Purification and Properties of the Inhibitory Guanine Nucleotide-binding Regulatory Component of Adenylate Cyclase*

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Attenuation of GTP-dependent inhibition of adenylate cyclase by islet-activating protein (pertussis toxin) is due to the ability of the toxin to catalyze the ADP-ribosylation of a 41,000/35,000-Da membrane-bound protein, which is thought to be the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase (Gi). We describe and document the purification of this protein from rabbit liver, and, in conjunction with evidence presented in the subsequent papers of the series, identify it as Gi.

Purified Gi serves as an excellent substrate for islet-activating protein and can be ADP-ribosylated to the extent of 1 mol of ADP-ribose/mol of protein. The extent of ADP-ribosylation of Gi correlates with the amount of guanine nucleotide that can be bound to the protein. Studies of the nucleotide binding site on the 41,000-Da subunit of Gi reveal a high affinity site that is specific for guanine nucleotides. Rank order of affinities for various nucleotides is GTP > GMP > GDP > Gpp(NH)p > ATP. High affinity binding of guanine nucleotides is dependent on Mg2+ and is essentially irreversible in the presence of divalent cations. Bound nucleotide readily dissociates from its site on the 41,000-Da subunit of Gi in the absence of Mg2+. This reversal of binding is markedly enhanced by the presence of the 35,000-Da subunit of Gi. The physical and chemical constitution (23, 24) of the IAP substrate is distinct from the identified components of Gi. The data presented here and in the subsequent papers of the series establish Gi as the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase.

The importance of GTP as a mediator of both hormonal stimulation and inhibition of adenylate cyclase is clearly established (1-3). In addition, GTP modulates the affinity of receptors for agonists in both stimulatory and inhibitory systems (1-3). The effects of GTP on hormonal activation of adenylate cyclase are mediated by a stimulatory, guanine nucleotide-binding regulatory protein, termed Gs, or G/F.

This protein has been purified to homogeneity and characterized in this laboratory (4-10). The mechanism by which GTP functions in hormonal inhibition of adenylate cyclase is less clear, and it has become important to determine if there are distinct stimulatory (Gs) and inhibitory (Gi) guanine nucleotide-binding regulatory components of the adenylate cyclase system.

Several experiments have suggested that Gs and Gi are, in fact, different. These include demonstrations that GTP-dependent inhibition of adenylate cyclase is differentially sensitive to inactivation by certain proteases (11), sulfhydryl reagents (12, 13), or manganese (11, 14, 15). However, the reagents used for such inactivation might only be affecting distinct domains within a single protein responsible for both stimulation and inhibition of adenylate cyclase. A more convincing case has been made by Uji and his associates, who have studied the mechanism of action of IAP, one of the toxins of Bordetella pertussis (16-22). IAP attenuates the ability of inhibitory hormones to inhibit adenylate cyclase, while often potentiating the effects of stimulatory hormones on the enzyme. These effects are mediated by the IAP-catalyzed ADP-ribosylation of a 41,000-Da membrane protein (19-22). This protein clearly differs in electrophoretic mobility from the 45,000-Da subunit of Gi (19-23), which is ADP-ribosylated by cholera toxin. Since inhibition of adenylate cyclase in many cells is blocked by the IAP-catalyzed ADP-ribosylation of this 41,000-Da protein, these data suggest that a protein that is distinct from the identified components of Gi is responsible for mediating GTP-dependent hormonal inhibition of adenylate cyclase.

We have recently described the isolation of the substrate for IAP from rabbit liver and have demonstrated that this protein is distinct from rabbit liver Gi, both in its specificity as a substrate for toxin-mediated ADP-ribosylation and in its physical and chemical constitution (23, 24). The IAP substrate exists as a heterodimer of 41,000- and 35,000-Da subunits, of which the larger (α) subunit contains specific sites for ADP-ribosylation and binding of guanine nucleotides (23). Dissociation of the two subunits occurs in the presence of guanine nucleotide analogs or fluoride (23). This finding was confirmed by Codina et al. (25). In this paper, we present data on the purification to homogeneity of the 41,000/35,000-Da IAP substrate and examine the properties of the purified protein in more detail. The data presented here and in the accompanying papers of this series identify this protein as Gi.

