Isolation of homologous and heterologous complexes between catalytic and regulatory components of adenylate cyclase by forskolin–Sepharose

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Homologous and heterologous complexes between catalytic and GTP-binding components can be isolated by means of immobilized succinyldeacetylforskolin (forskolin–Sepharose). A heterologous complex is formed by reconstitution of forskolin–Sepharose bound catalytic function from rabbit myocardial membranes with the homogeneous [^{3}H]methyl-GTP-binding protein from duck erythrocyte membranes. Analysis of the reconstituted complex by sodium dodecyl sulfate polyacrylamide gelelectrophoresis reveals that only the M_r 42 000 component of the GTP-binding protein's M_r 42 000/M_r 35 000 heterodimer contributes to the formation of active adenylate cyclase.

Adenylate cyclase *Forskolin* *G-protein* *Reconstitution* *Guanine nucleotide*

1. INTRODUCTION

It is now clear that the hormonally regulated adenylate cyclase of higher cells is made up of several distinct polypeptides: hormone receptors for stimulating or inhibitory hormones, regulatory GTP-binding protein(s) mediating the action of stimulatory or inhibitory hormones and a catalytic protein as a signal receiver generating cAMP [1–6]. Recently, some of these components have been purified to apparent homogeneity: the β-adrenergic receptor [7,8] and the GTP-binding component (G-protein, N_S or G/F) [9–11]. The latter has been shown to consist of either 55, 45, 35 kDa or 42, 35 kDa subunits [9–11]. There is evidence that the active species of adenylate cyclase represents a [G·C] complex: using [^{32}P]ADP-ribosylated G-protein, partially purified from pigeon erythrocyte membranes, we have observed that the radiolabeled 42 kDa subunit was sedimenting together with the formed holoenzyme [3]. However, we could not decide whether only the 42 kDa component or the 35 kDa subunit as well was involved. Two lines of evidence have been presented that only the larger entity was forming the activated complex together with C. First, it has been observed that the size of the regulatory G-protein is apparently reduced when it is activated by GTPγS or fluoride [3,12]. Second, it has been shown recently that excess 35 kDa subunit inhibits the rate and extent of reconstitution by the G-protein in the presence of GTPγS [12]. This has been interpreted to mean that following binding of the nucleotide dissociation of the 42 kDa/35 kDa dimer precedes formation of the active [G·C] complex. Purification of some of the components of the adenylate cyclase complex has been considerably facilitated by the use of suitable ligands for affinity labeling and affinity chromatography [2,12,13].

Such a specific ligand also seems to be available now for the catalytic portion of the enzyme since the discovery by Metzger and Lindner [14] and by Daly's group [15] that the hypotensive plant constituent forskolin activates adenylate cyclase from

Abbreviations: Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; GTPγS, guanosine 5′-(3-thiotriphosphate); Mops, 3-(*N*-morpholino)propanesulfonic acid

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a variety of sources obviously through bypassing the hormone receptor and the activating G-protein. We have introduced forskolin–Sepharose as a powerful tool for purification adenylate cyclase and have presented evidence that the catalytic portion rather than the G-protein is recognized by the diterpene [16].

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, GPP(NH)p, GTPγS, creatine phosphate and creatine kinase were purchased from Boehringer (Mannheim). Carbonyldiimidazole, DL-isoproterenol and Tween 60 were obtained from Serva (Heidelberg). Tween 60 was deionized by passage through a mixed bed ion exchanger (AG 501 × 8; BioRad, Munich). Lubrol PX, beef heart lactate dehydrogenase, pig heart malate dehydrogenase and cytochrome *c* (horse heart) were from Sigma (Munich). Ultrogel ACA 34 was a product from LKB; DEAE–Sephacel, Sepharose CL-4B and Sephadex G-25 were purchased from Pharmacia (Uppsala). Heptylamine-Sepharose CL-4B was prepared as in [18]. For swelling of polyacrylamide gel slices Tissue solubilizer TS 1 from Zinsser was used. [α-^{32}P]ATP (10–30 Ci/mmol) and [^{35}S]GTPγS (550 Ci/mmol) were from New England Nuclear (Dreicich), c[^{3}H]AMP (15–30 Ci/mmol), NaB^{3}H$_4$ (12.4 Ci/mmol) and 1-fluoro-2,4-dinitro[3,5-^{3}H]benzene (10–30 Ci/mmol) were from the Radiochemical Centre (Amersham). All other chemicals were of the highest purity available. Doubly distilled water was used throughout. Protein was determined as in [19] or, with fluorodinitro[^{3}H]benzene as in [20]. Soluble adenylate cyclase from cardiac muscle of New Zealand rabbits was prepared as in [16] using 5 mM Lubrol PX, 10 mM Mops, 1 mM Mg^{2+}/EDTA (pH 7.4) and membrane protein at 10 mg/ml. The Lubrol extract was centrifuged at 100 000 × *g* for 60 min. A floating turbid layer was discarded and the clear supernatant saved. Adenylate cyclase assays in the presence of 0.1 mM ATP, unless otherwise stated, were performed at 30°C for 10 min as in [16]. cAMP production was linear with time for at least 20 min. Forskolin was added to the assay mixtures as concentrated ethanolic solution. Control samples also received ethanol at 1% final concentration.

