Conformational Changes of Adenylate Cyclase Regulatory Proteins Mediated by Guanine Nucleotides*

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Cholera toxin, in the presence of [32P]NAD*, catalyzes the specific radiolabeling of a \( M_r = 42,000 \) subunit of a protein (G protein) which mediates guanine nucleotide regulation of hormone-sensitive adenylate cyclase in pigeon erythrocyte membranes. Treatment of labeled membranes with GMP and isoproterenol followed by extensive washing presumably releases tightly bound GDP from the G-protein regulatory site. In the absence of added guanine nucleotide, treatment of these membranes with trypsin results in the partial digestion of the \( M_r = 42,000 \) radiolabeled peptide and production of several minor trypsin-specific fragments which remain associated with the membrane. Incubation of membranes with guanosine-5'-O-(3-thiotriphosphate) (GTPyS) alters the partial trypptic digestion of the \( M_r = 42,000 \) radiolabeled peptide, resulting in a loss of the minor fragments and the quantitative generation of a \( M_r = 41,000 \) peptide. GTPyS exposes a new site to trypsic cleavage which releases from the membrane an inactive fragment of the G-protein containing the \( M_r = 41,000 \) labeled peptide.

Guanosine-5'-O-(2-thiodiphosphate) (GDP\( \beta S \)) fails to activate adenylate cyclase and does not cause the generation of the \( M_r = 41,000 \) trypptic fragment. Incubation of membranes with GDP\( \beta S \) does, however, decrease trypptic digestion of the \( M_r = 42,000 \) labeled peptide. The effects of GTP-S and GDP\( \beta S \) on the partial trypptic digestion of the G-protein are competitive suggesting their action is mediated by a common binding site.

Generation of the GTP\( \gamma S \)-specific \( M_r = 41,000 \) trypptic fragment from the \( M_r = 42,000 \) toxin labeled peptide exhibits a time-dependent increase upon incubation of membranes with GTP\( \gamma S \) which is similar to that for cyclase activation. Addition of isoproterenol to the GTP\( \gamma S \) incubation diminishes the lag time for cyclase activation and the generation of the \( M_r = 41,000 \) fragment.

Human erythrocyte membranes possess the G-protein but functionally lack both the \( \beta \)-adrenergic receptor and catalytic adenylate cyclase. Incubation of cholera toxin-labeled human erythrocyte membranes with GTPyS followed by exposure to trypsin also generates a specific \( M_r = 41,000 \) fragment.
examine differences in proteolytic susceptibility of the $M_r = 42,000$ peptide after incubation of membranes with guanine nucleotide. Our results indicate that guanine triphosphates cause a specific conformational change of the G-protein, and that hormone-receptor interactions affect the rate of guanine triphosphate-induced change in conformation.

**EXPERIMENTAL PROCEDURES**

**Methods**

Isolation of Plasma Membranes—Freshly collected pigeon erythrocytes were lysed in the presence of DNAse and membranes were prepared as described previously for turkey erythrocytes (19). Human erythrocytes were prepared similarly without the DNAse treatment.

Cholera Toxin Treatment of Membranes—Membranes were washed by centrifugation in 500 mM potassium phosphate (pH 7.0), and resuspended (5 mg of protein/ml) in 250 mM potassium phosphate (pH 7.0), 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine-HCl, 100 μM GTP, 400 μM ATP, 10 μg/ml of cholina toxin (activated with 20 mM diethiothreitol (20)), and 25 μM NAD. The reaction mixture was incubated for 30 min at 30°C and terminated by dilution and centrifugation in 10 mM of ice-cold 20 mM Hepes (pH 8.0), 2 mM MgCl₂, 1 mM EDTA, and 1 mM 2-mercaptoethanol (membrane buffer). For radiolabeling, the specific cholera toxin substrate ['³²P]NAD was added to the above incubation at a specific activity of 5 Ci/mM/mmol.

Protease Digestions—Digestions were performed in membrane buffer at 22°C. The membrane concentration was 4 mg of protein/ml. The reaction was inhibited by the addition of aprotinin followed by the addition of SDS and 2-mercaptoethanol to final concentrations of 1 and 5%, respectively. Peptide mapping using two-dimensional gels was performed by a modification of the method of Cleveland et al. (21) as described previously (18).