MgCl₂ and 10 mM NaF; TED, 20 mM Tris-HCl (pH 8.0), 1 mM sodium EDTA, 1 mM dithiothreitol; HED, 50 mM sodium Hepes (pH 8.0), 1 mM sodium EDTA, 1 mM dithiothreitol, Gpp(NH)p, guanyl-5'-5'-dithiophosphate; App(NH)p, adenosine-5'-[(S)-5-imino]triphosphate.
the inhibitory, guanine nucleotide-binding regulatory protein of adenylate cyclase.

EXPERIMENTAL PROCEDURES

Procedures have been developed for the simultaneous purification of G\textsubscript{i} and G\textsubscript{o} in substantial quantities and essentially free of each other. These procedures are modifications of the purification scheme for G\textsubscript{o}, previously described by this laboratory (4–7). Purification of G\textsubscript{i} and G\textsubscript{o} by the methods described has been carried out multiple times with comparable results.

Membrane Preparation and Extraction—Membranes were prepared from 1 to 1.5 kg of frozen rabbit liver (Type 1 from Pel-Freez), and the membranes were extracted by the addition of 1% sodium cholate, as described (5), except that the resulting extract was not supplemented with 1 mM ATP, 6 mM MgCl\textsubscript{2}, and 10 mM NaF.

DEAE-Sepharose Chromatography (Fig. 1)—The extract (total volume 1.5–1.8 liters) was applied to a column (5 x 60 cm) of DEAE-Sepharose (Pharmacia), which had been equilibrated with 2.5–3.0 liters of TED, 25 mM NaCl, 0.9% sodium cholate. The column was then eluted at ~200 ml/h with a linear gradient of NaCl (2 liters; 0–250 mM) in TED, 0.9% cholate. Fractions of 23–25 ml were collected. As shown in Fig. 1, a symmetrical peak of the IAP substrate eluted in essentially the same position as did G\textsubscript{i}.

Gel Filtration (Fig. 2)—The pooled DEAE fractions (170 ml) were concentrated to 25 ml by ultrafiltration with an Amicon PM-30 membrane. The concentrate was then fractionated on a column (6 x 60 cm) of Ultrogel AcA34 (LKB) in TED, 100 mM NaCl, 0.9% sodium cholate. The flow rate was maintained at 60–100 ml/h, and fractions of 14–16 ml were collected. Both the IAP substrate and G\textsubscript{i} eluted in a single symmetrical peak with a K\textsubscript{D} of about 0.55.

Heptylamine-Sepharose (C7-Sepharose) Chromatography (Fig. 3)—
The pooled peak from the AcA34 column (60 ml) was diluted with 120 ml of TED, 100 mM NaCl and applied to a 180-ml column (2.6 x 35 cm) of heptylamine-Sepharose that had been equilibrated with TED, 0.3% sodium cholate. Reduction of the concentration of sodium cholate to 0.3% allows G\textsubscript{i} and G\textsubscript{o} to bind to the hydrophobic matrix. The column was then washed successively with 150 ml of TED, 100 mM NaCl, 0.3% sodium cholate and with 150 ml of TED, 500 mM NaCl, 0.3% sodium cholate. Elution of G\textsubscript{i} and separation of G\textsubscript{i} from G\textsubscript{o} was accomplished by the application of a linear gradient (500 ml) starting with TED, 250 mM NaCl, 0.3% sodium cholate and ending with TED, 50 mM NaCl, 3% sodium cholate. Fractions of 6–7 ml were collected into siliconized glass test tubes to prevent loss of protein. G\textsubscript{i} eluted from the heptylamine-Sepharose column as a symmetrical peak just prior to the peak of G\textsubscript{o} (Fig. 3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of relevant fractions from this column is shown in Fig. 4. Fractions containing G\textsubscript{i} were pooled to minimize contamination with G\textsubscript{o}. In like fashion, fractions containing G\textsubscript{o} were pooled to avoid substantial contamination by G\textsubscript{i}. The heptylamine-Sepharose column was regenera-
ted by a final wash with 150 ml of TED, 500 mM NaCl, 1% sodium cholate plus 7 M urea to remove tightly bound protein. The preparation of G\textsubscript{o} documented in Table I, as well as that shown in Fig. 4, was chromatographed over a 50-ml column of heptylamine-Sepharose. This gives somewhat less resolution between G\textsubscript{i} and G\textsubscript{o} than does the C7-Sepharose chromatography over a second column as a symmetrical peak just prior to the peak of G\textsubscript{o}.