2.2. *Preparation of activated G-protein from duck erythrocyte membranes*

2.2.1. Membranes

Erythrocytes were collected from the blood of ducks (either sex) obtained from a local slaughterhouse. Plasma membranes were prepared as in [21] with minor modifications described in [22]. They were stored in 20 mM phosphate buffer (pH 7.4), containing 3 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl at −70°C.

2.2.2. Activation of G-protein and purification procedure

2.5 g of plasma membranes in 250 ml of buffer described above, were incubated with 20 μM Gpp(NH)p and 100 μM DL-isoproterenol for 20 min at 37°C. The membranes were spun down at 15 000 rpm for 20 min in an SS 34 rotor (Sorvall) and successively washed with 250 ml each of buffer A [20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTE] plus 500 mM NaCl, buffer A and buffer A containing 50 mM NaCl. Membranes were suspended in 250 ml of buffer A adjusted to 50 mM NaCl and 2.5% cholate. The suspension was stirred at 4°C for 60 min and nonsolubilized material separated by centrifugation at 100 000 × *g* for 60 min. The supernatant was diluted with buffer B (buffer A plus 1 mM ATP, 6 mM $MgCl_2$, 10 mM NaF) to yield a cholate concentration of 1% and was applied on top of a DEAE–Sephacel column (2.5 × 51 cm) which has been equilibrated with buffer B containing 1% cholate. The following 3 purification steps, DEAE–Sephacel, Ultrogel ACA gelfiltration, heptylamine–Sepharose chromatography, were essentially identical to those reported for the purification of hepatic G-protein [9,10]. In contrast to various other purification steps reported by these authors a sucrose density gradient centrifugation step was found to be most convenient, achieving more than 95% purity of the erythrocyte G-protein: The pooled eluates from the heptylamine Sepharose chromatography (10 ml) were diluted to 20 ml with buffer C [10 mM Mops (pH 7.4), 1 mM $MgCl_2$ 1 mM EDTA] and concentrated to 2 ml in an Amicon CF-25 centrifilo filter cone device. One ml each of the concentrate was layered on top of a linear 5–20% sucrose gradient (38 ml, in buffer C containing 1 mM Lubrol PX) and centrifuged in a VTI 50 (Beckman) vertical

rotor for 14 h at 42 000 rpm (4°C). Fractions of 1.3 ml were obtained by puncturing the bottom of the tubes and tested for reconstitutive adenylate cyclase activity. The maximum of activity appeared in fractions 8–9. Only those fractions were pooled which were devoid of excessive 35 kDa component and concentrated to about 200 μl in an Amicon CF-25 filter cone. The G-protein was stored at a concentration of about 500 μg/ml in the presence of 15% glycerol at −70°C. Reconstitutive activity of G-protein-containing fractions was routinely measured by combination with a crude non-activated adenylate cyclase preparation sulubilized from duck erythrocyte membranes (30 μg protein per assay). These were solubilized as in [23] using 5 mM Lubrol PX and a membrane concentration of 10 mg/ml. The fluoride concentration in the column eluates was diluted to <0.5 mM in the cyclase assay. Occasionally, fluoride (and cholate) in the fractions was removed by centrifugation through 2 ml of dehydrated Sephadex G-25 fine in buffer C containing 100 mM NaCl and 1 mM Lubrol PX. Considerably higher reconstitutive activity could be obtained by combination of G-protein with the catalytic portion of particulate membrane preparations (100 μg membrane protein per assay) from rabbit myocardium. In this case specific reconstitutive activity of the finally purified G-protein varied between 3 and 6 μmol·mg^{-1}·min^{-1} at 30°C using 1 mM Mg^{2+}/ATP as substrate.

