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Reconstitution of Catecholamine-Stimulated Guanosinetriphosphatase Activity†

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ABSTRACT: β-Adrenergic receptors were partially purified from turkey erythrocyte membranes by alprenolol–agarose chromatography to 0.25–2 nmol/mg of protein, and the stimulatory guanosine 5′-triphosphate (GTP) binding protein of adenylate cyclase (G_s) was purified from rabbit liver. These proteins were reconstituted into phospholipid vesicles by addition of phospholipids and removal of detergent by gel filtration. This preparation hydrolyzes GTP to guanosine 5′-diphosphate (GDP) plus inorganic phosphate (P_i) in response to β-adrenergic agonists. The initial rate of isoproterenol-stimulated hydrolysis is approximately 1 mol of GTP hydrolyzed·min^{-1}·mol^{-1} of G_s. This low rate may be limited by the hormone-stimulated binding of substrate, since it is roughly equal to the rate of binding of the GTP analogue guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) to G_s in the vesicles. Activity in the absence of agonist, or in the presence of agonist plus a β-adrenergic antagonist, is 8–25% of the hormone-stimulated activity. Guanosinetriphosphatase (GTPase) is not saturated at 10 μM GTP, and the response to GTP is formally consistent either with the existence of multiple K_m's or of a separate stimulatory site for GTP. The GTPase activity of G_s in vesicles is also stimulated by 50 mM $MgCl_2$ in the presence or absence of receptor. Significant GTPase activity is not observed with Lubrol-solubilized G_s, although [^{35}S]-GTPγS binding is increased by Lubrol solubilization.

Hormonal regulation of adenylate cyclase requires the presence of guanosine 5′-triphosphate (GTP)[1] or some closely related nucleotide, and poorly hydrolyzable analogues of GTP cause the persistent activation of the enzyme. These and related findings in many laboratories led to the speculation that hydrolysis of GTP might be involved in the regulation of adenylate cyclase activity [see Ross & Gilman (1980) for review]. This hormone-stimulated guanosinetriphosphatase (GTPase) activity was discovered by Cassel & Selinger (1976), who proposed an explicit model for the stimulation of adenylate cyclase by GTP and the termination of activation by hydrolysis. In this scheme, the receptor–hormone complex was proposed to act by facilitating release of guanosine 5′-diphosphate (GDP), thereby allowing another GTP molecule to bind and activate the enzyme. Considerable data consistent with this model have been obtained [Cassel & Selinger, 1976, 1977a,b; see Ross & Gilman (1980) for review], and it is likely that GTP hydrolysis is central to hormonal control of adenylate cyclase. In at least some cases, however, release of GDP is probably not the rate-limiting, hormone-stimulated step

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[1] Abbreviations: G_s, stimulatory GTP-binding protein of adenylate cyclase; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPase, guanosinetriphosphatase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); P_i, inorganic phosphate; NaHepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DMPC, dimyristoylphosphatidylcholine; PEI, poly(ethylenimine); Gpp(NH)p, guanyl-5′-yl imidodiphosphate; ITP, inosine 5′-triphosphate; ATP, adenosine 5′-triphosphate.

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(Iyengar, 1981; Neer & Salter, 1981; Northup et al., 1982; Tolkovsky et al., 1982).

The mechanism whereby GTP regulates adenylate cyclase was clarified by the discovery of a discrete GTP-binding regulatory protein, referred to here as G_s, that acts as a transducer between hormone receptor and the adenylate cyclase catalytic unit [see Ross & Gilman (1980)]. While it has been generally assumed that the GTPase catalytic site is located on G_s, Northup et al. (1982) did not detect GTPase activity in purified, Lubrol-solubilized G_s. They estimated that the rate of hydrolysis of GTP by G_s was less than 0.03 mol·min^{-1} (mol of G_s)$^{-1}$. They hypothesized that G_s might require incorporation into a membrane or some interaction with receptors or the adenylate cyclase catalytic unit in order to express hydrolytic activity.

Three groups (Pedersen & Ross, 1982; Citri & Schramm, 1980, 1982; Keenan et al., 1982) have reported that G_s and β-adrenergic receptors can be functionally reconstituted into phospholipid vesicles. We demonstrated efficient coupling of purified hepatic G_s with β-adrenergic receptors in such vesicles and used the known specific activity of G_s to estimate the rate and extent of the G_s–receptor interaction (Pedersen & Ross, 1982). However, this system and those developed by others have been too crude to allow interpretable studies of nucleotide binding or hydrolysis. We report here that partially purified β-adrenergic receptors and pure G_s can be reconstituted into phospholipid vesicles. This preparation displays hormone-stimulated guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) binding and hormone-stimulated GTPase activity, thereby establishing the biological activity of digitonin-solubilized receptors and definitely assigning the catalytic site of hormone-stimulated GTPase to G_s.

