Synthesis and Characterization of Fluorescently Labeled Bovine Brain G Protein Subunits†

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ABSTRACT: G proteins play an important role in transmitting hormonal signals, and fluorescence techniques would be useful to study their cellular distribution and mechanisms. To prepare active fluorescent G protein, G_o/G_i or $\beta\gamma$ subunits were reacted with fluorescein isothiocyanate (FITC) to label the α (F-α) and γ (F-γ/β) subunits or with (iodoacetamido)tetramethylrhodamine (TMR-IAA) to label the β subunit (TMR-$\beta\gamma$). Unreacted dye was removed from the labeled proteins by ultrafiltration, followed by further purification using HPLC gel filtration. The molar ratios of dye to protein were 0.96 ± 0.15, 0.59 ± 0.07, and 1.37 ± 0.09 for labeled α, β, and γ subunits, respectively. GTPγS binding to F-α and ADP-ribosylation by pertussis toxin of F-α were reduced to 63% and 78% of control, respectively. F-α was a heterogeneous population of α subunits. Active F-α containing less than one (0.7) label/subunit (F-α–Mono Q) was separated from unlabeled and multiply labeled F-α by Mono Q anion-exchange chromatography. F-α–Mono Q displayed reduced GTPase activity (turnover number was 46% of control), while GTPγS binding and ADP-ribosylation by pertussis toxin were only decreased to 78% and 82% of control, respectively. TMR-$\beta\gamma$ and F-γ/β retain full function compared to native $\beta\gamma$, as measured by three methods: (1) TMR-$\beta\gamma$ and F-γ/β are able to form heterotrimers with α_o subunits, (2) TMR-$\beta\gamma$ and F-γ/β support the ADP ribosylation of α_o subunits by pertussis toxin, and (3) TMR-$\beta\gamma$ and F-γ/β inhibit forskolin-stimulated adenylyl cyclase activity. The fluorescent G protein subunits will be valuable tools to study G protein mechanisms in reconstituted membranes and intact cells.

Cellular signal transduction occurs through a series of events initiated by the binding of extracellular ligands such as hormones and neurotransmitters to their cell surface receptors (Gilman, 1987). A family of structurally related receptors (Dohlman et al., 1987b, 1991) interact with a variety of G proteins[1] which deliver the biochemical signals initiated by the ligand-bound receptors to a host of different effector proteins. The elucidation of the molecular mechanisms of each step in the signal transduction pathway has been the focus of much recent research in cell biology.

The molecular cloning of the cDNAs of the visual phototransduction receptor, rhodopsin, the adrenergic receptors (AR), and other G protein-coupled receptors revealed that these proteins share a common topographical motif consisting of seven putative transmembrane regions joined by intracellular and extracellular loops (Dixon et al., 1986; Dohlman et al., 1987a; Peralta et al., 1987; Cotecchia et al., 1988). The critical regions of the AR involved in ligand and G protein binding have been studied. The ligand–receptor interaction site is proposed to involve multiple membrane-spanning domains on the basis of studies of chimeric receptors (Kobilka et al., 1988; Frielle et al., 1988) and photoaffinity labeling (Dohlman et al., 1988; Wong et al., 1988; Curtis et al., 1989). Studies with chimeric α_2/β_2 AR have indicated that the third intracellular loop of the receptor is critical for the receptor–G protein coupling (Kobilka et al., 1988). In addition, Dalman and Neubig(1991) reported that both the second cytoplasmic loop and the C-terminal part of the third cytoplasmic loop of the α_{2A}-AR are important in the interaction between the α_{2A}-AR and G_i protein, based on the effect of synthetic peptides from the α_{2A}-AR on the high-affinity binding state of the receptor.

A family of G proteins containing three subunits, α, β, and γ, is central in signal transduction. Upon activation by ligand-bound receptors, the GDP/GTP exchange takes place on the α subunit of G protein, and in model studies in detergent the GTP-bound α subunit then dissociates from the $\beta\gamma$ subunit. The dissociated α subunit usually interacts with an appropriate effector protein (Gilman, 1987). The role of dissociated $\beta\gamma$ subunits in interaction with effector proteins is controversial. Logothetis et al. (1987) proposed that $\beta\gamma$ subunits are responsible for activating the muscarinic-gated potassium channels, but Codina et al. (1987) show a major role for the α subunit. However the dissociated $\beta\gamma$ subunits, but not the α subunit, may activate phopholipase A_2 (Jelsema & Axelrod, 1987; Kim et al., 1989). Recently, Tang and Gilman (1991) demonstrated that $\beta\gamma$ subunits are able to activate Type II adenylyl cyclase.

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[1] Abbreviations: G protein, guanine nucleotide binding protein; G_o, G protein abundant in brain; G_i, G protein that mediates inhibition of adenylyl cyclase; GTP, guanosine triphosphate; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; AR, adrenergic receptors; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; FITC, fluorescein 5-isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; TMR-IAA, (iodoacetamido)tetramethylrhodamine; FIAA, (iodoacetamido)fluorescein; PT, pertussis toxin; NEM, *N*-ethylmaleimide; CHAPS, 3-[(cholamidopropyl)-diethylammonio]-1-propanesulfonate; F-α, fluorescein-labeled G protein α subunit; TMR-$\beta\gamma$, TMR-labeled β subunit; F-γ/β, fluorescein-labeled γ subunit; C7-Sepharose, heptylamine-Sepharose; TED buffer, 20 mM Tris, 1 mM EDTA, and 1 mM DTT, pH 8.0; HED buffer, 20 mM Hepes, 1 mM EDTA, and 1 mM DTT, pH 8.0; AMF, 20 μM $AlCl_3$, 10 mM $MgCl_2$, and 10 mM NaF.

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The molecular cloning of the cDNAs of G proteins reveals about 20 distinct α subunits as well as four β subunits and a similar number of γ subunits (Simon et al., 1991). The α subunit contains a high-affinity guanine nucleotide binding site, GTPase activity, and often an ADP-ribosylation site which is a substrate for either pertussis toxin or cholera toxin. On the basis of studies of trypsin cleavage, chemical modification, and genetically modified α_s (Fung & Nash, 1983; Winslow et al., 1986; Hingorani & Ho, 1987; Neer et al., 1988; Sullivan et al., 1987), the regions of the α subunit involved in GTP/GDP binding and in interactions with $\beta\gamma$ subunit, effector protein, and activated receptor have been tentatively assigned. The N-terminus of the α subunit is proposed to be the binding site for $\beta\gamma$, whereas the C-terminus is involved in the interaction with receptors. The putative binding sites for GTP and effector proteins have been predicted on the basis of the crystal structure of the GTP binding domain of elongation factor Tu (Jurnak, 1985) and $p21^{ras}$ (de Vos et al., 1988; Tong et al., 1989).