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography—Membranes in 1% SDS and 5% 2-mercaptoethanol were boiled for 5 min and 70°C glycerol was added to a final concentration of 12%. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (22) as described by O'Farrell (23) using a 12.5% acrylamide separating gel. Gels were stained and fixed in 2% Coomassie blue, 50% acetic acid, 10% ethanol, and destained in 10% acetic acid, 10% ethanol. Dried gels were used to expose Kodak X-Omat R film and DuPont lightning-plus intensifying screens at -70°C.

Adenylate Cyclase Assay—Adenylate cyclase was assayed in a mixture containing 0.4 mM [³²P]ATP (10 to 25 cpm/pmol), 50 mM Na Hepes (pH 8.0), 6 mM MgCl₂, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, 0.1 mg/ml of bovine serum albumin, 10 mM creatine phosphate, 10 units/ml of creatine phosphokinase (in a final volume of 100 μl). Effects of adenylate cyclase included in the reaction mixture when appropriate were 10 μM dl-isoproterenol, 100 μM GTPyS, 100 μM GDPyS. The assay was initiated by adding 25 μg of membrane protein to the reaction mixture and incubated at 30°C. The reaction was terminated by the addition of 1 ml of 1% SDS. [³²P]AMP was purified by sequential chromatography on Dowex and alumina.

Chromatographic Analysis of GDPyS—GDPyS (1 mM final concentration) was incubated either (a) for 30 min at 30°C in the adenylate cyclase reaction mixture containing pigeon erythrocyte membranes (0.4 mg of membrane protein/ml), or (b) for 30 min at 22°C in membrane buffer containing pigeon erythrocyte membranes (0.5 mg of membrane protein/ml). The membranes were removed by centrifugation and 15 μl of the supernatant was chromatographed on polyathylenimine cellulose sheets (Whatman CM 300) as described by Eckstein et al. (25). The developing solution contained 0.5 M NaCl and 0.75 M Tris-HCl (pH 7.5). The nucleotides were visualized by UV absorption. One major spot of $R_f = 0.23$ corresponding to GDPyS (25) and a minor spot (less than 10% of total) of $R_f = 0.07$ were observed in incubation a. Only the major GDPyS spot was observed in the supernatant of incubation b.

Materials

GTPyS and GDPyS were from Boehringer Mannheim. dl-Isoproterenol HCl and aprotinin were purchased from Sigma. Trypsin and chymotrypsin were purchased from Worthington. [³²P]NAD and $[γ-³²P]ATP$ were purchased from New England Nuclear. Cholera toxin was obtained from Schwarz/Mann. All other materials were reagent grade.

**RESULTS**

Fig. 1 shows limited tryptic digestion patterns of the $M_r = 42,000$ radiolabeled toxin substrate in the presence or absence of GDPyS or GTPyS after treatment of membranes with isoproterenol and GMP. The thio analogs of GDP and GTP are resistant to cellular nucleotide phosphatases, GTPyS being an activator of adenylate cyclase (24-26) and GDPyS being a competitive inhibitor or partial activator (25, 26). Incubation of membranes with guanine nucleotide does not affect the behavior of the $M_r = 42,000$ peptide on SDS acrylamide gels (not shown). In the absence of guanine nucleotide (Fig. 1, Lane 2), trypsin treatment of the membranes results in the partial digestion of the $M_r = 42,000$ labeled peptide. The labeled proteins at the top of the gel in Lanes 1 to 4 other than the $M_r = 42,000$ peptide are nonspecifically labeled proteins that generally migrate as broad diffuse bands. These nonspecifically labeled proteins are seen also in control preparations which are treated in exactly the same manner but without the addition of cholera toxin to the reaction mixture. When control preparations are partially digested with trypsin (Lanes 5 to 7), these nonspecifically labeled bands are partially digested but do not contribute any of the fragments designated by arrows in Fig. 1, and do not impair the analysis of digest products of the toxin-specific $M_r = 42,000$ radiolabeled peptide.

