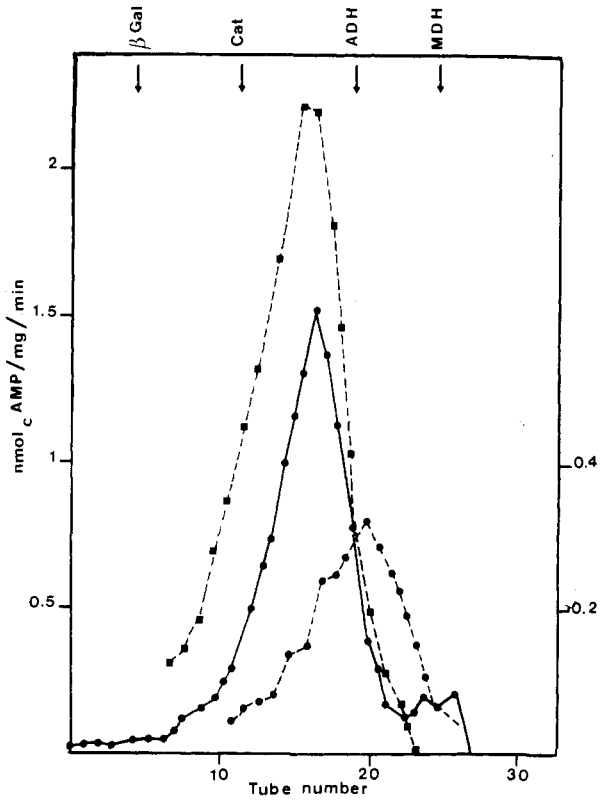

FIGURE 1: Sucrose gradient centrifugation of adenylate cyclase. Supernatants from membranes preincubated 20 min at 30 °C with or without 0.2 mM Gpp(NH)p were incubated with or without trypsin. The specific activities of adenylate cyclase in the supernatants (nmol of cAMP mg^{-1} min^{-1}) were the following: control, 1.3 (no trypsin) and 0.6 (4-min trypsin); Gpp(NH)p, 1.8 (no trypsin) and 2.2 (1-min trypsin). The *supernatants* were analyzed on 2.5–4% sucrose gradients [(■) gradient fractions from Gpp(NH)p treated membranes; (●) gradient fractions from control membranes; (—) gradient fractions from unproteolyzed supernatant; (---) gradient fractions from trypsin-digested supernatants). Adenylate cyclase was *assayed* in gradient fractions and supernatants in the presence of 10 mM $MgCl_2$, 2 mM $MnCl_2$, with (●---●) and without (■---■, ●—●) 0.2 mM Gpp(NH)p and 0.2 M $(NH_4)_2SO_4$. [The position of the peaks in the gradient was not influenced by the addition of 0.2 M $(NH_4)_2SO_4$ to the assay.] Note the difference in scales for enzyme specific activities (ordinates): left, values corresponding to (■---■) and (●—●), and right, to (●---●). Arrows point to water-soluble marker enzymes assayed according to published procedures.