Recent studies using fluorescence spectroscopic methods have contributed significantly to the understanding of the molecular basis for activation of G proteins and the interactions of the individual components in well-defined systems. Higashijima et al. (1987) reported the existence of different conformational states of the α subunit upon activation by GTPγS by correlating the kinetics of the enhancement of intrinsic fluorescence with the [^{35}S]GTPγS binding to the α subunit. The rhodopsin stimulated activation–deactivation cycle of transducin reconstituted in phospholipid vesicles has also been studied by use of intrinsic protein fluorescence (Guy et al., 1990). Signal transduction and ligand–receptor interactions in the human neutrophil have been extensively studied using fluorescein-labeled chemotactic N-formylated peptides and high-affinity antifluorescein antibody as a probe of the temporal relationships between the ligand–receptor interaction and the cell response and adaptation (Sklar et al., 1981, 1984; Fay et al., 1991). The interactions of the eosin-labeled α_T with the fluorescein-labeled retinal cyclic GMP phosphodiesterase (PDE) have been directly monitored using resonance energy transfer techniques (Erickson & Cerione, 1991).

In order to study G protein activation and subunit interactions with each other and with receptor or effector protein using fluorescence spectrosopic methods, we elected to label G protein subunits with extrinsic fluorescent probes. In this paper, we present detailed methods used to incorporate fluorescent labels into each of the three G protein subunits, report the effects of fluorescent modifications on the activities of the G protein subunits, and describe initial results on the location of the incorporated labels. In the accompanying paper, Remmers and Neubig (1993) report estimates of the distances between G protein subunits and lipid fluorescent probes using these fluorescently modified G protein subunits.

EXPERIMENTAL PROCEDURES

Reagents. Fluorescein 5-isothiocyanate (FITC) and (iodoacetamido)tetramethylrhodamine (TMR-IAA) were obtained from Molecular Probes (Eugene, OR). [^{35}S]GTPγS, [^{32}P]ATP, [^{32}P]GTP, and [^{32}P]NAD were obtained from New England Nuclear (Boston, MA). Cholic acid, L-cysteine, and GTP-agarose were obtained from Sigma Chemical Co. (St. Louis, MO). The cholic acid was purified by the method of Ross and Schatz (1978) except that chromatography was performed on DEAE-Sephacel. GTPγS and 3-[(cholamidopropyl)diethylammonio]-1-propanesulfonate (CHAPS) was purchased from Boehringer-Mannheim (Indianapolis, IN). Pertussis toxin was a gift from Dr. Nadine Cohen (Jamaica Plains, MA). Heptylamine-Sepharose (C7-Sepharose) was prepared as described (Shaltiel, 1974). Lubrol PX was obtained from ICI and deionized prior to use.

Synthesis of Fluorescently Labeled G Protein Subunits. Reaction of intact G protein with FITC or TRITC resulted in incorporation of label in the α and γ subunits. Reaction of resolved $\beta\gamma$ subunits with FIAA or TMR-IAA resulted primarily in labeling of β. We took advantage of this specificity to prepare proteins with individual subunits labeled.

(*1*) *TMR-$\beta\gamma$.* G_o and G_i from bovine brain were purified by the method of Sternweis and Robishaw (1984) as modified by Kim and Neubig (1987). Bovine brain G protein $\beta\gamma$ subunits were purified by running the AMF-activated G proteins over a C7-Sepharose column (1.0 cm × 30 cm) as described by Katada et al. (1984). Resolved $\beta\gamma$ subunits were purified from other contaminant proteins using HPLC gel filtration on tandem Dupont GF-450 and GF-250 columns at a flow rate of 1 mL/min in 25 mM Hepes, 1 mM EDTA, 2 mM $MgSO_4$, 50 mM Na_2SO_4, and 0.8% cholate. Purified $\beta\gamma$ subunits were gel-filtered into the reaction buffer (20 mM Hepes, 1 mM EDTA, and 0.5% cholate, pH 8.0) on a G-50 Sephadex column and concentrated to 2–3 mg/mL. $\beta\gamma$ subunits (150 μM) were reacted for 1 h at room temperature with a 5-fold excess of TMR-IAA added from a 10 mg/mL stock solution in *N*,*N*-dimethylformamide (DMF). The reaction was terminated by adding 750 μM cysteine (final concentration). The majority of free dye was removed by filtering the reaction mixture using Centricon 30, followed by HPLC gel filtration to further remove the free dye. HPLC gel-filtered TMR-$\beta\gamma$ was concentrated to about 5 mg/mL in TED buffer containing 0.5% cholate. Concentrated TMR-$\beta\gamma$ was stored at −70 °C until use. Identical labeling patterns were observed when fluorescein iodoacetamide was used to label the $\beta\gamma$ subunit.

(*2*) *F-γ/β.* Purified G proteins were exchanged into the reaction buffer (20 mM Hepes, 1 mM EDTA, 100 mM NaCl, 6 mM $MgCl_2$, 0.4 mM ascorbate, 20 μM GDP, and 0.1% Lubrol or 0.5% sodium cholate, pH = 9.0) on a G-50 Sephadex column and concentrated using Centricon-30. G proteins (20 μM) in 1 mL of reaction buffer were reacted for 1 h at 30 °C in the dark with 400 μM FITC (final concentration) added from a freshly prepared 10 mg/mL solution in DMF. The reaction was stopped by the addition of 0.3 mL of 1.8 M glycine. The majority of free dye was removed by filtering the reaction mixture three times in Centricon 30 with a 20-fold dilution each time. The remaining free dye was removed by HPLC gel filtration in 25 mM Hepes, 1 mM EDTA, 2 mM $MgSO_4$, 50 mM Na_2SO_4, and 0.1% Lubrol. The HPLC-purified fluorescein-labeled G_o/G_i was incubated overnight at 4 °C in TED buffer (20 mM Tris, 1 mM EDTA, and 1 mM DDT) supplemented with 0.5% sodium cholate and AMF to separate labeled α and $\beta\gamma$ subunits. Fluorescein-labeled $\beta\gamma$ subunits(F-γ/β) eluted from the C7-Sepharose column after the F-α and were pooled and concentrated to about 1 mg/mL using Amicon 30. Running F-γ/β on a 13% polyacrylamide gel indicated that the majority of label is located on the γ subunit. The purified F-γ/β was stored at −70 °C until use. Identical labeling patterns were observed when TRITC was used to label α and γ subunits of G protein.