2.3. *Reductive methylation of duck erythrocyte G-protein*

The procedure was adopted from [25] for the radiolabeling of ribosomal proteins. Briefly, 2 μl of an aqueous solution of formaldehyde (1%) were added to 45 μl of pure G-protein (210 μg/ml) in 100 mM borate buffer (pH 8.5), 1 mM Tween 60 in an Eppendorf tube at 0°C. Following addition of about 10 mCi NaB^3H_4 (solid) the reaction was left for 3 min at 0°C. Subsequently, 5 1-μl aliquots of aqueous formaldehyde (1%) were supplied every 2 min. In order to ensure completeness of reduction, 4 μl of a solution of unlabeled $NaBH_4$ (3 mg/ml) were added and left for another 3 min at 0°C. Excess borohydride (and methanol) was removed by centrifuging of the mixture in a plastic syringe through 1 ml of dehydrated Sephadex G-25 fine in buffer D (buffer C plus 1 mM Tween 60, 50 mM NaCl). The eluate containing [^{3}H]methyl-G-protein (0.825 μCi/μg) was quickly frozen together with 15% glycerol in liquid nitrogen. On storage at −70°C, no measurable decay of reconstitutive activity could be observed during 10 months.

2.4. *Forskolin–Sepharose*

Forskolin–Sepharose was prepared from aminoethyl–Sepharose CL-4B and the hydroxysuccinimide ester of 7-succinyl-7-deacetylforskolin [16] or by condensation with 7-succinyl-7-deacetylforskolin and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide in 75% dimethylformamide. Aminoethyl–Sepharose CL-4B was prepared as in [17] by the carbonyldiimidazole method. Due to the relatively high substitution by amino groups (20–40 μmol per ml) chromatography of forskolin–Sepharose was carried out in the presence of 0.5 M NaCl to prevent ionic interactions. The substitution by 7-succinyl-7-deacetylforskolin varied between 0.8 and 2 μmol per ml.

3. RESULTS AND CONCLUSIONS

Previous experiments from this laboratory have suggested that although forskolin–Sepharose does not retain the G-protein it may copurify with the catalyst provided it has been previously activated by GTP-analogs or NaF [16]. This is corroborated by the experiment in fig.1 which compares the sedimentation behavior of two different forms in a sucrose density gradient. It is evident that following affinity chromatography non-activated ($s_{20,w}$ = 7.0) and guanine nucleotide activated adenylate cyclase ($s_{20,w}$ = 8.7) significantly differ with respect to their apparent sedimentation coefficients. Since the forskolin is removed during gradient centrifugation the former returns to its low basal activity while the latter displays full activity in the absence of any activator. Moreover, if membranes have been activated with [^{35}S]GTPγS before solubilisation ^{35}S radioactivity exactly follows adenylate cyclase activity in the gradient fractions. This allows approximation of the turnover number of the nucleotide activated adenylate cyclase: Assuming a 1:1 stoichiometry between G-protein and catalyst, turnover numbers ranging from 1200 to 2000 min^{-1} could be calculated from four independent experiments. Such a value would be in good agreement with that estimated for the purified enzyme from *Brevibacterium liquefaciens* [26].