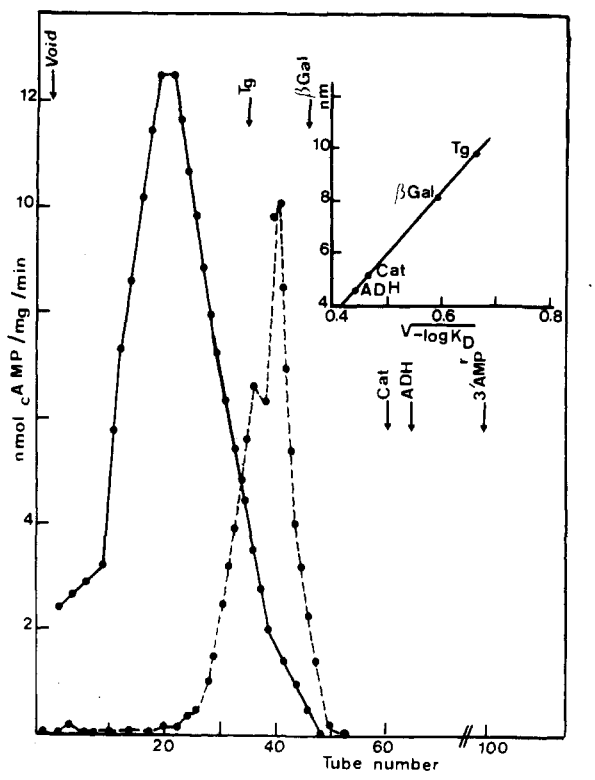

FIGURE 2: Gel filtration of adenylate cyclase on Bio-Gel A-5m. Eluates of the Lubrol supernatants of unproteolyzed synaptosomes (—) or trypsin-treated synaptosomes (---) were assayed for adenylate cyclase activity in the presence of 10 mM $MgCl_2$ and 2 mM $MnCl_2$, with (---) or without (—) 0.2 mM Gpp(NH)p and 0.2 M $(NH_4)_2SO_4$. [The position of the peaks did not depend upon the presence of 0.2 mM Gpp(NH)p and 0.2 M $(NH_4)_2SO_4$ in the assays.] Arrows point to proteins and enzymes used to calibrate the column. 3'AMP indicates V_t (total volume). Inset: Calibration of the column [$K_D = (V_e - V_0)/(V_t - V_0)$].

of dithiothreitol, and inclusion of 2 mM $MnCl_2$, largely decreased the adenylate cyclase activity.) The total enzyme recovery in the gradient was 100%.

Figure 2 shows the elution profile of the solubilized enzyme applied to a Bio-Gel A-5m column (●—●). Development of the column revealed a single peak of adenylate cyclase activity. From the position of the peak, we could deduce a Stokes radius of 11.5 ± 0.5 nm (n = 15), a value much larger again than those found in the literature for brain enzyme, or any solubilized adenylate cyclase [brain enzyme 7 nm (Neer, 1978) or, depending upon preincubation, and as measured on the graphs, 6.8–7.4 nm (Neer et al., 1980)]. Various changes in samples pretreatment, or in A medium composition, as described above, did not alter the Stokes radius of the enzyme. The specific activity of this enzyme is shown in Table I. (The recovery in the column was 100%, in the presence of either 0.2 M ammonium sulfate or 1 mg/mL azolectin in the assay.)

Properties of Adenylate Cyclase Solubilized from Samples Unprotected from Proteolysis. When no precautions were taken to avoid proteolysis (use of frozen brains, resulting in lysosome rupture upon thawing; omission of protease inhibitors during the preparative steps, repeated cycles of freezing and

Table II: Adenylate Cyclase Solubilized from Synaptosomes Degraded by Endogenous Proteases: Apparent s Values

pretreatment of synaptosomes		s values (S) measured in gradients containing: 0 mM $MgCl_2$	9 mM $MgCl_2$ (n = 2)
triethanolamine buffer:	0 °C	7.15 ± 0.1 (n = 5)	7.3 ± 0.2
	36 °C	6.0 ± 0.1 (n = 7)	7.1 ± 0.5
10 mM NaF:	0 °C	7.7 ± 0.25 (n = 3)	7.8 ± 0.5
	36 °C	6.5 ± 0.2 (n = 3)	7.8 ± 0.5
0.2 mM Gpp(NH)p:	0 °C	8.1 ± 0.5 (n = 3)	7.8 ± 0.5
	36 °C	6.3 ± 0.5 (n = 1)	7.8 ± 0.5

thawing of the samples; storage at room temperature for a few hours), the s values found for the soluble enzyme were smaller and covered a wider range, 6–7.3 S, than when "undegraded" samples were used. Contrary to the above results, the sizes of the enzyme-soluble complexes obtained from "unprotected" samples depended upon the membrane-bound enzyme level of activity at the time of solubilization, e.g., upon the nature of the enzyme's effector present. The lowest s value corresponded to the soluble enzyme obtained from samples not preactivated by Gpp(NH)p or NaF, whereas the largest s value was associated with enzyme obtained from samples preincubated with Gpp(NH)p (Table II). These findings are in accordance with several published reports (Pfeuffer, 1979; Goldhammer et al., 1980; Neer et al., 1980; Guillon et al., 1981). The s values also depended upon the divalent ion concentration in the gradients (Table I).

