GUANINE NUCLEOTIDE-CONTROLLED INTERACTIONS BETWEEN COMPONENTS OF ADENYLATE CYCLASE

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1. Introduction

Hormonally-stimulated adenylate cyclase consists of at least 3 components: a hormone receptor, a regulatory GTP-binding protein (abbreviated G-protein) mediating the hormonal signal to the third component, the catalytic unit [1–5]. The main, if not the only function of the hormone–receptor complex appears to be to enable exchange of GTP for GDP formed by a GTPase associated with the GTP-binding protein [6,7]. It was shown in pigeon erythrocyte membranes [8] that the 42 000 M_r GTP-binding component [5] is the target for cholera toxin-catalyzed ADP-ribosylation. Subsequently ADP-ribosylation of a protein of similar size, again in pigeon erythrocytes [9] and in mouse lymphoma and rat hepatoma cells [10] has been reported. It has been suggested that cholera toxin inhibits the GTPase and that this inhibition is responsible for the observed cholera toxin enhancement of cAMP production [11]. We took advantage of the specific labelling by the toxin of the 42 000 M_r G-protein of pigeon erythrocyte membranes for studying the interaction between this component and the catalytic moiety of adenylate cyclase and report on differences in the sedimentation behaviour of the G-protein dependent on the type of nucleotide bound. Furthermore, we show that binding of a triphosphate to the G-protein facilitates association and formation of the active holoenzyme.

Abbreviations: Gpp(NH)p, Guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); MOPS, 3-(*N*-morpholino) propane sulfonic acid; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. *Materials*

ATP, NAD^+, GTP, GDP, GMP, guanosine 5′-(β, γ-imido)triphosphate, (Gpp(NH)p), guanosine 5′-(3-thio)triphosphate, (GTPγS), creatine phosphate, creatine kinase, catalase (bovine liver), lactate dehydrogenase (rabbit muscle) and cytochrome *c* (horse heart) were obtained from Boehringer. Malate dehydrogenase was from Sigma. [α-^{32}P]ATP (8–15 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. Cholera toxin was from Schwarz/Mann. [^{32}P]NAD^+ (8–15 Ci/mmol, labelled in the AMP-moiety) was prepared as in [8]. GTP–Sepharose was prepared according to [5].

2.2. *Adenylate cyclase*

Pigeon erythrocyte membranes were prepared and adenylate cyclase activity solubilized with Lubrol PX as in [4,5]. Adenylate cyclase activity was assayed as in [8] and c[^{32}P]AMP isolated according to [12]. Pigeon erythrocyte membranes were treated with cholera toxin as in [8]. GTP-binding protein was prepared by affinity chromatography on GTP–Sepharose [5]. The catalytic function (C) was not adsorbed to this column and was subsequently purified by covalent chromatography on disulfide Sepharose; the procedure will be detailed in [13].

2.3. *Sucrose density gradient centrifugation*

Linear gradients (5–20%, 5 ml) were prepared in 10 mM MOPS, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Lubrol PX buffer (pH 7.4). Samples (200–250 μl) were centrifuged through the

gradient at 3°C in a Beckman SW 50 rotor for 16 h at 48 000 rev./min. Fractions were collected dropwise by puncturing the bottom of the tubes. Marker proteins with the following $s_{20,w}$ values were applied to each gradient: 40 μg catalase (bovine liver), 11.3 S [14]; 10 μg lactate dehydrogenase (rabbit muscle), 6.95 S [15]; 5 μg malate dehydrogenase (pig heart), 4.32 S [16]; 500 μg cytochrome *c* (horse heart), 2.1 S [14]. The enzymes were assayed according to [17]. Cytochrome *c* was determined by its A_{410}.

2.4. *[^{32}P]ADP-ribosylated G-protein*

[^{32}P]ADP-ribosylated 42 000 M_r protein was found to be > 95% radiochemically pure as based on SDS–polyacrylamide electrophoresis [8]. The radioactivity in the protein was determined by precipitation with 10% cold trichloroacetic acid on Whatman GF/F glassfiber discs. The filters were washed extensively with 10% trichloroacetic acid, dried and counted in Bray's solution for radioactivity [18]. Protein was determined by the Lowry method with bovine serum albumin as standard [19].