Residual F-α subunit was removed from F-γ/β by incubation with GTP-agarose which had been equilibrated with HED, 10 mM $MgCl_2$, 0.1 M NaCl, and 0.3% cholate (pH 8.0). Resin (0.5 mL) and G proteins (0.5 mg) in 4 mL of buffer were tumbled 30 min at room temperature in the dark.

The resin was pelleted (200g, 5 min) and the supernatant containing labeled γ was concentrated.

(3) *F-α.* The labeling procedure for F-α was the same as that for F-γ/β up through the C7-Sepharose column. Total F-α eluted from the C7 column was run on an SDS–13% polyacrylamide gel to test for the presence of free dye. Minor amounts of contaminant-free dye were completely removed by running F-α eluted from the C7-Sepharose column over HPLC gel-filtration columns once more. This preparation of total labeled F-α was concentrated to 2–3 mg/mL, frozen in liquid N_2, and stored at –70 °C until use. Resolution of singly labeled G_o α from F-α is described in Results.

GTPγS Binding. [^{35}S]GTPγS binding was performerd as described by Sternweis and Robishaw (1984). Briefly, varying amounts (0.05, 0.1, 0.15, and 0.2 μg) of G_o, G_o/G_i, F-$G_{o\,total}$, and F-G_o were incubated with 1 μM [^{35}S]GTPγS (12.5 mCi/mL) in HED, 0.1% Lubrol, 20 mM $MgCl_2$, and 100 mM NaCl in a total volume of 40 μL for 1 h at 30 °C. The reaction mixture was filtered on BA85 filters, and the filters were washed with 3 × 2 mL of cold wash buffer (20 mM Tris, 100 mM NaCl, and 25 mM $MgCl_2$, pH 8.0) using a Hoeffer filtering device. The amount of GTPγS bound was quantitated using a liquid scintillation counter.

Determination of Stoichiometry of Labeling. Absorbance measurements at 494 and 555 nm were obtained using a Perkin-Elmer UV spectrophotometer for fluorescein and tetramethylrhodamine at pH 8.0. Protein content was measured by amido black protein assay (Schaffner & Weissmann, 1973). The stoichiometries of fluorescein and tetramethylrhodamine incorporated into subunits were calculated using molar extinction coefficients of 64 800 and 75 000 cm^{-1} M^{-1}, respectively, and subunit molecular weights of 39 000 and 45 000 for α and $\beta\gamma$, respectively.

SDS–Polyacrylamide Gel Electrophoresis. Analysis of fluorescently labeled subunits was performed on 13% polyacrylamide gels prepared according to Laemmli (1970). The gels containing fluorescently labeled subunits were photographed before staining with a Polaroid camera on a UV light box (Fotodyne) as means of visualizing fluorescence. The gel was then stained with Coomassie Blue to visualize proteins. Tricine–SDS–polyacrylamide gels (Schagger & Von Jagow, 1987) were used as indicated in the figure legends to run TMR-$\beta\gamma$ and F-γ/β to yield a good separation of γ subunits from the dye front.

Tryptic Digestion. Ten micrograms of F-α, TMR-$\beta\gamma$, or F-γ/β was digested with 0.1 μg of trypsin for 30 min at 30 °C. Trypsin digestion was stopped by addition of 10 μL of 4X sample buffer, followed by boiling for 3 min. F-α and trypsin-digested F-α were run on an SDS–13% polyacrylamide gel. TMR-$\beta\gamma$ and F-γ/β and trypsin-digested TMR-$\beta\gamma$ and F-γ/β were run on a Tricine–SDS–13% polyacrylamide gel.

Sucrose Density Centrifugation. Linear sucrose density gradients (5 mL, 5–20% sucrose in 50 mM Tris, 1 mM EDTA, 1 mM DTT, 6 mM $MgCl_2$, and 0.1% Lubrol PX) were prepared according to Huff and Neer(1986). Briefly, 150-μL samples containing various combinations of fluorescently labeled subunits and their appropriate unlabeled subunits with marker proteins were layered on top of the gradients. The marker protein mixture consisted of 25–30 μg each of bovine serum albumin, fumarase, and carbonic anhydrase per gradient. Gradients containing samples were centrifuged at 51 000 rpm for 18 h in a Beckman SW Ti-55 rotor at 4 °C. Approximately 20–25 fractions were collected from each gradient. Eighty microliters of each fraction was boiled with 20 μL of 4X Laemmli sample buffer and applied to 10% polyacrylamide gels. Aliquots (100 μL) were used to determine fluorescence intensity.

ADP-Ribosylation by Pertussis Toxin. To assess the function of TMR-$\beta\gamma$ and F-γ/β, their ability to enhance ADP-ribosylation of the purified G_o/G_i α subunits by pertussis toxin (PT) was determined by the method of Bokoch et al. (1983). Purified α was incubated in a total volume of 36 μL containing 50 mM Tris, pH 7.6, 1 mM EDTA, 10 mM DTT, 2 μM [^{32}P]NAD (2 μCi/tube), 0.5 mM dimyristoylphosphatidylcholine, and 20 μg/mL PT. Varying concentrations of TMR-$\beta\gamma$, F-γ/β, and native $\beta\gamma$ were combined with 2 μg of α in the reaction mixture as indicated in the figure legends. The reaction was allowed to proceed for 1 h at 30 °C. The reaction was stopped by the addition of 30 μL of 4X sample buffer according to Laemmli. The reaction mixture was electrophoresed on an SDS–10% polyacrylamide gel. Gels were stained with Coomassie Blue and dried. Analysis of radioactivity was done by autoradiography using Kodak RP-Xomat film at –70 °C for 30 min. The single labeled band was cut out of the dried gels and counted in 4 mL of Scintiverse scintillation cocktail. To assess the function of F-G_o/G_i and F-G_o purified from a Mono-Q column, 0.2 μg of the appropriate control or fluorescein-labeled protein was assayed for incorporation of [^{32}P]ADP-ribose as described above.