Experimental Procedures

Materials. G_s was purified from rabbit liver according to Sternweis et al. (1981). Turkey erythrocyte plasma membranes were prepared as described (Pedersen & Ross, 1982). Alprenolol–agarose was prepared according to Caron et al. (1979). Polar lipids were prepared from turkey erythrocyte membranes by solubilizing the membranes in sodium deoxycholate as described (Pedersen & Ross, 1982; isoproterenol was omitted), dialyzing the extract against water to remove detergent, and extracting the residue into chloroform–methanol (2:1) from 0.74% KCl. [γ-^{32}P]GTP was prepared according to Johnson & Walseth (1979). [^{3}H]GTP and [^{35}S]GTPγS were purchased from New England Nuclear. Unlabeled GTPγS (Boehringer) was purified by elution from DEAE-Sephacel with a gradient of 0–1 M LiCl. [^{125}I]Iodocyanopindolol was prepared according to Engel et al. (1981). (±)-Cyanopindolol was a gift of G. Engel, Sandoz Pharmaceuticals, and alprenolol was a gift of Hässle Pharmaceuticals. Sources of other materials are listed elsewhere (Pedersen & Ross, 1982; Fleming & Ross, 1980).

Receptor Purification. β-Adrenergic receptors were partially purified by modification of methods of Shorr et al. (1982). Turkey erythrocyte plasma membranes were suspended at 5 mg/mL and stirred 60 min at 0 °C in 20 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (NaHepes) (pH 8)–0.1 M NaCl–2 mM ethylenediaminetetraacetic acid (EDTA)–1.4% digitonin. The supernatant (60 min at RCF_{max} = 1.4 × 10^5 g) was applied to alprenolol–agarose. The column was washed with 5–6 volumes of 20 mM NaHepes (pH 8)–2 mM EDTA–0.05% digitonin–0.1 M NaCl and eluted at room temperature with 10 μM alprenolol. The eluate was dialyzed for 24 h against 20 mM NaHepes (pH 8)–1 mM EDTA–0.05% digitonin and concentrated 50–100-fold by adsorption to DEAE-Sephacel in the same buffer and elution in 0.2 M NaCl (0.2–0.7 mg of protein/mL). Such preparations typically bound 0.25–1 nmol/mg [^{3}H]dihydroalprenolol, suggesting a purity of less than 10%. Digitonin-solubilized receptors are stable at 4 °C for several days throughout this procedure.

Reconstitution. Receptor (1 volume), G_s [0.6 volume in 10 mM NaHepes (pH 8)–1 mM EDTA–0.1 mM dithiothreitol (DTT)–0.1% Lubrol 12A9], and lipids [1.2 volumes, 0.4 mg/mL dimyristoylphosphatidylcholine (DMPC) plus 0.8 mg/mL turkey erythrocyte polar lipids in 3.6 mg/mL deoxycholate plus 0.4 mg/mL cholate] were combined and reconstituted by gel filtration on Sephadex G-50 according to Pedersen & Ross (1982). The elution buffer used was 20 mM NaHepes (pH 8)–1 mM EDTA–2 mM $MgCl_2$–1 mM dithiothreitol–0.1 M NaCl. The G_s to receptor ratio varied over the range 3–9 (Table II, for example).