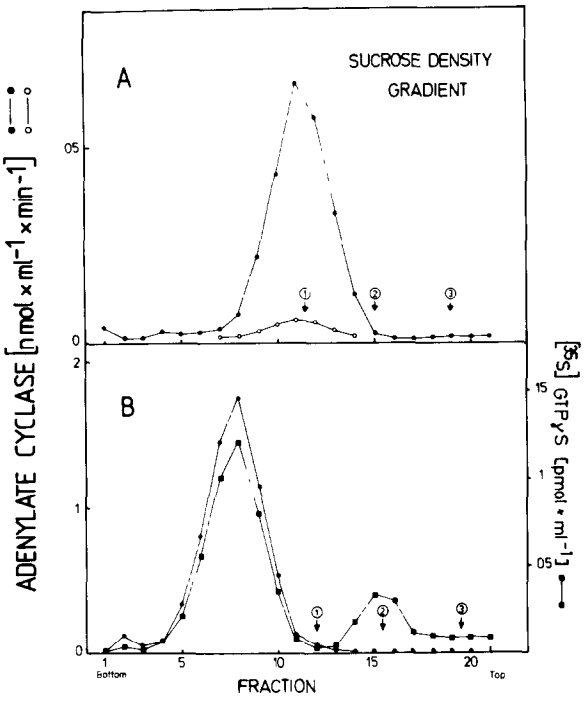

Fig.1. Isolation of a [^{35}S]GTPγS-labeled adenylate cyclase complex from rabbit myocardial membranes. 1.5 ml of rabbit myocardial membranes (10 mg/ml) were incubated with 10^{-7} M [^{35}S]GTPγS (340 Ci/mmol), 1×10^{-4} M DL-isoproterenol, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTE, 10 mM Mops (pH 7.4) for 20 min at 30°C. Following 3 washings with buffer C (5 ml each), solubilisation was performed in the same buffer containing 5 mM Lubrol PX at 0°C for 1 h as in [16]. For comparison membranes not treated with GTPγS/isoproterenol were solubilized under identical conditions (A). 1.2 ml of the solubilized preparations were adjusted to 500 mM NaCl and treated with 0.3 ml of packed forskolin–Sepharose in buffer D under gentle shaking for 90 min at 4°C. The mixtures were centrifuged at $100 \times g$ for 2 min and the supernatant removed. After washing the beads with buffer D (5×2 ml each) adenylate cyclase was released by adding 1.2 ml of buffer D containing 100 μM of forskolin. After gently agitating for 1 h at 4°C, the slurry was spun down as above and the supernatants removed. Forskolin–Sepharose eluates (1.2 ml each) were concentrated in an Amicon CF 25 filter cone device to 250 μl each and layered on top of a 5 ml 5–20% sucrose gradient in buffer E (buffer C plus 1 ml Tween 60) together with 300 μg LDH, 100 mg MDH and 500 μg cytochrome *c*. Centrifugation was performed at 4°C, 48 000 rpm using an SW 50 rotor for 13 h. Following centrifugation the gradient tubes were punctured and 240 μl fractions collected. Calibrating proteins were localized as in [3]. Adenylate cyclase activity was assayed in the presence (●—●) and absence (○—○) of 100 μM forskolin. Note the absence of stimulation by forskolin in B (GTPγS-treated membranes). [^{35}S]GTPγS was measured in 50 μl of the fractions in the presence of 500 μl tissue solubilizer TS-1. Numbers indicate the position of markers. 1, lactate dehydrogenase; 2, malate dehydrogenase; 3, cytochrome *c*.

We have purified the GTP-binding protein (stimulating) from duck erythrocyte membranes by a procedure very similar to that reported for the isolation of the respective proteins from liver and turkey erythrocyte membranes [9–11]. The G-protein is maintained in its Gpp(NH)p charged form throughout the purification procedure which may explain the excellent yield of 15–20%. Although activating nucleotides are supposed to reduce the apparent size of the G-protein due to dissociation of the 42 kDa/35 kDa dimer, the G-protein is isolated as a 80 kDa species as analyzed on a sucrose density gradient in the presence of Lubrol PX. Obviously, dissociation occurs only at higher dilution.

The G-protein can be easily radiolabeled by NaB^3H_4 via reductive methylation. More than 80% of the reconstitutive activity is preserved during this procedure. While from densitometric scans of Coomassie Blue stain both subunits of the G-protein preparation appear in a 1:1 stoichiometry, distribution of tritium label reveals a 1:0.65 ratio in favor of the 42 kDa component. For reconstitution experiments, we have chosen the catalytic moiety of rabbit myocardial membranes as the acceptor for the G-protein, since the homologous protein from duck erythrocyte membranes is only marginally activated by or bound to forskolin or forskolin–Sepharose, respectively. The flow diagram in fig.2 shows the reconstitution protocol where forskolin–Sepharose charged with catalytic protein is treated with and without tritiated G-protein. Another control experiment includes incubation of tritiated G-protein with uncharged forskolin–Sepharose: Following the reconstitution step and removal of non-absorbed material by washing, bound protein(s) are specifically released from the matrix by addition of free forskolin. In order to estimate the degree of reconstitution forskolin has