3. Results

In fig.1 the sedimentation behaviour of [^{32}P]ADP-ribosylated G-protein is shown to depend on the type of guanine nucleotide bound. The majority of the GTPγS-charged binding protein ($G_{GTP\gamma S}$) sediments with app. *S*-value (expt A) of 3.4*, while the GDP-form (G_{GDP}) in expt B has app. $S = 5.5$. SDS–polyacrylamide gel electrophoresis indicated that the [^{32}P]ADP-ribose was associated in all cases, including the trailing radioactivity in expt A, with the 42 000 M_r protein. The GTPγS-charged binding protein always had a shoulder with app. $S = 4.7$. This could reflect instability of the cholera toxin treated G-protein, because GTPγS-(or Gpp(NH)p)-charged binding protein from non-treated membranes gives a single symmetrical 3.4 S activity peak on subsequent reconstitution. In any event the 3.4 S component of the toxin-treated G-protein was much more potent than the 4.7 S component in reconstituting adenylate cyclase activity. When the [^{32}P]ADP-ribosylated binding protein with GTPγS bound was combined with a purified catalytic fraction and applied to the gradient, > 80%

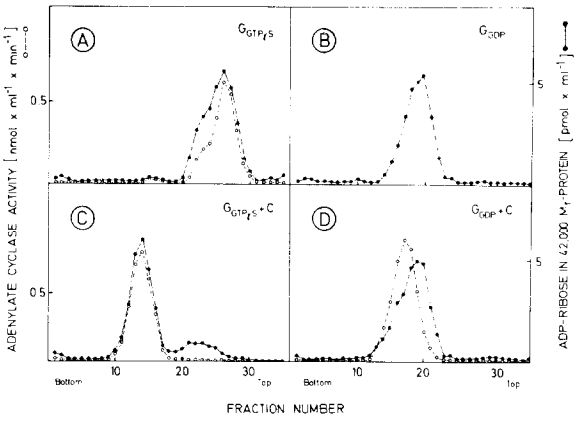

Fig.1. Sucrose density gradient centrifugation of separated and recombined components. Membranes were treated with cholera toxin (40 μg/ml) and [^{32}P]NAD$^+$ (20 μM, 2700 cpm/pmol) as in [8]. They were washed, incubated with 1 mM GMP and 50 μM isoproterenol and solubilized with 20 mM Lubrol PX [4,5]. The soluble preparation (1.2 mg/ml, 2 ml) was gently shaken with 1 ml GTP–Sepharose in 83 mM NaCl, 1 mM Mg^{2+}/EDTA, 1 mM dithiothreitol, 10 mM MOPS buffer (pH 7.4) (buffer A) at 10°C for 180 min. The supernatant was removed and the resin washed with the same buffer (A) containing 1 mM Lubrol PX. The bound material was desorbed by gentle agitation in buffer A containing 0.25 mM GTPγS or 0.25 mM GDP and 1 mM Lubrol PX for 45 min at 22°C. About 30% of [^{32}P]ADP-ribosylated 42 000 M_r protein was recovered. The yield was about the same using GTPγS or GDP for release. Aliquots (2.8 ml) of the G-fractions ($G_{GTP\gamma S}$ or G_{GDP}, respectively) were concentrated to ~300 μl by centrifugation at 1000 × *g* in a 'centriflo filter cone' device (CF 25 - Amicon) and 125 μl were combined with 125 μl buffer A (expt A, B) or with 125 μl (70 μg/ml) of purified catalytic fraction, depleted of G-protein ('C', see section 2) in expt C, D. After standing at 22°C for 10 min the material was layered onto a sucrose gradient and centrifuged. Fractions (140 μl) were collected of which 25 μl were used for adenylate cyclase activity measurements (○– –○) and 100 μl for counting the [^{32}P]ADP-ribosylated 42 000 M_r protein (●——●) and 10 μl for the detection of the marker enzymes. To measure adenylate cyclase in expt A, 50 μl purified catalytic fraction were added whereas in expt D, 50 μl GTP-binding fraction eluted with GTPγS, were added. In expt B [^{32}P]ADP-ribosylated 42 000 M_r protein in the inactive form (G_{GDP}) is present. This G-protein was therefore incapable of restoring adenylate cyclase activity on addition of the catalytic component. Adenylate cyclase activity in expt C was assayed without additions.