Assays. GTPase activity was assayed by incubating vesicles at 30 °C in a total volume of 0.1 mL containing 50 mM Hepes (pH 8), 1 mM EDTA, 2 mM $MgCl_2$ (unless shown otherwise), 1 mM dithiothreitol, 0.1 M NaCl, 0.1 mM adenyl-5′-yl imidodiphosphate, 0.1 mM ascorbic acid, and [γ-^{32}P]GTP (5–100 cpm/fmol). The concentration of GTP used in each experiment is given in the text or legends. The reaction was stopped by the addition of 0.25 mL of cold 5% Norite in 50 mM NaH_2PO_4 and rapid chilling. The mixture was centrifuged (1500 rpm, 10 min) and [^{32}P]P_i in the supernatant (0.2 mL) was determined. The zero-time blank, caused by contamination of substrate with [^{32}P]P_i, varied from 2.5% to 3% of the total radioactivity in the reaction mixture and is subtracted from all data except in Figure 1. Nonenzymatic hydrolysis of [γ-^{32}P]GTP during the assay was less than 5% of the hormone-stimulated activity (see Figure 1). The identity of the product as GDP was confirmed by the parallel use of [^{3}H]GTP as substrate. The reaction was stopped by the addition of cold methanol, and the mixture was chromatographed on poly(ethylenimine) (PEI)–cellulose plates in 1.2 M LiCl plus 0.15 M formic acid. The production of [^{32}P]P_i and of [^{3}H]GDP was equal. Data shown are averages of triplicate determinations that varied by less than 10%. All experiments have been performed at least twice with different batches of receptors and reconstituted vesicles. Data are expressed according to the volume of vesicles used in an assay, reflecting a 4.3-fold dilution of the initial reconstitution mixture. Since protein is donated by both G_s and the receptor fraction and because the protein concentration is low, the amount of vesicles used is normalized according to the number of high-affinity GTPγS binding sites [1 mol/mol of G_s; see Northup et al. (1982)].

Binding of [^{35}S]GTPγS was measured essentially as described by Northup et al. (1982). Vesicles were incubated at 30 °C in medium containing 20 mM NaHepes (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl and 1 mM ascorbic acid, and the amounts of [^{35}S]GTPγS and $MgCl_2$ shown in the text (0.05-mL total volume). $MgCl_2$ (50 mM)-stimulated binding in the presence of 0.1% Lubrol 12A9 was assayed after 30 min to estimate the total number of binding sites (B_{max}) (Northup et al., 1982; T. Asano and E. M. Ross, unpublished experiments). Isoproterenol-stimulated binding was assayed after 2–3 min, at which time bound nucleotide had reached a constant level. Samples were quenched in cold buffer containing 0.1 mM GTP and 10 μM propranolol, and bound nucleotide was separated by filtration on membrane filters as described by Northup et al. (1982). Nonspecific binding, determined in the presence of a 100-fold excess of GTP over

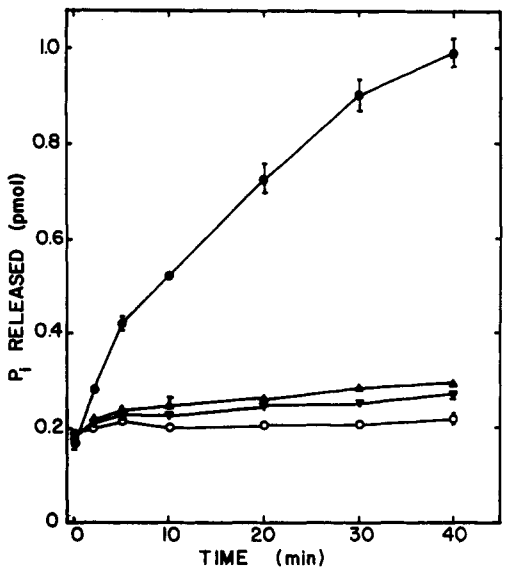

FIGURE 1: GTPase activity in receptor–G_s vesicles. Vesicles (3.4 μL; 74 fmol of [^{35}S]GTPγS binding sites) were incubated at 30 °C for the times shown. The concentration of [γ-^{32}P]GTP was 0.1 μM. Assays contained 1 μM (−)-isoproterenol (●), isoproterenol plus 10 μM (−)-propranolol (▲), or 0.1 μM propranolol (▼). Hydrolysis in the absence of vesicles is also shown (○). The zero-time, zero-protein value represents [^{32}P]P_i contaminating the [γ-^{32}P]GTP. The standard deviation of triplicate data is indicated by bars where significant.

[^{35}S]GTPγS, was 3–8% of total binding at the steady state. Agonist-stimulated [^{35}S]GTPγS binding will be described in more detail elsewhere (T. Asano and E. M. Ross, unpublished data).

Binding of [^{3}H]dihydroalprenolol to β-adrenergic receptors was assayed as described previously, and the binding of isoproterenol was assayed by competition with [^{125}I]iodocyanopindolol by the method described for [^{125}I]iodohydroxybenzylpindolol (Fleming & Ross, 1980). The stable activation of G_s by GTPγS was assayed according to its ability to stimulate the activity of the adenylate cyclase catalytic unit as described by Pedersen & Ross (1982). Other ancillary procedures are described elsewhere (Fleming & Ross, 1980; Pedersen & Ross, 1982).