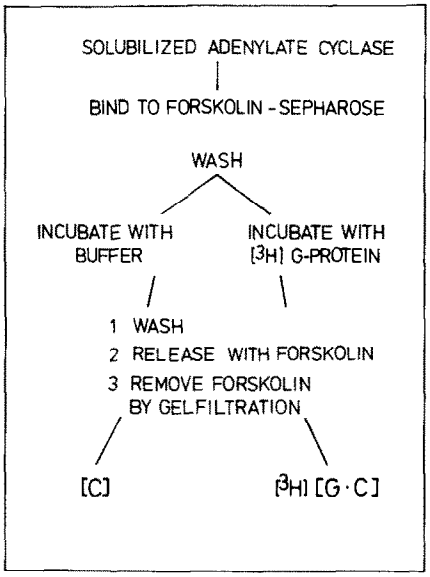

Fig.2. Reconstitution of [^{3}H]GTP-binding protein with forskolin–Sepharose-bound catalytic moiety. Two plastic syringes (2 ml) equipped with a nylon net were supplied with 0.3 ml of packed forskolin–Sepharose in buffer D and 1.5 ml of solubilized myocardial adenylate cyclase (4.5 mg) in 10 mM Mops (pH 7.4), 1 mM $MgCl_2$, 1 mM EDTA, 15 mM Lubrol PX, 500 mM NaCl (solubilized adenylate cyclase (non-activated) prepared as in [16] was concentrated 3-fold in an Amicon CF-25 filter cone). The syringes were stoppered at both ends and gently shaken for 90 min at 4°C. The fluid was removed and the resin was washed 4 times with 2 ml each of buffer D at 4°C. One of the syringes received 300 μl of buffer D containing 0.2 μg [^{3}H]GTP-binding protein, while the other syringe received 300 μl of buffer only. Another control was set up by mixing 0.2 μg [^{3}H]GTP-binding protein in 300 μl of buffer D with the same amount of packed forskolin–Sepharose. The syringes were stoppered and shaken for 10 min at 22°C and 50 min at 4°C. After removal of the fluid phase the resins were washed 6 times with 1.5 ml of buffer D at 4°C. For specific release, 1 ml of buffer D containing 100 μM forskolin was added to the resin and the slurry gently shaken for 60 min at 4°C. The fluid was separated from the beads which were rinsed with another 400 μl buffer D containing 100 μM forskolin.

to be removed by gel filtration. In the non-reconstituted control an adenylate cyclase [C] is obtained which was shown functionally to be resolved from proteins required for guanyl nucleotide and fluoride activation. This adenylate cyclase is however stimulated by forskolin and by activated GTP-binding protein (table 1). On the other hand, if the matrix-bound catalytic subunit is reconstituted with the purified G-protein a fully active adenylate cyclase preparation is obtained [^{3}H][G·C]. No further activation occurs on addition of more G-protein. If such reconstituted adenylate cyclase is now analyzed by SDS–polyacrylamide gel electrophoresis (fig.3) only the radiolabeled 42 kDa component of the G-protein dimer is observed. When [^{3}H]G-protein was incubated with forskolin– Sepharose not charged with catalytic protein much less radioactivity (3%) was released. This is ascribed to nonspecific binding, since no preference for either the 42 kDa or 35 kDa component was observed nor was the

Table 1

Activities of crude (I) and forskolin–Sepharose purified adenylate cyclase treated with (III) and without (II) [^{3}H]G-protein

	Adenylate cyclase activity (nmol·mg^{-1}·min^{-1}) in the presence of		
	None	G-Protein	Forskolin
Adenylate cyclase crude (I)	0.15	1.5	1.83
Adenylate cyclase, forskolin–Sepharose purified (II)	83.2	620.0	610.2
[^{3}H]G-Protein-reconstituted adenylate cyclase, forskolin–Sepharose purified (III)	845.3	830.2	1110.2

In order to remove the forskolin, 150 μl each of the eluates of preparations B and C were chromagotraphed on a small column (1 × 2.7 cm) of Sephadex G-25 fine equilibrated with 10 mM Mops, 1 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl (pH 7.4) at 4°C and 0.2 ml fractions collected. Protein-containing fractions were pooled (260–280 μl) and 40 μl aliquots assayed for adenylate cyclase activity in the presence and absence of 40 ng of pure Gpp(NH)p activated G-protein or 100 μM forskolin. The data are representative of 5 independently conducted experiments. In these experiments 70–75% of the applied adenylate cyclase activity (based on the reconstitutive activity with G-protein) was retained by the forskolin column, while 22–30% of the activity could be recovered following specific desorption by forskolin.