Assay of Adenylyl Cyclase Activity. The ability of TMR-$\beta\gamma$ and F-γ/β to inhibit forskolin-stimulated adenylyl cylase activity in human platelet membranes (Neubig & Szamraj, 1986) was examined. Adenylyl cyclase activity was assayed in a medium containing the final concentrations of 25 mM Tris-HCl (pH 7.6) 2.5 mM EGTA, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM cAMP, 0.1 mM isobutylmethylxanthine, 10 μM GTP, 5 mM Phosphocreatine, 50 units/mL creatine phosphokinase, 10 μM propranolol, 0.2 mM ATP (0.5 μCi of [α-^{32}P]ATP/tube), and 10 μM forskolin. After incubation for 15 min at 30 °C, the reaction was terminated, and [^{32}P]-cAMP formed was measured by the method of Salomon et al. (1974).

GTP Hydrolysis. GTPase activity was measured as described by Higashijima et al. (1987) with the following modifications. G_o/G_i α subunit and F-α (75 nM) or 24 nM G_o or F-G_o purified using the Mono-Q column were incubated in HED buffer containing 0.1% Lubrol, 10 mM $MgSO_4$, and 1 μM [γ-^{32}P]GTP (6000 cmpm/mol) for 10 min at 20 °C. One-hundred-microliter aliquots of the GTPase reaction were quenched with 1 mL of activated charcoal (25% w/v) in 50 mM phosphoric acid (0 °C) and vortexed. After 30 min on ice, the charcoal was pelleted at 2000 rpm for 10 min. A 300-μL aliquot of the supernatant was counted in 10 mL of Scintiverse. The results are normalized to the amount of GTPγS binding (picomoles of P_i/picomole of GTPγS bound) to determine turnover number.

RESULTS

Modification of the α and γ Subunits of Bovine G Protein by FITC and the $\beta\gamma$ Subunit by TMR-IAA. Purified bovine G_o/G_i was reacted with FITC at pH 9.0 to ensure the selective modification of the ϵ-amino group of the lysine residues. FITC incorporated mainly into α and γ subunits with no significant labeling on the β subunit based on the separation of the subunits by SDS–polyacrylamide gel electrophoresis (Figure 1). Labeling purified $\beta\gamma$ subunits with a 5-fold excess of TMR-IAA resulted in the incorporation of TMR-IAA mostly on the β subunit (Figure 1).

A major difficulty in the preparation of fluorescently labeled G protein subunits was separation of contaminating dye from

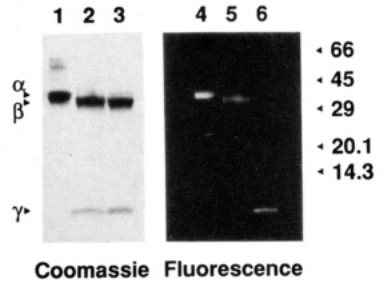

FIGURE 1: SDS–PAGE of fluorescently labeled G protein subunits. G protein α, β, and γ subunits were labeled with fluorescent reagents as described in the Experimental Procedures section. Five micrograms of each labeled subunit was run on a Tricine–SDS–10% polyacrylamide gel as described in Experimental Procedures. The left panel shows Coomassie staining and the right panel shows a fluorescence photograph. Lanes 1 and 4; F-α; lanes 2 and 5, F-$\beta\gamma$; lanes 3 and 6, F-γ/β.

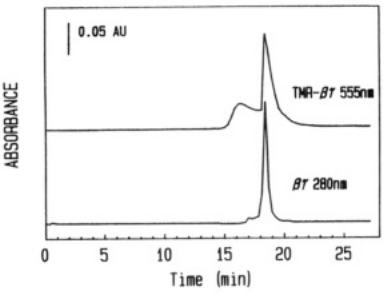

FIGURE 2: Purification of TMR-$\beta\gamma$ using HPLC gel filtration. HPLC gel filtration was used for a standard purification step to remove contaminating dye from the labeled proteins. Tandem Dupont GF450 and GF250 columns(1.0 cm × 25 cm) were used at a flow rate of 1 mL/min. Bottom trace: 300 μg of $\beta\gamma$ in TED/0.5% cholate was injected. Protein was detected at 280 nm. Top trace: 300 μg of TMR-reacted $\beta\gamma$ in TED/0.5% cholate was injected. Labeled protein and contaminating dye were detected at the TMR absorbance wavelength, 555 nm. The HPLC running buffer was HED, 100 mM Na_2SO_4 and 0.8% cholate.

the labeled proteins. The majority of free dye was removed by ultrafiltration and low-pressure gel filtration but a substantial amount of free dye, however, still remained with the labeled G protein subunits based upon the presence of fluorescence in the dye front on SDS–polyacrylamide gels. The HPLC gel-filtration step (Figure 2) with tandem Dupont GF 250 and GF 450 columns was critical to remove contaminating dye completely from the labeled proteins. The bottom trace shows the elution profile of native $\beta\gamma$ subunits at 280 nm with its retention time of 18.5 min. The top trace shows the elution profile of TMR-reacted $\beta\gamma$ subunits at 555 nm, which is the absorption wavelength for TMR. The elution profile of TMR-reacted $\beta\gamma$ subunits resulted in two peaks with retention times of 15 and 18.5 min. Collecting the two peaks and running them on an SDS–polyacrylamide gel identified the second peak as labeled $\beta\gamma$ protein. The broad first peak contained some labeled high molecular weight contaminant proteins and aggregated free dye. The difficulty in removing contaminating fluorescence is partially explained by its apparent high molecular weight on gel filtration. The HPLC gel-filtration traces for F-α and F-γ/β resulted in similar profiles as in Figure 1 but with different retention times and sizes of the first shoulder peaks.