Results

When a mixture of detergent-solubilized G_s, β-adrenergic receptors, and phospholipids was gel filtered on Sephadex G-50 in the absence of detergent, protein and phospholipid eluted in the void volume. This fraction contained unilamellar vesicles and some amorphous material according to electron micrographs of negatively stained samples. Recovery of [^{125}I]-iodocyanopindolol binding activity in this fraction varied up to 35%, and recovery of G_s varied up to 70%. Loss of receptors probably reflects denaturation, since total recovery was unchanged if receptors were assayed with [^{3}H]dihydroalprenolol in the presence or absence of digitonin. This preparation displayed efficient receptor-catalyzed binding of [^{35}S]GTPγS (see below) and activation of G_s by GTPγS. The extent of receptor-catalyzed activation and binding ranged from 60% to 120% of the total G_s, indicating that most or all G_s-containing vesicles also contained active receptors.

When this preparation, referred to as receptor–G_s vesicles, was incubated with 0.1 μM [γ-^{32}P]GTP, it expressed catecholamine-stimulated GTPase activity (Figure 1). Basal activity, defined as that observed in the presence of 1 mM free Mg^{2+}, was stimulated up to 15-fold by β-adrenergic agonists. GTP hydrolysis was not linear with time but declined during the assay period, probably reflecting denaturation of G_s at 30

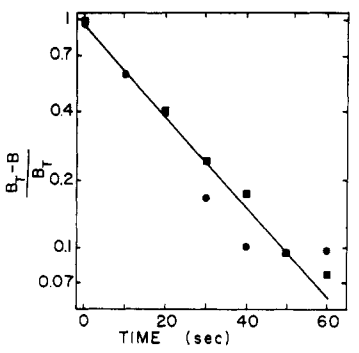

FIGURE 2: Catecholamine-stimulated binding of [^{35}S]GTPγS to receptor–G_s vesicles. Data are from two experiments using two different vesicle preparations (●, ■). Vesicles (3 μL) were incubated at 30 °C in a binding assay mixture containing 2 mM $MgCl_2$ (1 mM free Mg^{2+}), 0.1 μM [^{35}S]GTPγS, and either 1 μM (−)-isoproterenol or 100 nM (−)-propranolol. The apparent K_d for [^{35}S]GTPγS to G_s in vesicles is 5–10 nM, and the concentration of isoproterenol giving half-maximal stimulation of binding is 3.5 nM (not shown). [^{35}S]-GTPγS was allowed to associate with the vesicles for the times shown, as well as for 90, 120, and 180 s. The data shown are the incremental amounts of binding due to isoproterenol. Bound [^{35}S]GTPγS at 90, 120, and 180 s was averaged for each experiment to yield B_T [26.2 fmol (●), 33.4 fmol (■)] and used to normalize binding at shorter times. The data shown [log $[(B_T - B)/B_T]$ vs. t] were fit by an unweighted linear least-squares formula.

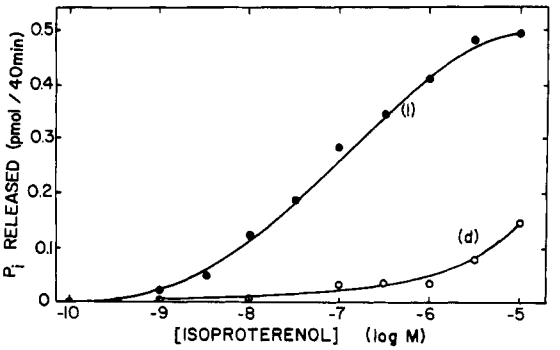

FIGURE 3: Response of reconstituted GTPase to *l*-(−)- and *d*-(+)-isoproterenol. Reactions were carried out for 40 min with 0.1 μM [γ-^{32}P]GTP. The volume of vesicles used was 2 μL/assay.

°C. However, assays were frequently carried out for more than 5 min to enhance the signal. Omission of adenyl-5′-yl imidodiphosphate increased the basal GTPase rate by about 10% and did not decrease the increment caused by agonist.