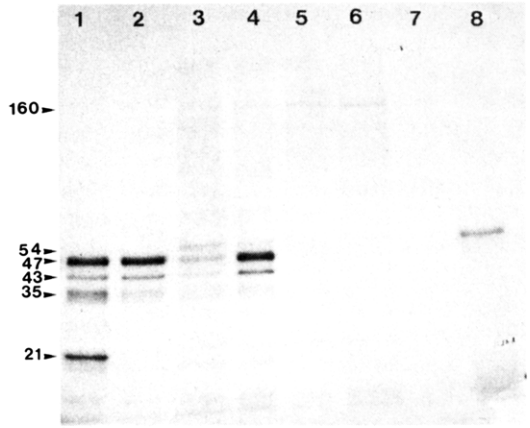

FIGURE 3: Distribution of ADP-ribosylated, Lubrol-soluble proteins in a sucrose gradient (10–18%). The gradient was divided in seven fractions, which were analyzed on a 7.5–12% acrylamide gel. The deposits on the gel were normalized with respect to protein concentration. The calibrated gel was autoradiographed. Lanes 1–7: Sucrose gradient fractions 1–7, from the top of the tube (fraction 1 = lane 1) to the bottom of the tube (fraction 7 = lane 7). Lane 8: Resuspended pellet of the gradient. Fraction 2 (lane 2) corresponds to proteins of 2–4S sedimentation coefficient. Fraction 4 (lane 4) corresponds to proteins of 8–10 S and contains the adenylate cyclase activity.

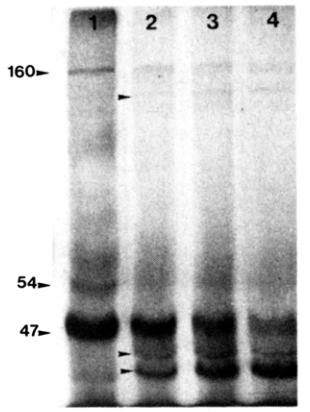

FIGURE 4: Time course of trypsin digestion of ADP-ribosylated, Lubrol-soluble membrane proteins. Membranes were ADP-ribosylated and then solubilized with Lubrol. The unfractionated supernatant was digested by trypsin (18 μg/mL) at 30 °C, and aliquots were withdrawn at 1, 2, and 4 min (lanes 2, 3, and 4), while an aliquot was incubated 4 min at 30 °C with a mixture of trypsin and trypsin inhibitor (lane 1). Electrophoresis of the aliquots was performed on a 10%, calibrated slab gel which was autoradiographed. Specific activities of the supernatant (nmol mg^{-1} min^{-1}): 0.9 (control), 1.35 (1-min trypsin), 0.98 (2 min), and 0.56 (4 min).

Since these smaller brain adenylate cyclase complexes consistently had a decreased specific activity[2] as compared to the 9.1S enzyme (by at least 60%), we suspected some degree of proteolysis within the adenylate cyclase complex. In order to test this hypothesis, we studied trypsin-treated fractions.

Properties of the Adenylate Cyclase Derived from Trypsin-Treated Material. Proteolysis exerts a biphasic action on adenylate cyclase activity; it first activates it and then quickly reduces it (Ryan et al., 1975; Hanoune et al., 1977). We studied this phenomenon by subjecting membranes, preincubated in buffer with or without Gpp(NH)p or NaF, or the derived supernatants, to trypsin treatment.

When the proteolytic attack was stopped during its activation step, the corresponding adenylate cyclase *s* value did not shift (Figure 1, 1-min trypsin treatment, ■---■).

As trypsin digestion proceeded, the enzyme *s* and *r* values decreased, as well as its specific activity. Enzyme obtained from membranes not preincubated with Gpp(NH)p or NaF yielded upon trypsin trypsin treatment (4 min) a soluble complex of 6 S (Figure 1, ●---●) and 8-nm Stokes radius (Figure 2, ●---●) and low specific activities (Figures 1 and 2). Enzyme obtained from Gpp(NH)p-stimulated membranes yielded a 7–7.5S soluble complex of intermediate specific activity upon proteolysis, and the digestion kinetics were slower (not shown).

Proteolysis of ADP-Ribosylated Proteins. Cholera toxin is known to stimulate adenylate cyclase through the ADP-ribosylation of the G/F regulatory subunits (Ross & Gilman, 1980). Up to now, ADP-ribosylation of brain membrane-bound proteins had not been shown, although the brain enzyme is activated by cholera toxin (Watkins et al., 1980). We recently succeeded in ADP-ribosylating brain membrane proteins with cholera toxin, without adding any cytosolic factor, presumably because we took extensive precautions to avoid proteolysis of the samples (Berthillier et al., 1982). Indeed, when membranes were *first* briefly treated with trypsin, to produce activation of adenylate cyclase, and then submitted to cholera toxin in the presence of labeled NAD, absolutely no ADP-ribosylated proteins could be detected (whereas a control protein, creatine kinase, was ADP-ribosylated). Hence, the ADP-ribosylated sites were probably very susceptible to proteolysis.