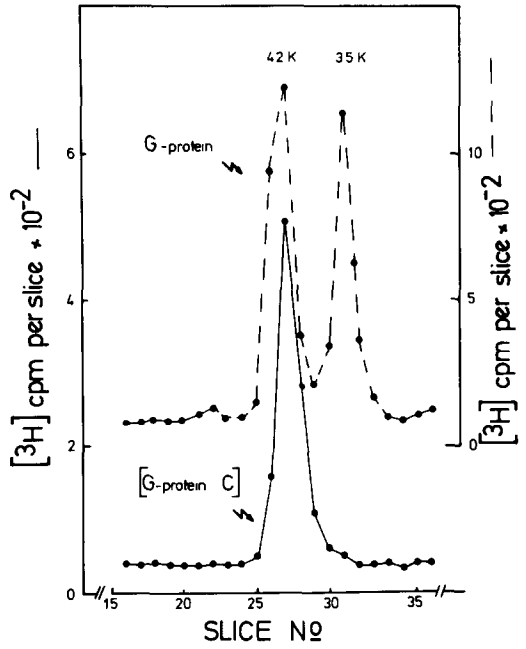

Fig.3. SDS–polyacrylamide gelectrophoresis of subunits of the [^{3}H]GTP-binding protein before and after reconstitution with the catalytic component. Purified [^{3}H]G-protein from duck erythrocyte membranes was reconstituted with forskolin–Sepharose bound catalytic function (rabbit myocardium) as outlined in fig.2. Samples from the forskolin–Sepharose eluates were diluted 5-fold with buffer C (to reduce the salt) and concentrated by centrifugation in an Amicon CF 25 filter cone. Samples of [^{3}H]G-protein and of [^{3}H]G-protein-reconstituted adenylate cyclase from forskolin–Sepharose were dissociated by addition of 200 μl of Laemmli's sample buffer for 12 h at room temperature and electrophoresed on 10% polyacrylamide rod gels as in [27]. Following electrophoresis gels were cut into 2 mm slices which were subsequently treated with 100 μl water and 500 μl Tissue-Solubilizer TS-1 for 4 h at 50°C. After cooling 10 ml of scintillation fluid was added for liquid scintillation counting. K, kDa.

released radioactivity significantly enhanced by forskolin. These results strongly suggest that the active adenylate cyclase complex contains only the larger (43 kDa) subunit of the GTP-binding protein. This feature strikingly parallels the light-driven GTPase of the retina where the analogous GTP-binding, α-component of transducin is sufficient to activate the cGMP-phosphodiesterase [28] and therefore adds to several other functional and structural homologies of these two GTP-regulated systems.

While it seems reasonable that the activated species of adenylate cyclase represents a complex made up of the catalytic protein (which by itself may consist of several subunits) and the 42 kDa component of the G-protein, the situation with the non-activated enzyme appears to be less clear.

Several lines of evidence suggest that the enzyme purified by forskolin–Sepharose lacks the G-protein:

(i) It is virtually insensitive to agents acting on this regulatory entity, namely guanyl nucleotides, NaF and vanadate (see [16]);
(ii) It can be reconstituted with a purified G-protein which becomes tightly associated as demonstrated by the use of radiolabeled G-protein (fig.3);
(iii) Its apparent sedimentation coefficient is significantly smaller than that of the activated adenylate cyclase complex (fig.1).

On the other hand, the sedimentation rates of nonactivated and activated enzymes may not indicate differences in mass but could reflect a conformational change and consequently altered detergent binding. Moreover, the failure of ligands to G-protein to activate purified adenylate cyclase from non-activated membranes could be due to the absence of the 35 kDa component. The fact that interaction of the 42 kDa subunit of the G-protein and the catalyst is strong enough to resist affinity chromatography conditions would seem to make it unlikely that functional and physical reconstitution involved an exchange between free and bound 42 kDa subunits. Such a mechanism has been considered recently by authors in [29] because an activation mechanism involving dissociation/association of G-protein and catalyst was not compatible with their kinetic studies. Whether or not catalytic and regulatory subunits are in association/dissociation equilibrium in solution or more important in their native membrane environment must await more detailed investigations.

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