The rate of hormone-stimulated GTPase can be expressed as a molar turnover number by measuring the total number of G_s molecules according to [^{35}S]GTPγS binding (Experimental Procedures). When normalized thus, reconstituted hormone-stimulated GTPase has a maximal observed turnover number of 1–1.5 mol of GTP hydrolyzed·min^{-1}·(mol of G_s)$^{-1}$ at short times (≤5 min). At longer times, the turnover number is as low as 0.4 mol of GTP·min^{-1}·(mol of G_s)$^{-1}$, exceptionally slow for a typical enzyme. However, the rate of nucleotide binding to G_s, which is an upper limit on the rate of hydrolysis, is also slow. In the experiments shown in Figure 2, the pseudo-first-order association rate constant for the agonist-stimulated binding of the substrate analogue GTPγS to vesicle-bound G_s was found to be 2.9 min^{-1}. This value, which has varied from 1.7 min^{-1} to 3.4 min^{-1} in five vesicle preparations, was not significantly faster than the turnover number for the GTPase.

Stimulation of GTPase activity in the receptor–G_s vesicles displays typical β-adrenergic specificity. (−)-Isoproterenol was much more potent than the (+) isomer (Figure 3). Half-maximal stimulation was observed at about 100 nM (−)-iso-

Table I: Inhibition of Hormone-Stimulated GTPase by Purine Nucleotides[a]

nucleotide	% activity: 1 μM nucleotide	% activity: 10 μM nucleotide
none	(100)	(100)
GTPγS	7	3
GTP	27	6
GDP[b]	56	17
Gpp(NH)p	76	57
ITP	89	54
ATP[b]	84	78

[a] Activity was measured at 200 nM [γ-^{32}P]GTP in the presence of 1 μM (−)-isoproterenol. Total activity was 0.89 pmol of P_i/40 min, and each assay contained 4 μL of vesicles (63 fmol of GTPγS-binding sites). [b] No effort was made to ensure that GDP was not converted to GTP during the assay. Purchased ATP (Sigma) may be contaminated with GTP.

proterenol, similar to the K_d (0.14–0.34 μM in three experiments). The curve shown in Figure 3 is shallow and can be described by an apparent Hill coefficient of about 0.6–0.65. The basis of this behavior is unclear, but the binding of (−)-isoproterenol yields a similarly shallow curve. Neither the K_d for isoproterenol nor the concentration dependence for activation of the GTPase was a sensitive function of the concentration of GTP present in the assay. Stimulation by isoproterenol is blocked by the antagonist propranolol (Figure 1) with about 70-fold selectivity for the (−) over the (+) isomer (not shown).

Saturation of the reconstituted GTPase activity by substrate has not been observed in several experiments using up to 10 μM GTP (Figure 4). The failure to saturate was noted whether total hormone-stimulated activity or the agonist-induced increment above basal was considered. These data are consistent with the formal existence of two sites for GTP binding with affinities (K_m's) of about 30 nM and 3 μM but might reflect some other heterogeneity (environmental, well coupled vs. poorly coupled, etc.). Since assay of GTPase activity above 10 μM GTP is difficult, these issues have not yet been resolved. Similar behavior has been noted for prostaglandin-stimulated GTPase in platelet membranes (Lester et al., 1982). Hydrolytic activity is relatively specific for GTP (Table I). GTPase is inhibited by unlabeled GTP or GTPγS, more weakly by GDP, guanyl-5′-yl imidodiphosphate [Gpp(NH)p], or inosine 5′-triphosphate (ITP), and only slightly by adenosine 5′-triphosphate (ATP).

The data of Table II indicate that the observed GTPase activity is localized on hepatic G_s. Denaturation of G_s by

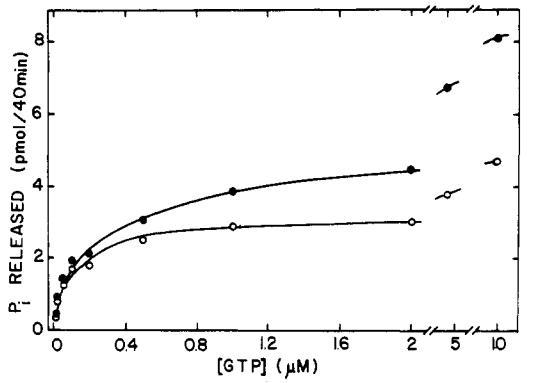

FIGURE 4: Substrate dependence of reconstituted GTPase. GTPase was assayed over 40 min at varying concentrations of [γ-^{32}P]GTP. The specific activity varied from 2.1 to 195 cpm/fmol. Each assay contained 10 μL of vesicles, equivalent to 0.23 pmol of GTPγS binding activity. Assays were performed in the presence of 50 μM (−)-isoproterenol (●) or 0.1 μM (−)-propranolol (equivalent to no addition). The increment in GTPase activity due to isoproterenol is also shown (○).