* *S*-values are apparent and not corrected for detergent binding. They are given relatively to the $s_{20,w}$ values of the soluble marker proteins

of the radioactivity appeared in a peak with 7.6 S (expt C). This peak represents the reconstituted holoenzyme and supports the notion that the 42 000 M_r component is the regulatory component of the adenylate cyclase complex. The smaller, more slowly sedimenting [^{32}P]ADP-ribose peak (fractions 20–26 in expt C) does not shift even when more of the catalytic fraction is added and probably represents denaturated G-protein incapable of reassociation. No material capable of reconstitution remained at the position of the isolated G-protein. Not shown are experiments with a purified catalytic moiety [13] which is not stimulated by nucleotides and Mg^{2+}/F^- and which sediments with app. $S = 6$. The catalytic moiety has basal activity, which is stimulated by Mn^{2+} [20] and which is further characterized by being functionally reconstituted with the G-protein*. In contrast to expt C the GDP-liganded G-protein has the same $S = 5.5$ with or without addition of the catalytic moiety (cf. B, D). Moreover the adenylate cyclase activity in expt D sediments like the separate catalytic preparation. Thus the GTP-form of the G-protein ($G_{GTP\gamma S}$) has much greater affinity for the catalytic moiety than the GDP-form. This is supported by the experiments in fig.2: Unfractionated Gpp(NH)p- or GTPγS-treated soluble adenylate cyclase centrifuged through a sucrose gradient sediments with $S = 7.6$ like the reconstituted enzyme (cf. fig.1C,2A), whereas solubilized adenylate cyclase treated with GDP or GTP (with or without a GTP-regenerating system) yields only Mn^{2+}-stimulated adenylate cyclase activity with the same $S = 6$ as the purified catalytic moiety (fig.2B). Since the same inactive species is formed with GTP and GDP it follows that GTP is hydrolyzed to GDP at the guanyl-nucleotide regulatory site of the pigeon erythrocyte adenylate cyclase as was shown with turkey erythrocyte adenylate cyclase [6]. However, the catalytic moiety and the G-protein which barely interact in the presence of GDP can still form an active higher molecular weight complex with $S = 7.6$ when pretreated with Mg^{2+}/F^- (fig.2C). The persistence of Mg^{2+}/F^- activation of soluble adenylate cyclase from

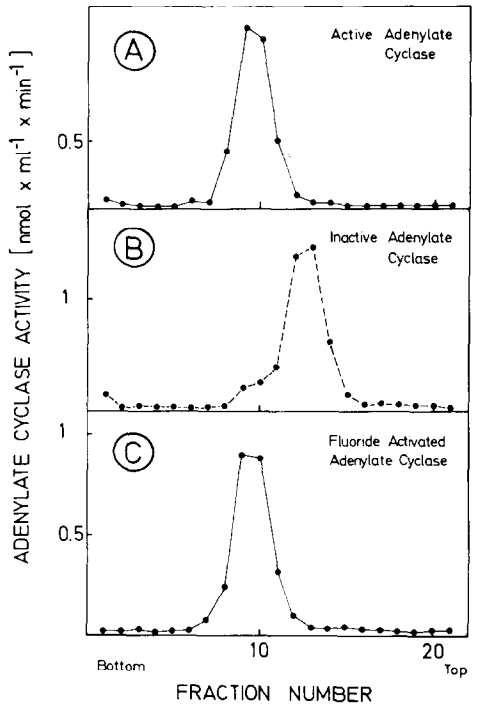

Fig.2. Effects of nucleotides and Mg^{2+}/F^- on sedimentation of soluble adenylate cyclase in sucrose gradients. Membranes (5 mg/ml) were treated with 1 mM GMP and 50 μM isoproterenol and solubilized with 20 mM Lubrol PX as in [4,5]. Soluble preparation (0.5 ml each) was treated with either 0.2 mM GTPγS (expt A), or 0.2 mM GDP (expt B) or with 0.2 mM GDP plus 10 mM NaF and 5 mM $MgCl_2$ (expt C) for 30 min at 22°C. The preparations were concentrated to 250 μl by centrifugation at 1000 × *g* in a 'centriflo filter cone' device (CF 25-Amicon) and layered on top of a sucrose gradient. Fractions (250 μl) were collected of which 10 μl were used for detection of marker enzymes and 25 μl for measurement of adenylate cyclase activity. Activity was determined in expt B after adding 50 μl of a GTP-binding fraction (GTPγS-form) (•– – –•), whereas in expt A, C no additions were made (•——•).

pigeon erythrocyte membranes following gel filtration or dialysis [4] explains that the 'holo' enzyme does not require the continuous presence of Mg^{2+}/F^- during centrifugation. As expected, a soluble adenylate cyclase from cholera toxin-treated membranes which has become refractory to F^- no longer forms the higher molecular weight holoenzyme species with Mg^{2+}/F^- (not shown).