Both GDPyS and GTPyS affect the digest pattern produced.
by trypsin. Incubation of membranes with GDPβS (Lane 3)
causes a decrease in tryptic digestion of the $M_r = 42,000$
peptide. Similar effects of GDPβS and GTPγS on
tryptic digestion of the $M_r = 42,000$ labeled peptide are ob-
served with Lubrol PX-solubilized G protein only if the mem-
brares are preincubated with isoproterenol and GMP (data
not shown).

Fig. 2 shows the densitometric tracings of the autoradiograms (Lanes 1 to 4) of Fig. 1. It is clearly shown that in the
absence of added guanine nucleotide, trypsin treatment of
membranes reduces the label in the $M_r = 42,000$ band by
approximately 50%, resulting in the generation of several
minor fragments. Addition of GDPβS to the incubation
mixture results in a marked protection of the radiolabeled peptide
from trypsin digestion. In contrast to the effect of GDPβS, the
presence of GTPγS in the incubation results in the generation
of a $M_r = 41,000$ fragment with a corresponding quantitative
decrease in the $M_r = 42,000$ radiolabeled peptide, suggesting that the $M_r = 41,000$ fragment is generated by trypsin digestion
of the $M_r = 42,000$ peptide.

Fig. 3 provides further evidence that the $M_r = 41,000$ peptide
is a tryptic digest product of the $M_r = 42,000$ toxin-specific
band. Partial proteolytic digestion using Staphylococcus aureus
V8 protease indicates that the $M_r = 41,000$ peptide
generated by trypsin digestion of GTPγS-treated membranes
produces similar labeled peptides as the $M_r = 42,000$
toxin-specific peptide. The presence of one or two apparently unique
peptides in the $M_r = 41,000$ peptide digest pattern is not
unexpected, since this peptide is a tryptic digestion product of
the $M_r = 42,000$ peptide. Significantly, none of the nonspecifically
labeled peptides in other regions of the gel generate peptide fragments that correspond to any of the fragments in
the maps of the cholera toxin-specific tryptic peptides. These
results substantiate the findings that the nonspecifically la-
beled proteins do not contribute any of the peptide fragments
designated by arrows in Fig. 1.

Previously, it was demonstrated that GDPβS is either a
competitive inhibitor or a weak partial activator of adenylate
cyclase (25, 26). Table I confirms these findings with the
pigeon erythrocyte system. Membranes pretreated with
isoproterenol and GMP followed by thorough washing are mark-
ely stimulated by GTPγS, whereas GDPβS is ineffective.
In addition, GDPβS is able to inhibit GTPγS activation of ade-
nylate cyclase in a concentration dependent manner. Fig. 4
shows that GDPβS also is able to inhibit GTPγS-mediated
formation of the $M_r = 41,000$ tryptic fragment. When mem-
brares are incubated with a 2-fold excess of GDPβS relative
to GTPγS, there is a marked decrease in the ability of trypsin
to generate the $M_r = 41,000$ peptide, relative to GTPγS alone.
GDPβS alone causes little or no generation of the $M_r = 41,000$
tryptic fragment. The $M_r = 41,000$ fragment is therefore a
specific marker for guanine triphosphate-specific changes in
conformation of the G-protein.

In order to observe an effect of hormone on the susceptibil-
ity of the $M_r = 41,000$ labeled peptide, we used membranes
which were not preincubated with isoproterenol and GMP.
Fig. 5 shows the adenylate cyclase activity of pigeon erythro-
cyte membranes incubated for different periods of time in the
presence or absence of GTPγS and isoproterenol, followed by
washing of the membranes and subsequent assay of cyclase
activity. As demonstrated previously (12), hormone acceler-
ates the rate at which maximal activation of adenylate cyclase
is achieved by hydrolysis-resistant GTP analogs. With suffi-
cient time, the cyclase activity obtained by incubation with
GTPγS alone is similar to that achieved in the presence of
isoproterenol and GTPγS. Furthermore, the activation is sta-
ble to washing. The addition of GTPγS or isoproterenol to
the adenylate cyclase assay mixture has little effect on enzyme
activity once maximal activation is achieved.