incubation at 37 °C in medium containing 50 mM $MgCl_2$ (Smigel et al., 1982) prior to reconstitution destroyed the GTPase activity in the vesicles. A small amount of adenylate cyclase activity that cofractionated with purified receptors in some preparations of receptors (about 1–10% of the specific activity of plasma membranes) could be denatured by heating or by thiol reagents without decreasing GTPase activity. Purified rabbit hepatic G_s also displays GTPase activity upon stimulation with $MgCl_2$ (Table III). Activity did not require either β-adrenergic receptors or reconstitution with phospholipid. However, Lubrol 12A9, the detergent in which pure G_s is usually prepared and stored, inhibited GTPase activity, even at 1 mg/mL. This is in contrast to its enhancement of measurable binding of [^{35}S]GTPγS. GTPase activity was high even in the absence of phospholipid and detergent, conditions under which G_s is presumably aggregated (Sternweis et al., 1981). The inhibition of GTPase by Lubrol and the labilization of G_s by $MgCl_2$ probably account for the failure of Northup et al. (1982) to detect significant GTPase activity in purified G_s. In the presence of Lubrol and at lower concentrations of Mg^{2+}, the GTPase activity we measure is consistent with their data.

Discussion

The results reported here indicate that reconstitution of purified G_s and partially purified β-adrenergic receptors into phospholipid vesicles enhances GTPase activity of G_s and

Table II: Inactivation of G_s, Receptor, and Contaminating Adenylate Cyclase Prior to Reconstitution

treatment: receptor	treatment: G_s	activity (% of control): receptor binding[a]	agonist-stimulated GTPase[b]	[^{35}S]GTPγS bound: agonist-stimulated[c]	[^{35}S]GTPγS bound: total[d]	G_s activation[e]	adenylate cyclase[f]
30 °C, 75 min	NT[g]	89	79	96	89	108	9
70 °C, 10 min	NT	0	0	0	82	105	0
10 mM *N*-ethylmaleimide, 0 °C, 60 min	NT	335	104	94	115	121	0
NT	50 mM $MgCl_2$, 37 °C, 120 min	95	6	19	12	2	104

[a] By [^{125}I]iodocyanopindolol binding (260 pM ligand; K_d = 25–35 nM). 100% = 3.5 pmol/mL. [b] 100% = 315 pmol·40 min^{-1}·mL^{-1}. Assayed at 200 nM GTP. Data shown are increments over basal caused by 1 μM isoproterenol. [c] 100% = 7.2 pmol/mL. Assayed at 0.1 μM GTPγS. Data shown are for the increment at 2 min caused by 1 μM isoproterenol. [d] 100% = 26 pmol/mL. Assayed at 10 μM GTPγS and 50 mM $MgCl_2$ for 30 min. [e] 100% = 20 nmol of cyclic AMP·min^{-1}·mL^{-1}. Activated G_s was assayed according to its ability to activate the catalytic unit of adenylate cyclase [see Pedersen & Ross (1982)]. [f] 100% = 7.1 pmol of cyclic AMP·min^{-1}·mL^{-1}. Assayed in the presence of 3 mM $MnCl_2$ and 0.15 mM forskolin. [g] NT = not treated.

Table III: Mg^{2+}-Stimulated GTPase Activity of Purified G_s

medium[a]	GTPase[b] (nmol·30 min^{-1}·mg^{-1})	(GTPase + Lubrol)/(GTPase − Lubrol)	[^{35}S]-GTPγS bound[c] (nmol/mg)	(binding + Lubrol)/(binding − Lubrol)
buffer	21.6	0.27	0.8	5.0
buffer + Lubrol	5.8		4.1	
DMPC	20.5	0.34	1.1	3.3
DMPC + Lubrol	7.1		3.6	
EL/DMPC	13.3	0.17	2.4	1.3
EL/DMPC + Lubrol	2.3		3.3	

[a] G_s was slowly diluted with 51.5 volumes of 20 mM NaHepes (pH 8)–1 mM EDTA–1 mM DTT–0.1 M NaCl (buffer) containing 0.1 mg/mL dimyristoylphosphatidylcholine (DMPC) or a mixture of 0.05 mg/mL erythrocyte lipids (EL) plus 0.05 mg/mL DMPC or these mixtures containing 1 mg/mL Lubrol 12A9. 10 μL of these preparations was assayed in a 100-μL volume. [b] GTPase was assayed for 30 min at 30 °C in the presence of 50 mM $MgCl_2$ and 2 μM [γ-^{32}P]GTP. [c] GTPγS binding was assayed in medium containing 50 mM $MgCl_2$ and 10 μM [^{35}S]GTPγS.