ADP-ribosylated, Lubrol-soluble proteins from samples protected from proteolysis were followed in sucrose gradients. All the gradient fractions were analyzed by autoradiograms of slab gels (Figure 3). The labeled polypeptides were mainly found in the 2–4S region of the gradient (Figure 3, lanes 1 and 2). However, the adenylate cyclase containing fraction (8–10 S) was slightly enriched in M_r 43 000 and 47 000 labeled proteins (Figure 3). ADP-ribosylated M_r 54 000 and 65 000 proteins were also present in the adenylate cyclase containing fraction.

We have studied the effects of trypsin treatment *upon the ADP-ribosylated proteins.* The supernatant from ADP-ribosylated membranes was subjected to trypsin digestion, and then adenyate cyclase activity and apparent molecular weights of the labeled proteins were followed as a function of time (Figure 4). The ADP-ribosylated polypeptides were quickly degraded. Cuts in the polypeptide chains were already apparent during the enzyme activation step (Figure 4, lane 2). No major changes could be seen in silver-stained gels corresponding to the same experiments.

It is likely that at least some of the brain ADP-ribosylated proteins participate in the adenylate cyclase complex. There is indeed a good correlation of the time course of membrane ADP-ribosylation and adenylate cyclase activation (Berthillier et al., 1982). The brain M_r 47 000 protein is very similar in size with the regulatory, ADP-ribosylated G/F subunit of M_r 45 000 purified by Sternweis et al. (1981). Preliminary data show that fractions from columns or gradients, which contain the bulk of the M_r 47 000 protein, restore the responsiveness of adenylate cyclase catalytic subunit from different sources to G/F-mediated effectors such as Gpp(NH)p.

Discussion

The nature and organization of the multiple components of eucaryotic adenylate cyclase is largely unknown [for reviews,

[2] The notion of enzyme "specific" activity in the gradient or eluate fractions is debatable. Indeed, the ratio of the protein content of the enzyme-enriched fractions to the total protein content of the supernatant varied with the experimental conditions. However, since the enzyme activity recovered in the fractions amounted in each case to 100% of the total activity subjected to analysis, comparison of the specific activities of the various enzyme complexes was valid.

see Ross & Gilman, (1980) and Farfel et al. (1981)]. As a result, the understanding of how this complex machinery functions is largely based on hypothesis. A cascade of changes in the individual conformation of the components and in their states of association is likely to occur and may control the enzymatic activity [for reviews, see Rodbell (1980), Limbird (1981), Schramm et al. (1981), and Sternweis et al. (1981)]. Estimation of the size of adenylate cyclase corresponding to given levels of enzyme activity is one way to pursue this study. Target analysis performed on isolated membranes has been used for this purpose. The results suggest that in several systems (liver and adipocytes), the ground state of the enzyme corresponds to large oligomeric structures containing multiple catalytic and regulatory subunits (Rodbell et al., 1981). Upon activation of the enzyme, these complexes seem to break down to smaller units, the size of which depends upon the complexity of the regulation. Although this method cannot relate the target size to specific components and thus may lead to divergent interpretations (Martin et al., 1979), it nevertheless hints at the existence, within the plasma membrane of these tissues, of organized clusters of adenylate cyclase components as large as $(0.5–1.3) \times 10^6$ daltons.

Our findings concerning the brain enzyme tend to corroborate this view. The approach we used here consisted of controlling proteolysis as much as possible, because proteolysis would undoubtedly affect subunit associations. Upon detergent solubilization, the enzyme complex would then possibly retain an organization closest to the in situ situation, compatible with detergent effects upon protein associations. A well-known result of detergent solubilization is to separate the adenylate cyclase from the relevant receptors (Schramm et al., 1981). Several polypeptides (subunits), however, often coexist within a micelle. This indeed seems to be the case for the brain large-sized adenylate cyclase complex obtained from samples protected from proteolysis. A single polypeptide chain of 9.1 S and 11.5-nm Stokes radius is indeed unlikely to occur. Nonspecific protein aggregation could possibly explain this result. However, the size of the large adenylate cyclase complex was constant, whether obtained from whole brain homogenate or from synaptosomes or membranes. Also the size of the large complex did not vary with the enzyme effectors present at the time of solubilization. The only prerequisite for obtaining adenylate cyclase as a large complex was to avoid substantial proteolysis. The relative discrepancy between the enzyme's s and r values suggest a high degree of asymmetry of this complex. Studies of brain adenylate cyclase in conjunction with other types of detergents confirm these findings (unpublished experiments).