Hydroxyapatite Chromatography (Fig. 5)—Although G\textsubscript{i} and G\textsubscript{o} can be almost fully resolved by heptylamine-Sepharose chromatography and each can be pooled to minimize contamination by the other, it is often desirable to achieve more complete resolution of the two proteins. This can be accomplished by hydroxyapatite chromatography (Fig. 5B). Although this step is illustrated for the pooled fractions containing G\textsubscript{i}, it can be utilized for either pool from the heptylamine-Sepharose column. The pooled protein was applied to an 8-ml column of hydroxyapatite (Bio-Rad HTTP), which had been equilibrated with TED (0.1 mM EDTA), 100 mM NaCl, 0.8% sodium cholate. After application, the column was washed once with 10 ml of equili-

Preparation for Storage—Both G\textsubscript{i} and G\textsubscript{o} can be successfully stored for extended periods at ~80 °C after elution from hydroxyapatite. It is desirable, however, to remove the proteins from phosphate- and cholate-containing solutions and to store them in a Lubrol-containing buffer. Stabilities of both G\textsubscript{i} and G\textsubscript{o} in Lubrol are similar to that of G. To accomplish this, proteins were filtered through an 8-ml column of Sephadex G-25 in HED, 0.1% Lubrol prior to storage at ~80 °C.

ADP-ribosylation of G\textsubscript{i} by IAP—Samples from various columns to be assayed for IAP substrate activity were diluted at least 20-fold with TED, 0.05% Lubrol. This ensured that each sample was in a comparable environment for the ADP-ribosylation reaction and that conditions (Tris buffer, 0.05% Lubrol) were optimal for quantitative engineering.

FIG. 3. Heptylamine-Sepharose chromatography of G\textsubscript{i}. See text for description.

FIG. 1. DEAE-Sepharose chromatography of G\textsubscript{i}. See text for description.

FIG. 2. AcA-34 chromatography of G\textsubscript{i}. See text for description.
**FIG. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of heptylamine-Sepharose fractions containing G and G. Samples from the heptylamine-Sepharose column fractions in the region of elution of G and G (Fractions 30-46) were diluted into 4 volumes of (5/4X) Laemmli sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures"; proteins were stained with silver (25). The vertical arrow indicates that AcA-34 pool that was applied to the column. Note that this figure depicts the profile of a 50-ml heptylamine-Sepharose column; consequently, the resolution between G and G is somewhat less than that seen with a 180-ml column, as depicted in Fig. 3.

**TABLE I**

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<th>Purification step</th>
<th>Volume</th>
<th>Protein</th>
<th>G</th>
<th>G</th>
<th>Recovery</th>
<th>Activity</th>
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<td>1.3</td>
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<td>6,840</td>
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<td>14</td>
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<tr>
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<td>34.2</td>
<td>45</td>
<td>1.3</td>
<td>15</td>
<td>6,840</td>
<td>200</td>
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* A unit of G activity is 1 nmol/min.

**FIG. 5.** Final steps in the purification of G and G. See text for explanation. A, second DEAE chromatography of G, pool from the heptylamine-Sepharose column. B, hydroxyapatite chromatography of G, pool from the heptylamine-Sepharose column. Sodium dodecyl sulfate gels of these purified fractions were stained with silver.

**Guanine Nucleotide Binding—**Methods utilized for study of the binding of guanine nucleotides to G were essentially those developed previously for the study of G (8).
Purification and Properties of $G_i$

**RESULTS**

A representative purification of $G_i$ and $G_e$ is documented in Table I. Both proteins were recovered with an approximate yield of 5% after purification (from the extract) of 330-fold ($G_i$) and 680-fold ($G_e$). The extract shown in Table I had an unusually high specific activity of $G_e$, accounting for lower fold purification than normal (4, 5). Major losses of the proteins of interest occurred at the first DEAE purification step; this is partially due to the narrow pool taken from this column, as well as to the relative instability of $G_i$ and $G_e$ in buffers that do not contain fluoride (see "Discussion"). One milligram of essentially homogeneous $G_i$ was obtained, as shown in Fig. 5A. Contamination of $G_i$ with $G_e$ was less than 5% in the preparation shown in Table I and can be reduced to insignificant levels by chromatography on hydroxyapatite. The lower yield of $G_e$ appears to reflect the lesser abundance of this protein in the membrane.