allows β-adrenergic stimulation of that activity. This system represents several technical advances over previous reconstituted preparations. Primarily, the increased purity of the receptors allows us to assay both the GTPase activity of G_s and its specific guanine nucleotide binding activity without interference from spurious nucleoside triphosphatases and other enzymes that bind GTP. We can therefore use ligand-binding assays to quantitate the number of receptor and G_s molecules present, their fractional saturation with ligands, and the moles of GTP hydrolyzed per mole of G_s. The reconstituted receptors and G_s are well coupled, as defined by the ability of receptors to promote the activation of multiple molecules of G_s by GTPγS (Pedersen & Ross, 1982). Such activation parallels the quasi-irreversible binding of [^{35}S]GTPγS (Northup et al., 1982). In our earlier preparation (Pedersen & Ross, 1982), only a small fraction of G_s could be activated in a receptor-catalyzed fashion, and we hypothesized that the limiting factor was that relatively few of the G_s-containing vesicles also contained a receptor. In the preparation used here, essentially all of the G_s molecules can be stimulated by receptor to bind [^{35}S]GTPγS (T. Asano and E. M. Ross, unpublished experiments).

The maximum observed turnover number of the GTPase, 1–1.5 min^{-1}, is small in comparison to that of typical metabolic enzymes, and it is unlikely that this low value merely reflects the use of subsaturating concentrations of GTP. However, the estimated rate of the catecholamine-stimulated GTPase in turkey erythrocyte membranes, 5 pmol·min^{-1}·mg^{-1}, is also low (Cassel & Selinger, 1976). Hanski et al. (1981) purified G_s from these membranes about 4000-fold to apparent homogeneity, implying that the turnover number in membranes is (5 pmol·min^{-1}·mg^{-1})·4000·(80 000 g·mol^{-1}) = 1.6 min^{-1}. This value is closely approached in reconstituted vesicles. The rate of the reconstituted GTPase can also be compared with the association rate of GTP, approximated in Figure 2 with GTPγS. The pseudo-first-order rate constant for GTPγS binding, which has varied from 1.7 to 3.4 min^{-1}, should reflect an upper limit on the turnover number of the GTPase. Thus, two independent estimates of an appropriate rate of GTP hydrolysis correspond to the rate determined here. Since β-adrenergic specificity is displayed and the enhancement of activity by agonist is large, we feel that the reconstituted system diplays good fidelity to the in vivo activity.

Two qualitative conclusions also emerge from these experiments. First, G_s alone can display GTPase activity (Tables II and III). While hormone-liganded receptor can stimulate that activity dramatically, so can Mg^{2+}. There is no evidence that the catalytic unit of adenylate cyclase stimulates the GTPase, although this has been widely assumed. The denaturation of the small amount of contaminating adenylate cyclase in the vesicles has no effect on GTPase activity (Table II). This implies that the actual hydrolysis of G_s-bound GTP, the "turn-off reaction" of Cassel & Selinger (1977a,b), may occur at a constant rate and that the only hormone-regulated step in the GTPase cycle may be substrate binding (or product release). Alternatively, physiologic concentrations of the catalytic unit might actually stabilize G_s–GTP to hydrolysis, thus prolonging the lifetime of the active adenylate cyclase complex.

A second conclusion of this work is that β-adrenergic receptors that have been solubilized in digitonin and purified by affinity chromatography on antagonist-substituted agarose retain their biological regulatory activity as well as their ability to bind ligands. This is vital, since this strategy has provided the only useful preparations of purified receptors (Shorr et al., 1982, and references cited therein). While we have not reconstituted receptors with purity greater than 2 nmol/mg (9% pure if M_r = 45 000; Shorr et al., 1982), our data using such 4000-fold purified receptors do not suggest the presence of any novel, readily dissociable factor needed for the productive interaction of receptors with G_s.