Fig. 6 shows the tryptic digestion products of the $M_r = 42,000$
radiolabeled peptide during the time course of cyclase
activation depicted in Fig. 5. The autoradiographs in Fig. 6A
were exposed for only 18 h. It is readily apparent that there is
a time-dependent GTPγS-mediated increase in the generation
of the $M_r = 41,000$ tryptic fragment (Fig. 6A, Lanes 2, 6, and
10) and that isoproterenol accelerates the rate of formation of
this digest product (Lanes 4, 8, and 12). Densitometry of the
autoradiographs shown in Fig. 6A indicates a quantitative
relationship between the formation of the $M_r = 41,000$
fragment and a corresponding decrease in the $M_r = 42,000$ peptide.
TABLE I

GDPβS competition of GTPγS activation of adenylate cyclase

Pigeon erythrocyte membranes were incubated with 10 μM isoproterenol and 100 μM GMP for 20 min at 30°C. Membranes were then washed three times by dilution with 10 ml of membrane buffer and centrifugation for 10 min at 30,000 × g. Membranes were resuspended in membrane buffer and adenylate cyclase activity was measured during a 30-min assay in the presence of various concentrations of GTPγS or GDPβS. Each value represents the mean of triplicate determinations and is representative of three separate experiments.

<table>
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<tr>
<th>GTPγS (μM)</th>
<th>GDPβS (μM)</th>
<th>Adenylate cyclase activity (pmol/min/mg protein)</th>
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<tr>
<td>50</td>
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<td>138</td>
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<tr>
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<tr>
<td>0</td>
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<td>46</td>
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These results are similar to those observed when GTPγS was included in the incubation as shown in Fig. 2. The GTPγS-mediated change in tryptic digest patterns follows a time course similar to that for adenylate cyclase activation. Isoproterenol accelerates both effects with maximal activation of cyclase, the ability to generate the M, = 41,000 tryptic fragment being virtually complete after a 2-min incubation. With GTPγS alone in the absence of isoproterenol, maximal activation of adenylate cyclase and the ability to generate the M, = 41,000 tryptic fragment are coordinately slower in onset and reach maximal levels only after the 15-min preincubation with GTPγS. Thus, there is a close parallel in the time course of GTPγS activation of cyclase and the time course of conformational change of the G-protein resulting in the ability of trypsin to generate the M, = 41,000 fragment.

Fig. 6B represents 72-h autoradiographs of the gels shown in Fig. 6A. Several new lower abundance fragments are ob-
served whose formation is affected by either GTP$_y$S or isoproterenol. A new $M_r = 30,000$ fragment is observed whose intensity is decreased by GTP$_y$S. In addition, isoproterenol in the absence of GTP$_y$S causes a time-dependent enhancement of at least two specific bands (designated by arrows in Lanes 3, 7, and 11). The addition of GTP$_y$S or the removal of isoproterenol causes a marked reduction in the intensity of these bands. In other experiments (not shown), propranolol, a $\beta$-adrenergic antagonist, does not cause the appearance of these fragments and inhibits the effect of isoproterenol. These results indicate that the effect of isoproterenol is mediated through the $\beta$-adrenergic receptor.

We demonstrated previously (18) that tryptic fragments generated from the $M_r = 42,000$ peptide using membranes not incubated with GTP$_y$S remain bound to the membrane. This

buffer at 5 mg/ml containing the following additions: A, none; B, 10 $\mu$m isoproterenol; C, 50 $\mu$m GTP$_y$S; D, 10 $\mu$m isoproterenol and 50 $\mu$m GTP$_y$S. The membranes were incubated at 30°C for 2, 5, and 15 min, and were then washed three times by dilution in ice-cold membrane buffer and centrifugation. The membranes were then resuspended in membrane buffer and adenylate cyclase activity was measured during a 30-min incubation in the presence of no addition ($\bigcirc$), 50 $\mu$m GTP$_y$S ($\triangle$), or 50 $\mu$m GTP$_y$S and 10 $\mu$m isoproterenol ($\Delta$). Values are the means of triplicate determinations which varied by less than 10%, and are representative of four separate experiments.