Analysis of purified $G_i$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is shown in Fig. 5A. After staining with either silver or Coomassie blue, $G_i$ was found to consist of approximately equal amounts of two polypeptides with molecular weights of 41,000 and 35,000, corresponding to those reported previously (23). However, some protein appeared to run with the dye front in the silver-stained gel from this preparation of $G_i$. To examine this further, samples from three separate preparations of $G_i$ and a single preparation of $G_e$ (taken after the heptylamine-Sepharose column step) were subjected to electrophoresis on a 15% polyacrylamide gel prior to staining with silver. As seen in Fig. 6, there is a polypeptide in these preparations of $G_i$ with an apparent molecular weight of about 10,000; this polypeptide is poorly visualized with Coomassie blue. We have observed this protein in every preparation of $G_i$ examined to date. It is not removed by chromatography on hydroxyapatite or the second DEAE column.

ADP-ribosylation of $G_i$ by IAP—As we have previously demonstrated, the 41,000/35,000-Da protein is a specific substrate for ADP-ribosylation by IAP (23). This property enabled us to use IAP-catalyzed ADP-ribosylation in the presence of $[^{32}P]NAD$ as a means to detect and quantitate $G_i$ throughout its purification, as indicated above. Fig. 7, C and D, demonstrates that incorporation of $^{32}P$ from $[^{32}P]NAD$ into $G_i$ is linear with protein for both the cholate extract of rabbit liver membranes and purified $G_i$. Representative time courses of labeling are shown in Fig. 7A. ADP-ribosylation proceeded to a plateau value by 45 min with both the extract and the pure protein. The rate of the reaction also depended on the concentration of IAP, as shown in Fig. 7B. Incorporation of $^{32}P$ into purified $G_i$ reaches a value of approximately 1 mol/mol of $G_i$ (assuming a molecular weight of 80,000). There thus appears to be one site for ADP-ribosylation per 41,000/35,000-Da dimer.

The conditions utilized for labeling $G_i$ with IAP have been optimized to obtain the best quantitative estimate of the protein under the varied conditions necessitated by the use of a number of chromatographic procedures during purification. Detergents affect labeling of $G_i$ by IAP, and samples are thus diluted to control detergent concentrations. Required components in the labeling reaction for purified $G_i$ obviously include IAP and NAD. Dimyristoyl phosphatidylcholine enhances labeling somewhat at the concentration used (0.5 mM),

![Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of various preparations of $G_i$ and $G_e$. Samples were prepared and subjected to electrophoresis as described under "Experimental Procedures," except that the acrylamide content of these gels was increased to 15%. The gels were stained with silver (25). Lanes 1, 2, and 3 are $G_i$ samples from three different preparations taken after the heptylamine-Sepharose column. Lane 4 is a preparation of $G_e$.](image-url)
but it is not an absolute requirement. This effect may reflect the ability of the lipid to sequester detergent. Dithiothreitol was also necessary for ADP-ribosylation; this presumably is required for activation of the toxin itself (33). Interestingly, with the purified substrate, there is no apparent effect of ATP on the ADP-ribosylation reaction (data not shown). Both Katada and Ui (19, 20) and Bokoch et al. (23) reported that ATP enhanced ADP-ribosylation of the IAP substrate in C6 glioma cell membranes. It is possible that ATP inhibits the hydrolysis of NAD by crude preparations.

**Binding of Guanine Nucleotides to G**

Use of the photoafinity probe 8-azido-GTP indicated that G has a specific binding site for guanine nucleotides on the 41,000-Da subunit (23). The characteristics of the interaction of guanine nucleotides with G were examined in more detail with [\(^{35}\)S]GTPγS. The experiment in Fig. 8 was carried out to examine the kinetics and stoichiometry of binding of [\(^{35}\)S]GTPγS at various concentrations of this ligand. The concentration of G in this experiment was 80 μg/ml or about 1 μM. When the concentration of GTPγS was 2–10 μM, binding proceeded until approximately 0.7 mol of GTPγS/mol of G was bound; this was the maximal value attainable with this preparation. Binding was slow, taking more than 60 min to reach maximal values. At concentrations of GTPγS below the concentrations of G, an approximately constant fraction of the ligand was bound. When 10 μM GTPγS was added to a sample of G after incubation with 0.1 μM nucleotide for 60 min, about 75% of the maximal number of binding sites was recovered. Such apparent denaturation of G confuses the analysis of these data. This situation becomes worse as the protein concentration is lowered. Similar difficulties were encountered with G, which is considerably less stable under these conditions (8).