The three basic points which emerged from our study were the following: (1) The Lubrol supernatant containing reasonably high adenylate cyclase specific activity gave rise, upon fractionation, to a sharp enzyme peak of large size. (2) This size was constant, unrelated to the states of activity of the membrane-bound enzyme. (3) The small size and low specific activity of enzyme complexes produced by proteolysis were correlated with the membrane-bound enzyme states of activity defined by the presence of enzyme effectors. This phenomenon is a likely explanation for the frequently noticed polydispersity of brain soluble adenylate cyclase.

The last finding with the brain enzyme can be compared with numerous reports in the literature showing changes in the apparent size of soluble adenylate cyclase as a function of effectors preincubated with the enzyme (at least in higher mammal tissues). In the thyroid (Goldhammer et al., 1980), brain (Neer et al., 1980), kidney, liver, and brain (Guillon et al., 1981), membranes preincubated with Gpp(NH)p yielded soluble enzyme of larger size than that obtained from unpreactivated or NaF-preincubated membranes. Likewise, reconstitution assays recombining isolated G/F subunits with G/F-depleted samples containing adenylate cyclase catalytic activity led to an increase in the enzyme apparent s value (Pfeuffer, 1979). Numerous other studies also clearly demonstrate the association of activated G/F subunits with the catalytic subunit (Ross & Gilman, 1980; Schramm et al., 1981).

The association of an activated G/F regulatory subunit with the adenylate cyclase catalytic subunit (or catalytic subunit containing complex) has been well documented in several systems (Pfeuffer, 1979; Kaslow et al., 1979; Ross & Gilman, 1980; Schram et al., 1981). In the brain case, *only* if some degree of proteolysis has occurred can this dissociation or reassociation be demonstrated by changes in the enzyme physicochemical parameters. Otherwise the two subunits are permanently part of a larger complex.

We propose that proteases are able to loosen or disrupt specific associations of adenylate cyclase components. This of course calls for great care in avoiding proteolysis during purification of adenylate cyclase. Whether the proteases act only during subcellular fractionation, as has been described in other systems (Kellermann et al., 1979), or whether their action is part of the physiological regulation of the enzyme, as hypothesized by Adnot et al. (1982), is not yet known.

Brain adenylate cyclase in situ is probably much more than a simple bimolecular complex (G/F subunit and catalytic subunit). In our view, the large-sized soluble enzyme of 9.1 S and 11.5-nm Stokes radius either contains additional subunits or has a different kind of structure than the relatively simple one inferred from published data. Both properties might render the soluble enzyme fully operational, whereas the smaller complexes would represent incomplete, partially active enzyme forms. Brostrom & Wolff (1981) have clearly demonstrated the participation of calmodulin in brain adenylate cyclase activation. Our preparations probably contain a mixture of calmodulin-sensitive and calmodulin-insensitive adenylate cyclases. However, the calcium-binding protein is of small size, and its association with the enzyme complex is probably not reflected in the Stokes radius or s value of the complex. Either additional stimulatory subunits or inhibitory subunits are likely to remain associated with the enzyme catalytic subunit upon solubilization. Inhibitory subunits have been considered as more sensitive to proteases than the others (Pinkett et al., 1980; Stiles & Lefkowitz, 1982), a result which might explain the activation of the enzyme by proteolysis. For the brain enzyme, however, this explanation is satisfactory only during the activating phase of proteolysis since the large enzyme complex had a higher adenylate cyclase activity than the smaller, presumably proteolyzed complexes. Later on, degradation of stimulatory subunits should also occur. The high sensitivity of brain ADP-ribosylated proteins to proteolysis might be a clue. The brain M_r 47 000 labeled polypeptide was very quickly degraded. A similar finding with the pigeon ADP-ribosylated erythrocyte M_r of 42 000 has been reported (Hudson et al., 1981). We now hope to be able to specifically analyze the components of the brain adenylate cyclase complex by making use of cross-linking agents, which might possibly prevent dissociation upon further purification (Monneron & d'Alayer, 1980a,b).

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