We speculate that the crucial feature in our restoration of GTPase activity to G_s is the stabilization against denaturation that reconstitution into a bilayer provides. The efficient stimulation of GTPase activity by the agonist-bound receptor probably derives from the ability of receptors to promote binding of substrate and, perhaps, to stabilize G_s further. This is in marked contrast to Mg^{2+}, which promotes thermal denaturation of G_s as it promotes binding (Northup et al., 1982; T. Asano and E. M. Ross, unpublished data). Smigel et al. (1982) have postulated that Mg^{2+} promotes high-affinity GTPγS binding and the activation of G_s by initiating the dissociation of its two subunits. Our data so far are consistent with hormone receptors also promoting the dissociation of the G_s subunits. We feel that the present reconstituted preparation of pure G_s and substantially purified receptor should allow us to study the mechanism of these processes quantitatively in molecular detail.

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Imino Proton Assignments in the Proton Nuclear Magnetic Resonance Spectrum of the λ Phage O_R3 Deoxyribonucleic Acid Fragment[†,1]

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ABSTRACT: The 17 base pair duplex d(TATCACCGCAAGGGATAp)·d(TATCCCTTGCGGTGATAp) corresponding to the O_R3 operator site of λ phage has been synthesized and studied by ^1H nuclear magnetic resonance spectroscopy at 470 MHz. The 13 imino proton resonances observed at 20 °C have been assigned to specific base pairs at positions 3-15 on the basis of nuclear Overhauser effect measurements and studies of the temperature dependence of peak intensities. Resonances from the A-T base pairs at positions 1, 2, 16, and 17 are assumed to be absent from the spectrum because of terminal fraying. Resonances from many of the base pairs suggested by Ohlendorf et al. [Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y., & Matthews, B. W. (1982) *Nature (London) 298*, 718-723] to be involved in specific binding of the λ phage cro repressor are well resolved.

A λ phage infection of *Escherichia coli* may follow either a lytic or a lysogenic life cycle (Ptashne et al., 1980; Echols, 1980; Herskowitz & Hagen, 1980). The choice depends on a delicate balance between the binding of the λ cI and cro repressors to the phage right-hand operator and several other phage and host factors (Herskowitz & Hagen, 1980; Little & Mount, 1982). Three repressor binding sites exist in the λ right-hand operator, O_R1, O_R2, and O_R3 (Ptashne et al., 1980). The cro repressor (as a dimer) binds preferentially to the O_R3 site (Ptashne et al., 1980) and represses transcription of genes from the P_{rm} promoter. Repression at P_{rm} turns off transcription of the cI gene and other λ genes required for a lysogenic life cycle. Transcription from the P_r promoter is allowed and leads to a lytic λ infection. Biophysical studies of this system are of great interest since they may illuminate how specific DNA-protein interactions occur and how they can serve as a switch for the control of biological functions.

The cro repressor structure has been determined by X-ray crystallography (Anderson et al., 1981). Through extensive model building exercises, Ohlendorf et al. (1982) have proposed several DNA-amino acid interactions they believe are involved in the specific binding of the cro repressor to the O_R3 DNA site. Their model is consistent with DNA chemical modification experiments (Ptashne et al., 1980; Johnson et al., 1979) and other studies of cro repressor binding to DNA (Boschelli, 1982; Boschelli et al., 1982). Four NMR studies of the cro repressor (Kirpichnikov et al., 1982; Kurochkin & Kirpichnikov, 1982) and its nonspecific binding to DNA have been published (Iwahashi et al., 1982; Arndt et al., 1983). We report here assignments for the imino proton resonances in the ^1H NMR spectrum of the λ phage O_R3 DNA fragment. A preliminary report of these data has been presented (Ulrich et al., 1983). The assignments were made through an analysis of imino-imino proton nuclear Overhauser enhancement (NOE) experiments, a technique originally developed for studies of tRNA (Roy & Redfield, 1981) and recently applied to duplex DNA molecules (Patel et al., 1982a,b, 1983; Scheek et al., 1983) and the temperature dependence of the imino proton peak intensities.

Materials and Methods

Chemical Synthesis of Oligodeoxyribonucleotides. The two fully protected oligonucleotides d(TATCACCGCAAGGGATA)rC and d(TATCCCTTGCGGTGATA)rC were synthesized by sequential condensations of tetramer and pentamer

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[1] *Editorial note*: Acceptance of this paper as an accelerated publication was initially declined upon the recommendation of the primary reviewers. This decision was appealed by the authors after a similar paper by Chou et al. [Chou, S.-H., Hare, D. R., Wemmer, D. E., & Reid, B. R. (1983) *Biochemistry 22*, 3037-3041] was published upon the recommendation of two different reviewers. The appeal was granted upon the advice of an ad hoc member of the Editoral Advisory Board who recommended that the abstract or the full paper be published post facto.

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