The rate of reversal of binding of GTPγS to G was examined after removal of free ligand by gel filtration (Fig. 9A). In the absence of MgCl₂ at 30 °C, there was extremely rapid dissociation of bound [\(^{35}\)S]GTPγS from G. However, in the presence of as little as 1 mM MgCl₂, there was no loss of bound nucleotide over a 3-h time period.

The rate of dissociation of GTPγS from the resolved 41,000-Da subunit of G was also examined (Fig. 9B). In the presence of as little as 0.7 mM free MgCl₂, there was no detectable dissociation of bound nucleotide from the protein. In the absence of free Mg²⁺ (excess EDTA), bound ligand dissociated at a slow, apparently linear rate. Addition of

![Fig. 8. Time course of binding of various concentrations of GTPγS to G. G (80 μg/ml) was incubated at 30 °C for the indicated times in TED, 0.1% Lubrol with 4 mM MgCl₂ and the indicated concentrations of [\(^{35}\)S]GTPγS. At the indicated times, aliquots were withdrawn, diluted 100-fold into TED, 50 mM NaCl, 25 mM MgCl₂, and filtered as described. The arrow indicates the point at which sufficient GTPγS was added to the incubation to raise the concentration from 0.1 to 10 μM.](http://www.jbc.org/)

![Fig. 9. Reversal of GTPγS binding to G. A, G (80 μg/ml) was incubated with 10 μM [\(^{35}\)S]GTPγS in TED, 0.1% Lubrol, 20 mM MgCl₂ for 150 min at 30 °C. Binding reached a final level of 0.6 mol of GTPγS/mol of protein. The protein (125 μl) was then applied to an 8-ml column of Sephadex G25 equilibrated with TED, 0.1% Lubrol and maintained at 4 °C in order to remove unbound nucleotide. The column was eluted with the same buffer and 150-μl fractions were collected. Aliquots of 10 μl were counted to determine the position of the peak of [\(^{35}\)S]GTPγS-protein complex, and fractions containing](http://www.jbc.org/)
increasing amounts of the 35,000-Da subunit of Gi caused enhanced rates of dissociation of nucleotide from the 41,000-Da subunit. A maximal rate of dissociation of ligand was achieved at roughly stoichiometric amounts of 35,000-Da subunit. In the presence of 0.7 mM MgCl₂, the effect of the 35,000-Da subunit on dissociation of nucleotide was totally prevented.

The effect of MgCl₂ concentration on [³⁵S]GTPγS binding to Gi is shown in Fig. 10; binding was almost completely dependent on the presence of MgCl₂. Increasing the concentration of MgCl₂ over the range of 1 to 25 mM resulted in more rapid rates of guanine nucleotide binding and progress curves that were more hyperbolic. These differences in rates of binding were most evident at high concentrations of Gi (50–100 µg/ml). At low concentrations of Gi, binding was rapid even at the lower concentrations of MgCl₂.

As we have indicated previously (23), there is competition for the binding of 8-azido-GTP to its site on the 41,000-Da subunit of Gi by GTPγS but not by ATP. The specificity of the binding site for GTPγS on Gi was examined in more detail (Fig. 11). Because of the essentially irreversible nature of the binding reaction in the presence of Mg⁺⁺, competition of unlabeled nucleotides for the GTPγS binding site was examined at 10 min, a time at which the rate of binding of [³⁵S]GTPγS was nearly linear. The apparent Kᵦ for GTPγS under the experimental conditions utilized was estimated from the initial rates of binding to be approximately 12 nM. GTP, GDP, and Gpp(NH)p (not shown) were all effective competitors and were nearly equipotent (EC₅₀ ≈ 1 μM; apparent Kᵦ ≈ 25 nM). GMP had much less affinity for the binding site, while ATP and App(NH)p had no detectable affinity.