![Fig. 5. Persistent activation by GTP$_y$S of adenylate cyclase activity in pigeon erythrocyte membranes. Membranes were treated with cholera toxin and NAD and then washed three times in membrane buffer. Membranes were then resuspended in the same buffer at 5 mg/ml containing the following additions: A, none; B, 10 $\mu$m isoproterenol; C, 50 $\mu$m GTP$_y$S; D, 10 $\mu$m isoproterenol and 50 $\mu$m GTP$_y$S. The membranes were incubated at 30°C for 2, 5, and 15 min, and were then washed three times by dilution in ice-cold membrane buffer and centrifugation. The membranes were then resuspended in membrane buffer and adenylate cyclase activity was measured during a 30-min incubation in the presence of no addition ($\bigcirc$), 50 $\mu$m GTP$_y$S ($\triangle$), or 50 $\mu$m GTP$_y$S and 10 $\mu$m isoproterenol ($\Delta$). Values are the means of triplicate determinations which varied by less than 10%, and are representative of four separate experiments.]

![Fig. 6. Tryptic digestion products of the $M_r = 42,000$ radiolabeled cholera toxin substrate during the time course of adenylate cyclase activation. Pigeon erythrocyte membranes were labeled with [$^{32}$P]NAD and cholera toxin as described under "Experimental Procedures." Labeled membranes were washed three times in membrane buffer and then treated exactly as described in the legend to Fig. 5. After the appropriate preincubation time, membranes were washed three times and treated with 25 $\mu$g/ml of trypsin for 12 min at 22°C. The samples were then electrophoresed on 12.5% SDS-acrylamide gels and autoradiographed. The samples in each lane correspond to membranes that were used for the assay of adenylate cyclase activity with no further additions in the second incubation (Fig. 5, open circles). Lanes represent the following additions in the preincubation: 1, 5, and 9, none; 2, 6, and 10, 50 $\mu$m GTP$_y$S; 3, 7, and 11, 10 $\mu$m isoproterenol; 4, 8, and 12, 50 $\mu$m GTP$_y$S plus 10 $\mu$m isoproterenol. A, an 18-h exposure; B, an autoradiograph of the same gel exposed for 72 h. Large arrows without tails designate an approximate $M_r = 30,000$ tryptic fragment of the $M_r = 42,000$ peptide. Small arrows with tails designate two fragments that appear to be specific for the interaction of receptor-hormone complex with the G protein in the absence of guanine nucleotide.]

Coupling Components of Adenylate Cyclase
with control or trypsin-treated membranes in the presence or absence of proteolytic digestion in the supernatant after centrifugation. This finding indicates that the release by trypsin of a G-protein fragment from the membrane only after GTPyS treatment indicates that guanine triphosphate changes the site of trypsin digestion resulting from a conformational change of the G-protein.

Fig. 8 shows that with human erythrocytes, GTPyS but not GDPβS is capable of mediating the conformational change of the G-protein, resulting in a \( M_r = 41,000 \) fragment when the membranes are subsequently exposed to trypsin. This finding is significant, because human erythrocytes possess the G-protein of adenylate cyclase but functionally lack both hormone receptors and catalytic adenylate cyclase (27–29). Therefore, the ability of guanine triphosphates to cause a conformational change of the G-protein does not require its interaction with either hormone receptor or catalytic cyclase.

**DISCUSSION**

We have demonstrated guanine nucleotide-specific changes in susceptibility of the \( M_r = 42,000 \) cholera toxin substrate to trypsin digestion. A unique \( M_r = 41,000 \) peptide is generated in the presence of GTPyS. In some experiments, a very small amount of the \( M_r = 41,000 \) fragment could be detected in the presence of GDPβS. However, this is always less than 5% of that generated in the presence of GTPyS. The formation of the \( M_r = 41,000 \) fragment can therefore be used to measure guanine triphosphate interactions with the G-protein.