In order to assess heterogeneity of the labeling of the α subunit, an alternative purification of F-α was performed. G_o was prepared using Mono-Q (HR 5/5, Pharmacia) anion-exchange chromatography to resolve G_o from bovine brain G_o/G_i as described by Katada et al. (1987a) except that the NaCl gradient was 125–225 mM over 30 min. Purified G_o

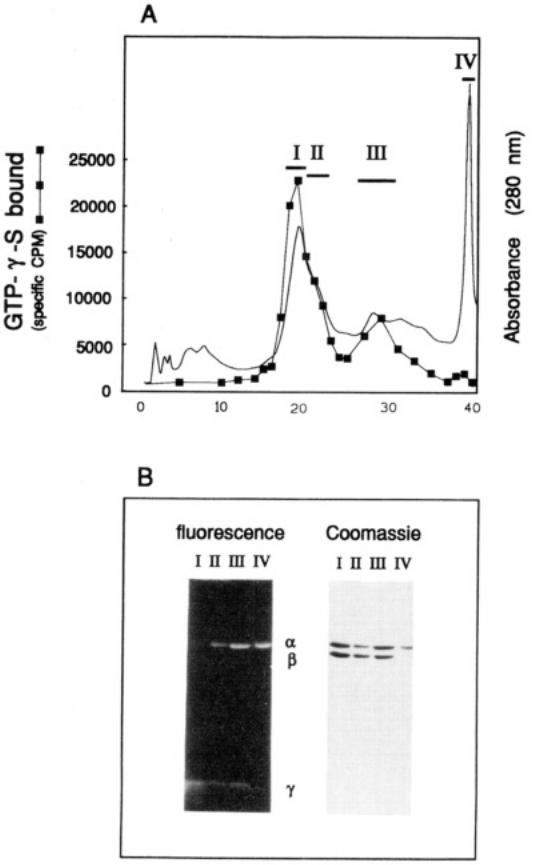

FIGURE 3: Resolution of F-α using Mono-Q anion-exchange chromatography. G_o was resolved from the G_o/G_i mixture and labeled with FITC as described in Experimental Procedures Methods. Three milligrams of FITC-G_o was run on a Mono-Q anion-exhange column as described in Experimental Procedures. (A) The 280-nm absorbance trace (solid line) as well as GTPγS binding (■) in each fraction are shown. (B) Fractions were pooled as indicated (I–IV) and run on an SDS–13% polyacrylamide gel. The Coomassie stain and fluorescence photograph are shown for each of the pooled fractions (I–IV).

was reacted with FITC and gel-filtered to remove unreacted dye as described in Experimental Procedures except that the G-50 column was equilibrated with TED and 0.7% CHAPS. The FITC-G_o was concentrated and run on the Mono-Q column as described above. When FITC-labeled G_o was run on a Mono-Q anion-exchange column, three main protein peaks were seen (Figure 3A). The first could be resolved into a main peak (I) and a shoulder (II). The last peak (IV) contained highly fluorescent but inactive α subunits. The broad middle peak (III) bound GTPγS but contained multiple labels per protein (range 3–4, data not shown). SDS–PAGE analysis showed that peak I contained label predominantly in the γ subunit while peak II had label in both α and γ (Figure 3B). Unlabeled, purified G_o eluted from the column as a single peak with a retention time of 20 min, the same as peak I (data not shown). The shoulder (peak II) was further resolved into singly labeled α subunit by activation and C7-Sepharose chromatography as described above. This singly labeled active protein will be called F-α–Mono Q. The free dye bound tightly to the Mono-Q column and was eluted after all of the labeled proteins with 0.5 mL of 75% acetic acid. F-α–Mono Q (fraction II) contained 0.7 label/subunit (Table I).

Table I shows the molar ratio of dye to protein as determined by absorbance (see Experimental Procedures). For the determination of the stoichiometry between TMR and $\beta\gamma$ subunit, another method was used to confirm the results

Table I: Determination of Stoichiometry[a]

labeled subunit	F-α	F-α–Mono Q	TMR-βγ	F-γ/β
dye:protein ratio	0.96 ± 0.15	0.7	0.58 ± 0.07	1.37 ± 0.09

[a] The molar ratio of dye to protein was determined by measuring the UV absorbance at 494 and 555 nm for fluorescein and TMR, respectively (pH 8.0), and the amount of protein was determined by the amido black protein assay. The extinction coefficients used were 64 800 cm^{-1} M^{-1} for fluorescein and 75 000 cm^{-1} M^{-1} for TMR. Data are mean ± SEM of measurements on three preparations of each subunit except for Mono-Q purified F-α, where $n = 1$.

obtained by the measurement of the absorbance of the fluorophore. We examined the decrease in [^{3}H]-*N*-ethylmaleimide (NEM) incorporation into βγ following reaction with TMR-IAA. The number of the free sulfhydryl groups on the βγ subunit accessible to NEM under nondenaturing conditions is reported to be 2.0 (Ho & Fung 1984; Winslow et al., 1987; Kwon and Neubig, unpublished results). Treatment with TMR-IAA reduced [^{3}H]NEM labeling by 30% (data not shown) consistent with the molar ratio of 0.6 TMR/βγ, obtained by absorbance measurements.

Effects of Fluorescent Modification on the Function of α and βγ Subunits. The function of F-α was determined using four different assays. The GTPγS binding and ADP-ribosylation by pertussis toxin for F-α are 63% and 78% of control, respectively (Figure 4A,B). However, there was no significant difference between F-α and native G_o/G_i α subunit for GTPase turnover number (Figure 4C). These results were surprising in that FITC-transducin lost 75% of its GTPase activity (Hingorani & Ho, 1987). Despite the overall 1:1 ratio of dye to protein, we showed (Figure 3) that the F-α was a mixture of active and inactive, labeled and unlabeled α subunits.

The function of F-α–Mono Q was somewhat different from the total pool of F-α (Figure 4D,E). Both GTPγS binding and the ability of PT to ADP-ribosylate F-α–Mono Q were minimally decreased (Figure 4D,E) while a 54% decrease in GTPase was observed (Figure 4F), similar to the results of Hingorani and Ho (1987) with FITC-transducin.

The sucrose density sedimentation experiment shows that fluorescein-$α_{total}$ forms a heterotrimer with the βγ subunit (Figure 5A). Fluorescein-α alone migrated near the top of the gradient with a sedimentation coefficient of 2 S. In the presence of βγ subunits, the fluorescence peak shifted toward the bottom of the gradient with sedimentation coefficient of about 4 S, which corresponds to the published value of the sedimentation coefficient for the heterotrimer form (Huff & Neer, 1986). The ability of F-α to form heterotrimers with βγ appears to be somewhat attenuated as compared to the ability of TMR-βγ and F-γ/β to form heterotrimers as assessed by relative shifts in the sucrose density gradient (Figure 5). This may be due to the presence of inactive labeled α subunit (Figure 3, peak IV) in F-α.