**Binding of GTPγS and ADP-ribosylation**—The data of Figs. 7 and 8 indicate that one site is ADP-ribosylation bound nucleotide were pooled on ice. Portions of the nucleotide-protein complex (108 µl) were then combined with 5 µl of 2.5 mM GTP and 12 µl of H₂O (O), 12 µl of 20 mM MgCl₂ (A), or 12 µl of 50 mM MgCl₂ (C). At the indicated times, 10-µl aliquots were withdrawn and diluted with 1 ml of 25 mM Tris-Cl, pH 8.0, 50 mM NaCl, 25 mM MgCl₂. Samples were held on ice until all time points were accumulated. The mixtures were then combined with 12 µl of 20 mM GTP and 12 µl of 20 mM [³⁵S]GTPγS. The nucleotide-protein complex was separated from unbound nucleotide by Sephadex G25 chromatography in HED (with 0.1 mM EDTA), 0.1% Lubrol with 0.5 mM MgCl₂. The GTPγS-ligated 41,000-Da subunit (43 pg/ml) was diluted 10-fold on ice into HED (with 0.1 mM EDTA). Binding reached a final level of 0.5 mol of [³⁵S]GTPγS/mol of protein.

**Fig. 10.** Binding of GTPγS at various concentrations of MgCl₂. Gi (50 µg/ml) was incubated with 20 µM [³⁵S]GTPγS at the indicated concentrations of MgCl₂ in HED, 100 mM NaCl, 0.1% Lubrol at 30 °C. Aliquots were withdrawn at the indicated times and assayed for bound GTPγS, as described under "Experimental Procedures."

**Fig. 11.** Competition by nucleotides for [³⁵S]GTPγS binding sites on Gi. Gi (5 µg/ml) was incubated in TED, 0.1% Lubrol with 10 mM MgCl₂, 0.5 µM [³⁵S]GTPγS, and the indicated nucleotide for 10 min at 30 °C. At this time, the rate of binding of GTPγS to Gi is nearly linear. Duplicate samples were then assayed for bound GTPγS as described under "Experimental Procedures." Symbols are as follows: GTPγS (A), GTP (B), GDP (D), GMP (O), App(NH)p (O), and ATP (Δ).

**DISCUSSION**

We have described the purification of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase from rabbit liver, and we have characterized it as a substrate for IAP and as a guanine nucleotide-binding protein. Preparations of Gi are more than 90% pure and contain mg quantities of protein. Reasonable quantities of purified Gi, can
be obtained by the same techniques, which are modifications of those described originally (4-7). The primary modifications that enable purification of Gi entail (a) the absence of AMF in the column buffers during purification, and (b) changes in the conditions for loading and eluting the heptylamine-Sepharose column. AMF was originally included in the buffers for purification of Gi, as a stabilizer of Gi activity. We have determined, however, that AMF causes dissociation of the subunits of Gi during purification (9, 10), with much of this dissociation taking place prior to or during the initial chromatographic step on DEAE. Resolution of the subunits of Gi takes place particularly during chromatography on heptylamine-Sepharose in the presence of AMF. In the absence of AMF, Gi remains in the form of a 41,000/35,000-Da dimer, and yields of the protein are much increased. The Gi dimer then co-migrates with G, during the first two steps of purification.

The modifications of the heptylamine-Sepharose chromatographic step involve two significant changes. Loading the Aca34 pool onto the heptylamine-Sepharose column in 0.3% cholate allows Gi to bind to the resin more readily. This facilitates resolution of Gi from G, and other contaminating proteins. The modified gradient used to elute the heptylamine-Sepharose column provides adequate resolution of Gi from G, and other contaminates, yet still yields rather concentrated peaks of both proteins.

The appearance of Gi on sodium dodecyl sulfate-polyacrylamide gels was used as the primary criterion for purity. Such gels indicate that Gi consists at least predominantly of 41,000- and 35,000-Da subunits. As reported previously, hydrodynamic measurements support this conclusion, since the protein in the basel state behaves as a particle with $M_0 = 80,000$. It is possible or perhaps likely, however, that Gi contains an additional polypeptide with $M_0 = 10,000$. This polypeptide appears in all preparations of Gi. Furthermore, when Gi is incubated with AMF, a condition that causes dissociation of the 41,000- and 35,000-Da subunits, the 10,000-Da peptide appears to remain associated with the 35,000-Da subunit. Variable amounts of a 10,000-Da peptide can also be found in some preparations of Gi (see Fig. 6); however, it is difficult to rule out contaminating Gi, or the 35,000-Da subunit of Gi, as a source of this protein in preparations of Gi, since the β subunits of the two regulatory proteins are very similar or identical. The poor ability of Coomassie blue to stain the 10,000-Da peptide, coupled with routine precipitation of samples with trichloroacetic acid prior to gel electrophoresis, precluded its prior detection in preparations of Gi. The possibility of an additional subunit of Gi becomes more attractive when one compares Gi with transducin, the guanine nucleotide-binding regulatory protein of the retinal rod outer segment. Gi, G, and transducin constitute a family of homologous proteins (24). In particular, analysis of amino acid compositions and of gels of proteolytic digests of the three proteins suggest that Gi and transducin are remarkably similar. The presence of a 10,000 Da γ subunit in Gi, would extend this homology still further, since transducin has been shown to contain a γ subunit with $M_0 = 10,000$ (34, 35).