Hormone-receptor interaction was shown to affect the rate of the specific conformational change induced by GTPyS, i.e. the generation of the \( M_r = 41,000 \) trypsin fragment. This finding supports the Cassel and Selinger hypothesis (5) that hormone acts by making the G-protein regulatory site available to guanine triphosphate. In addition to its effects on guanine nucleotide accessibility, isoproterenol in the absence of guanine nucleotide results in a partial trypsin digest pattern of \( M_r = 42,000 \) labeled peptide distinguishable from that generated in the presence of GTPyS. The formation of the \( M_r = 41,000 \) fragment can therefore be used to measure guanine triphosphate interactions with the G-protein.

The simplest explanation for this finding is that hormone-receptor complex physically interacts with the G-protein. This notion is substantiated by the recent findings of Limbird et al. (30), which demonstrate that the binding of the \( \beta \)-adrenergic agonist hydroxybenzylisoproylterenol to membranes followed by detergent solubilization resulted in an increase in the Stokes radius of the soluble \( M_r = 42,000 \) toxin-labeled peptide on gel filtration columns, indicating the association of receptor with the G-protein. The addition of guanine triphosphate resulted in the dissociation of receptor from the G-protein. Our results extend this finding, in that we have directly demonstrated that GTPyS alters the conformation of the G-protein so that it is capable of activating adenylate cyclase in a quasi-reversible manner, and greatly diminishes the influence of hormone-receptor interaction on G-protein trypsin digestion.

The simplest interpretation of our results is that guanine triphosphates cause a conformational change of the G-protein resulting in altered partial trypsin digest patterns. Pfeuffer (2) and Howlett and Gilman (31) have recently reported changes in the sedimentation behavior of the G-protein in sucrose gradients following incubation with guanine triphosphates, supporting the notion of conformational changes of the G-protein mediated by GTPyS. The fact that Pfeuffer (2) was able to observe this effect of guanine triphosphates using
pigeon erythrocyte G-protein resolved from catalytic adenylate cyclase suggests that the conformational change is a result of guanine nucleotide interaction with the G-protein, and not interaction of the protein with the catalytic unit. Our results showing the change in tryptic digest pattern mediated by GTPγS of the G-protein from human erythrocytes confirm this hypothesis (Fig. 8).

The finding that incubation of membranes with GTPγS results in the release from the membrane of a tryptic fragment of the \( M_r = 126,000 \) G-protein supports the hypothesis first suggested by Ross et al. (1) and substantiated by others (2, 16, 18) that the G-protein has a small hydrophobic domain and is probably a stalked intrinsic membrane protein (16, 18). It is tempting to speculate that the small fragment lost from the \( M_r = 42,000 \) toxin-labeled peptide after tryptic digestion of the GTPγS-activated G-protein is part of the stalk that binds the protein to the membrane. The fact that the protein released from the membrane is inactive is not surprising, based on our previous observation (18) that there appears to be a second peptide, probably a subunit of the G-protein, which is more sensitive to trypsin than the \( M_r = 42,000 \) peptide which was required for reconstitution with cyc1 membranes.

A major question still to be answered is how hormone receptors interact with the G-protein. The work of Limbird et al. (30) suggests that \( \beta \)-adrenergic receptors can physically interact with the G-protein. The findings we have presented here indicate that guanine triphosphates cause a specific conformational change of the G-protein, hormone increases the rate of guanine triphosphate-mediated conformational change, and the receptor-hormone interactions induce changes in tryptic digest patterns of the toxin-labeled \( M_r = 42,000 \) peptide in the absence of guanine triphosphate. This last observation indicates that receptor-mediated events alter the conformation of the G-protein. The ability to measure conformational changes of the G-protein mediated by guanine nucleotides and hormone-receptor interactions by partial tryptic digestion and peptide mapping provides a new approach to study the regulation of hormone-sensitive adenylate cyclase. We are now using this technique of partial proteolytic digestion together with reconstitution procedures to combine \( \beta \)-adrenergic receptors and the G-protein into defined membrane environments in an attempt to gain insight into the molecular mechanisms of coupling.

REFERENCES