The function of TMR-βγ and F-γ/β was determined using three different assays. First, TMR-βγ and F-γ/β were able to form heterotrimers with α subunits according to the sucrose density sedimentation method as shown in Figure 5B,C. Second, the heterotrimer form of G protein is a better substrate for ADP-ribosylation of the α subunit by pertussis toxin compared to the α subunit alone (Neer et al., 1984; Katada et al., 1986). Therefore, we assessed the function of fluorescently modified βγ subunits by examining their ability to support the pertussis toxin catalyzed ADP-ribosylation of purified bovine $α_o$. Figure 6 shows that both fluorescently modified βγ subunits were able to support the ADP-

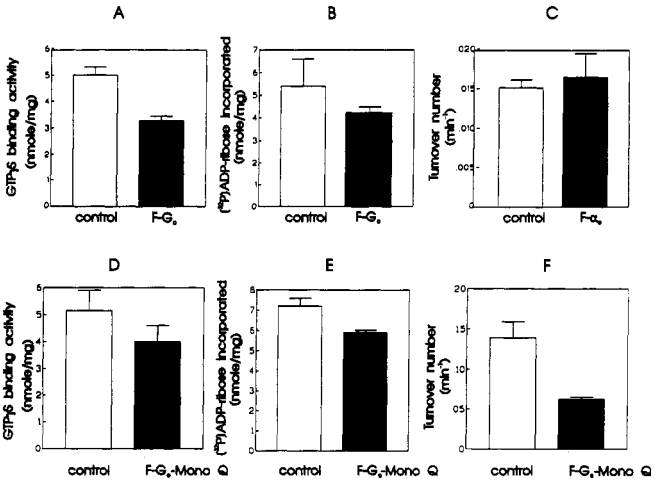

FIGURE 4: Effect of fluorescent modification on the function of Gα. (A) Binding of [^{35}S]GTPγS to G_o/G_i and F-G_o/G_i. [^{35}S]GTPγS binding to control G_o/G_i and F-G_o was determined as described in Experimental Procedures. (B) [^{32}P]ADP-ribosylation of G_o/G_i by pertussis toxin. Varying amounts (0.1, 0.2, and 0.4 μg) of G_o/G_i were incubated with 2 μM [^{32}P]NAD and the amount of [^{32}P]NAD incorporated was quantitated as described in Experimental Procedures. (C) Hydrolysis of [^{32}P]GTP by the α subunit of G_o/G_i and F-G_o/G_i. The α subunits (75 nM) were incubated in HED buffer containing 1 μM [γ-^{32}P]GTP and the release of [^{32}P] P_i was quantitated as described in Experimental Procedures. (D) Binding of [^{35}S]GTPγS to G_o and F-G_o. Binding to Mono-Q purified G_o and F-G_o was determined as described in panel A. The F-G_o contained 0.9 label in γ and 0.7 label per α subunit. Incorporation of label in the γ subunit did not alter [^{35}S]GTPγS binding (data not shown). (E) [^{32}P]ADP-ribosylation of G_o and F-G_o by pertussis toxin. The amount of labeling by pertussis toxin was determined as described in panel B on the Mono-Q purified G_o and F-G_o. (F) Hydrolysis of [^{32}P]GTP by Mono-Q purified G_o and F-G_o. Turnover numbers were determined using 24 nM G_o and F-G_o as described in Experimental Procedures. Data in panels A–C are the mean ± SEM of three different experiments from three preparations of FITC-G_o. Data in panels D and E are the mean and range of two different experiments performed in triplicate from two preparations of Mono-Q purified G_o and F-G_o.

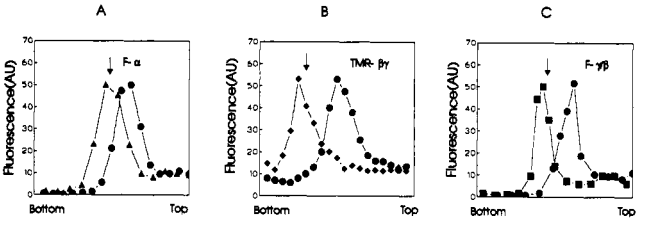

FIGURE 5: F-α, TMR-βγ, and F-γ/β form heterotrimers with their respective unlabeled subunits based upon sucrose density gradient sedimentation in Lubrol. The abilities of fluorescently modified G protein subunits (15 μg) to form heterotrimers with their appropriate unlabeled subunits (30 μg) were examined to assess their function using a sucrose density centrifugation method. (A) F-$α_o$ alone (●) or F-α in the presence of βγ (▲) were incubated for 20 min at 30 °C. Marker proteins were added to the samples after 20 min of incubation and the mixtures were layered on top of 5-mL gradients. After centrifugation, fractions were collected from the bottoms of the tubes. The detailed procedure is described in Experimental Procedures. (B) The same procedure was followed with different subunits. (●) TMR-βγ alone; (◆) TMR-βγ + α. (C) (●) F-γ/β alone; (■) F-βγ + α. The arrows indicate the peak of bovine serum albumin, one of the internal standards, which has the sedimentation coefficient 4.2 S. The data are from a single experiment representative of three different experiments with each subunit.

ribosylation of α subunit as well as the native βγ subunits. The maximal incorporation was ~20–25 pmol, which is near the expected value of 51 pmol for 2 μg of α. Third, we assessed the interaction of TMR-βγ and F-γ/β with the effector adenylyl cyclase by examining their ability to inhibit adenylyl

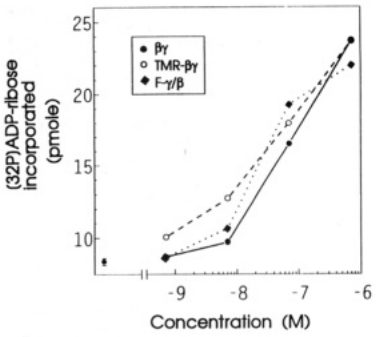

FIGURE 6: TMR-$\beta\gamma$ and F-γ/β support the ADP-ribosylation of the α_o as well as the native $\beta\gamma$ subunit by pertussis toxin subunit. The ability to TMR-$\beta\gamma$ and F-γ/β to support the ADP-ribosylation of α_o catalyzed by pertussis toxin was determined to assess the interaction of TMR-$\beta\gamma$ and F-γ/β with α_o. Two micrograms of α_o in the presence of varying amounts of native $\beta\gamma$, TMR-$\beta\gamma$, or F-γ/β (1, 10, 100, or 1000 ng) was reacted with pertussis toxin and 2 μM [^{32}P]NAD for 1 h at 30 °C. The reaction was terminated by the addition of 30 μL of 4× sample buffer. The reaction mixture was applied to an SDS–13% polyacrylamide gel. The closed circle at the lower left corner shows the amount of [^{32}P]ADP-ribose incorporated to the α_o alone. (●) Control $\beta\gamma$; (○) TMR-$\beta\gamma$; (◆) F-γ/β.