* This adenylate cyclase form may be the same as that in the adenylate cyclase deficient (AC^-) variant of S49 lymphoma cells (cf. [20])

4. Conclusions

The different sedimentation of the G-protein on binding a guanosine triphosphate analog as compared to guanosine diphosphate is consistent with its proposed function in GTP hydrolysis [6].

Whether the higher S-value of the GDP-liganded G-protein 5.5 S reflects dimerisation of the 3.4 S form or its association with another as yet unknown component of the adenylate cyclase complex needs to be clarified. Preliminary estimates of the hydrodynamic properties of the two forms of the G-protein in D_2O and H_2O seem to indicate that the G-protein as compared with the catalytic fraction binds much less detergent [13]. Assuming, $s_{20,w}$ 3.4, the GTPγS-charged G-protein is compatible with a 42 000 M_r monomer and the 5.6 S GDP-form with a dimer.

The differences in affinity of the G-protein for the catalytic moiety depending on the binding of a nucleoside triphosphate or a diphosphate may be interpreted to mean that the cycle of activation–deactivation of adenylate cyclase [7] is primarily governed by the GTPase activity of the binding protein geared to an association–dissociation cycle of the G-protein with the catalytic unit (fig.3). Further work should clarify details of this GTP-driven conformational transition and might also provide information on possible reciprocal interactions of hormone receptor, and catalytic unit with the G-protein depending on whether GTP or GDP is bound. The hypothetical scheme shown in fig.3 would account for the collision coupling model according to which the β-adrenergic receptor [3] activates adenylate cyclase by forming transient complexes. It also incorporates the conclusions [7] that the GTP-binding protein oscillates between a GTP- and a GDP-form without leaving the nucleotide binding site empty.

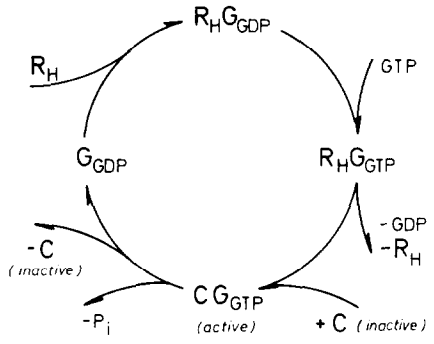

Fig.3

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References

[1] Orly, J. and Schramm, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4410–4414.
[2] Haga, T., Haga, K. and Gilman, A. G. (1977) J. Biol. Chem. 252, 5776–5782.
[3] Tolkovsky, A. M. and Levitzki, A. (1978) Biochemistry 17, 3795–3810.
[4] Pfeuffer, T. and Helmreich, E. J. M. (1975) J. Biol. Chem. 250, 867–876.
[5] Pfeuffer, T. (1977) J. Biol. Chem. 252, 7224–7234.
[6] Cassel, D. and Selinger, Z. (1976) Biochim. Biophys. Acta 452, 538–541.
[7] Cassel, D. and Selinger, Z. (1978) Proc. Natl. Acad. Sci. USA 75, 3050–3054.
[8] Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA 75, 2669–2673.
[9] Gill, D. M. and Meren, R. (1978) Proc. Natl. Acad. Sci. USA 75, 3050–3054.
[10] Johnson, G. L., Kaslow, H. R. and Bourne, H. R. (1978) J. Biol. Chem. 253, 7120–7123.
[11] Cassel, D. and Selinger, Z. (1977) Proc. Natl. Acad. Sci. USA 74, 3307–3311.
[12] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[13] Pfeuffer, T. (1979) in preparation.
[14] Sober, H. A. ed (1970) Handbook of Biochemistry, Chemical Rubber Co., Cleveland, OH.
[15] Schwert, G. W. and Winer, A. D. (1963) in: The Enzymes (Boyer, P. D. et al. eds) 2nd edn, vol. 7, pp. 127–148, Academic Press, New York.

[16] Thorne, C. J. R. (1962) Biochim. Biophys. Acta 59, 624–633.
[17] Bergmeyer, H. U. ed (1970) Methoden der enzymatischen Analyse, 2. Auflage, Verlag Chemie, Weinheim Bergstraße.
[18] Bray, (1960) Anal. Biochem. 1, 279–285.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Ross, E. M., Howlett, A. G., Ferguson, K. M. and Gilman, A. G. (1978) J. Biol. Chem. 253, 6401–6412.