Gi is a highly specific substrate for ADP-ribosylation by IAP (18, 21); Gi cannot be ADP-ribosylated by IAP under any conditions identified to date (20, 23). We have used this property of Gi to locate and quantitate the protein throughout its purification. Under the conditions of the assay, IAP-catalyzed incorporation of $[^3H]FAD$ into Gi, is linear with protein and reaches a maximal level of incorporation with similar time courses for both the extract and the purified product. ADP-ribosylation of Gi, with IAP at each step of purification has been found to correlate exactly with the positions of the subunits determined by functional assays, which will be described in the subsequent papers of this series (36, 37). Incorporation of ADP-ribose into Gi appears to occur at a single site. ADP-ribosylation of Gi correlates very well with the ability of the protein to bind $[^3H]GTPγS$, suggesting that only native protein can serve as a substrate for IAP.

Studies of the guanine nucleotide-binding site on Gi reveal a single class of high affinity binding sites on the purified protein. We can detect a maximum of 1 mol of sites/mol of protein. This site resides on the 41,000-Da subunit of Gi, as determined with the photoaffinity probe, 8-azido-GTP (23), and by radiolabeled nucleotide binding to the resolved subunit (36). The site is specific for guanine nucleotides and does not bind either ATP or App(NH)p. Guanine nucleotide binding to the site occurs with the order of affinities $GTP > GDP > Gpp(NH)p > GMP$. The kinetics of binding of $[^3H]GTPγS$ observed in these studies is consistent with the model proposed previously to explain binding of $[^3H]GTPγS$ to Gi (8, 38) and suggests that this model can be extended to Gi. Basically, the model proposes that Gi exists as a dimer of 41,000- and 35,000-Da subunits in its basal state. This dimer serves as the species to which guanine nucleotide is bound rapidly and with relatively low affinity. Activation of the protein involves the divalent cation-promoted dissociation of the subunits and high affinity binding of guanine nucleotide to the 41,000-Da subunit. That subunit dissociation does occur in the presence of guanine nucleotides or fluoride is supported by examination of the hydrodynamic properties of the purified protein (23). Other data presented above also provide evidence for this model of nucleotide binding to and activation of Gi. (a) There is a marked enhancement of the rate of high affinity binding of guanine nucleotide to Gi, by concentrations of Mg$^{2+}$ that are well in excess of that of nucleotide (Fig. 10). (b) Dissociation of nucleotide is extremely slow in the presence of divalent cation (Fig. 8A). (c) The 35,000-Da subunit of Gi promotes release of nucleotide from the 41,000-Da subunit. This effect is maximal at equal concentrations of the two subunits (Fig. 8B). (d) The rate of nucleotide binding is dependent on protein concentration (not shown), although this effect is less marked than with Gi (8). This is presumably because of the
ease with which \(G_i\) dissociates at relatively low concentrations of \(\text{Mg}^{2+}\). Such dissociation may explain the rapid phase of binding evident in Fig. 10.

The differences between the reactions of \(G_i\) and \(G_t\) with guanine nucleotides appear to be important determinants of the relative functions of the two proteins. The affinity of \(G_t\) for guanine nucleotides appears to be somewhat greater than that of \(G_i\). This property is consistent with the characteristics of inhibition of adenylate cyclase by guanine nucleotides. Binding of \(G_i\) is that of \(G_t\), which is consistent with a model of activation of \(G_i\) (and \(G_t\)) involving a cation-promoted dissociation of the protein's units. As we shall show in the following papers of this series, the physical characteristics of \(G_i\) and \(G_t\) involving divalent cation-promoted dissociation of the protein's subunits. As we shall show in the following papers of this series, the physical characteristics of \(G_i\) and \(G_t\) involving divalent cation-promoted dissociation of the protein's subunits.

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