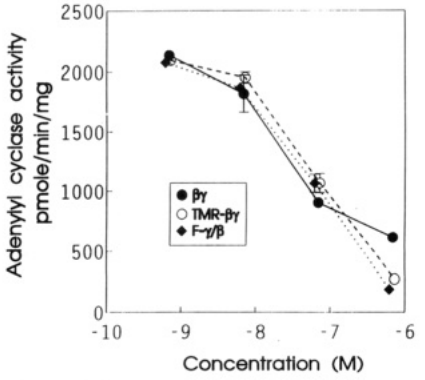

FIGURE 7: TMR-$\beta\gamma$ and F-γ/β inhibit forskolin-stimulated adenylyl cyclase activity. The ability of TMR-$\beta\gamma$ and F-γ/β to inhibit adenylyl cyclase activity in the platelet membranes was examined. Varying concentrations of native $\beta\gamma$, TMR-$\beta\gamma$, or F-γ/β (0.66, 6.6, 66 or 655 nM) were incubated with platelet membranes in the assay buffer (Experimental Procedures) in the presence of 0.5 μCi of [α-^{32}P]ATP for 15 min at 30 °C. [^{32}P]cAMP formed was isolated and quantitated. (●) Control $\beta\gamma$; (○) TMR-$\beta\gamma$; (◆) F-γ/β.

cyclase activity in human platelet membranes. Figure 7 shows that TMR-$\beta\gamma$ and F-γ/β inhibit forskolin-stimulated adenylyl cylase activity in the platelet membranes as well as native $\beta\gamma$ subunits. TMR-$\beta\gamma$ and F-γ/β at 655 nM inhibited over 90% of adenylyl cyclase activity in the platelet membranes.

Localization of the FITC and TMR Sites by Limited Tryptic Peptide Mapping. Figure 8A shows the tryptic fragmentation pattern of F-α_{total} as revealed by FITC fluorescence(left) and Coomassie Blue staining (right). The partial tryptic digestion of F-α results in the cleavage of a 2-kDa fragment from the amino teminus (Figure 8A), resulting in a 37-kDa peptide which contained fluorescence. Further digestion resulted in two fragments, 24- and 15-kDa peptides. The 15-kDa peptide contained the majority of fluorescent label. Further tryptic digestion of F-α also occurred, as is evident by the fluorecence in the dye front of the trypsin-treated protein. Figure 8B shows the tryptic fragmentation pattern of TMR-$\beta\gamma$ and F-γ/β. TMR mainly incorporated into the 26-kDa fragment of the β subunit with minor incorporation into γ subunit. Interestingly, the TMR-IAA appears to label a different γ subunit than does the FITC. The former has a higher apparent molecular weight and may correspond to γ_3 (Tamir et al., 1991). F-γ/β was not cleaved

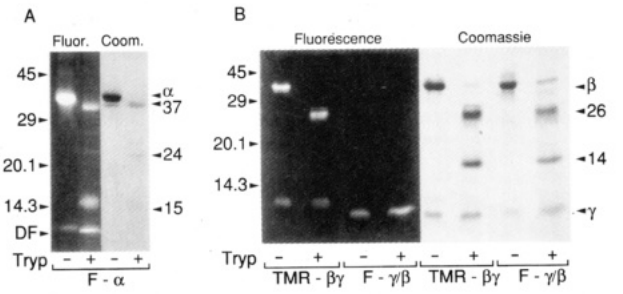

FIGURE 8: Localization of the FITC and TMR sites by tryptic peptide mapping. Ten micrograms of F-α, TMR-$\beta\gamma$, or F-γ/β was digested with 0.1 μg of trypsin for 30 min at 30 °C. Trypsin digestion was stopped by addition of 10 μL of 4× sample buffer, followed by boiling for 3 min. (A) F-α and trypsin-digested F-α were run on an SDS–13% polyacrylamide gel. Lanes 1 and 2 show the photographs of fluorescence, and lanes 3 and 4 show protein bands stained with Coomassie Blue. Lanes 1 and 3 contain control F-α, and lanes 3 and 4 contain trypsin-digested F-α. (B) TMR-$\beta\gamma$, F-γ/β, and trypsin-digested TMR-$\beta\gamma$ and F-γ/β were run on a Tricine–SDS–13% polyacrylamide gel to have a good separation of γ subunits from the dye front. Lanes 1–4 show the photographs of fluorescence, and lanes 5–8 show protein bands stained with Coomassie Blue. Lanes 1 and 5, TMR-$\beta\gamma$ lanes 3 and 7, $\beta\gamma$; F-γ/β; lanes 2 and 6, trypsin-digested TMR-$\beta\gamma$; lanes 4 and 8, trypsin-digested F-γ/β.

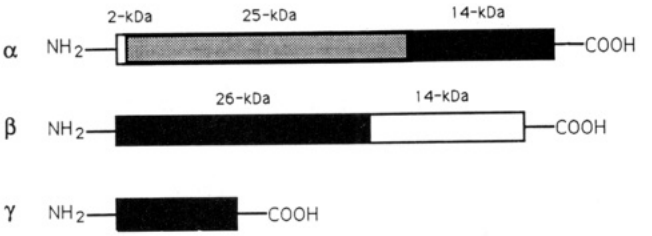

FIGURE 9: Schematic diagram indicating fluorescent label sites on α, β, and γ subunits. The possible fluorescent label sites on each subunit are shown by the black areas. The stippled area indicates minor labeling.

by trypsin. Figure 9 shows a schematic diagram indicating the likely locatioins of fluorescent label sites in α, β, and γ chains.

DISCUSSION

We report here detailed methods used to label all three G protein subunits with fluorescent reagents, the effects of fluorescent modifications on the activities of the G proteins, and initial data on the location of incorporated labels. Hingorani and Ho (1987) initially reported the selective modification of α_T subunit with an isothiocyanate reagent, FITC. The chemical modification of an amine residue on the α_T subunit with FITC had no effect on the interaction of α_T with rhodopsin or on the binding of guanosine 5'-(β,γ-imidotriphosphate) to transducin in the presence of photolyzed rhodopsin. It did, however, decrease the intrinsic GTPase activity of α_T as well as the ability of activated α_T to activate cGMP phosphodiesterase. Erickson and Cerione (1991) reported the chemical modification of α_T with eosin 5-isothiocyanate, which exhibited the same labeling kinetics as that described for the FITC modifications. We labeled bovine G_o/G_i with FITC using a modification of the procedure reported by Hingorani and Ho (1987). The labeling pattern and the effect of FITC labeling on G_o/G_i were somewhat different from those of transducin, even though similar conditions of labeling were used. The incorporation of FITC was found only on the α_T subunit of transducin, whereas a substantial amount of labeling was also found on the γ subunit of bovine brain G_o/G_i (Table I). In addition, we observed the FITC labeling of G_o/G_i resulted in a heterogeneous preparation

of labeled and unlabeled α subunit despite incorporation of one label on average per α subunit. Using Mono-Q anion-exchange chromatography followed by AMF activation and C7-Sepharose chromatography, we were able to prepare singly labeled α subunit. The stoichiometry of 0.7 fluorescein/mol of α_o protein may be due to incomplete resolution of the shoulder containing F-α_o from unlabeled α_o. F-α–Mono Q was able to serve as PT substrate, suggesting that it is able to form a functional heterotrimer. Although the modification of crude G_o/G_i with FITC had no effect on the GTPase turnover number, when F-α–Mono Q was purified, reduced GTPase activity was observed (Figure 4), which is similar to the results for FITC-labeled transducin. The GTPase turnover numbers are similar for F-α_o and native α_o (Figure 4C). The rates of the GTPase activities were an order of magnitude lower than those measured here for heterotrimer G_o (Figure 4F) and by other groups (Higashijima et al., 1987). The large difference in control turnover numbers may be due to a loss of catalytic activity during the resolution of α from βγ.

The labeled βγ subunits (TMR-βγ and F-γ/β) can both bind efficiently to α_o since the fluorescence peak shifted toward the bottom of the gradient in the presence of α subunit based on the sucrose gradient sedimentation. This could not be due to residual unlabeled subunit, as we measured a shift in the mobility of the fluorescence as well as a shift in protein mobility. The mechanism of the effects of the G protein βγ subunits on the effector protein, adenylyl cyclase, are not clear. Katada et al. (1986) initially reported that the βγ subunits inhibited the adenylyl cyclase via a direct protein–protein interaction, although the concentrations of the βγ required for the inhibition seemed to be too high to be safely responsible for an inhibition under physiological conditions. Katada et al. (1987b) also reported that much lower concentrations of the βγ subunit of G_o/G_i inhibited 80% of calmodulin-stimulated, but not forskolin-stimulated, adenylyl cyclase partially purified from rat brain membranes. They proposed a direct interaction of the βγ with calmodulin as a mechanism involved in βγ-induced inhibition of the calmodulin-stimulated adenylyl cyclase. Mangels et al. (1992) have shown that a direct stoichiometric effect of βγ on calmodulin cannot account for the inhibition of adenylyl cyclase since βγ can inhibit the effect of a 1000-fold excess of calmodulin. In this report, low concentrations of both the native βγ subunit of G_o/G_i and the fluorescently modified βγ subunit inhibited over 90% of forskolin-stimulated adenylyl cyclase in the platelet membranes. Thus the labeled βγ subunits maintain normal effector function (either directly on the catalytic subunit or via α_s). Tang and Gilman (1991) studied the effects of the βγ subunit on different types of adenylyl cyclases in transfected Sf9 cell membranes. They reported that in the presence of $G_s\alpha$ the βγ subunit inhibited one form of adenylyl cyclase(Type I), stimulated another form, and had no effect on some forms. They propose that the inhibitory effect of βγ on Type I adenylyl cyclase is induced by a direct protein–protein interaction.

Casey et al. (1989) reported that substoichiometric amounts of the G protein βγ subunits from bovine brain were sufficient to support the ADP-ribosylation of the α subunit of G_o. We also observe that substoichiometric amounts of either fluorescently modified βγ subunits or native βγ subunits were sufficient to support the ADP-ribosylation of the α subunits. These data support their speculation that the ADP-ribosylated G protein oligomers can dissociate into their respective α and βγ subunits in the absence of activating regulatory ligands.

The sites of tryptic cleavage on the α_o subunit were identified by Hurley et al. (1984). The linear peptide map of the α_o subunit constructed by Hurley et al. illustrates two trypsin cleavage sites, resulting in 2-, 24-, and 15-kDa fragments from N-terminus to C-terminus. Under the same condition, the β_T subunit was cleaved into 26- and 14-kDa fragments and γ_T was not cleaved. Pines et al. (1985) reported that the tryptic fragments of purified brain G-β are identical to those of β_T based on the immunoblot analysis.

The limited tryptic digestion of F-α_o resulted in 25- and 14-kDa fragments, which are comparable to the 23- and 9-kDa fragments for transducin. Hingorani and Ho (1987) observed that both the 23- and 9-kDa fragments contained the FITC label, with the majority of label incorporating to the 23-kDa fragment (72%). The FITC label sites for F-α_o was different from those for transducin, having the majority of label associated with the 14-kDa fragment instead of the 24-kDa fragment. The FITC incorporation at two different sites of α_o subunit implies that there are at least two separate lysine residues on the α_o subunit molecule that can be labeled by FITC. The molar ratio of the dye to the α_o subunit, however, is close to unity instead of 2. This discrepancy between the stoichiometry and the possible label sites for the α_T was also observed for both fluorescein- and eosin-labeled α_T. Labeling may be imcomplete or labeling of the two sites on α subunit may be mutally exclusive. Indeed, when F-α was resolved using Mono-Q anion-exchange chromatography, we found that F-α was composed of unlabeled α subunit (associated with F-γ/β) and singly and multiply fluorescein-labeled α subunits.

In this paper we present a detailed study of the preparation of fluorescently modified G protein subunits which retain full activities for GTP binding, subunit–subunit, and effector interactions. These fluorescently modified G protein subunits will be valuable tools in studying the molecular mechanisms of subunit interactions with each other and with either receptors or effector proteins in a lipid environment. In the accompanying paper, Remmers and Neubig (1993) report the distance estimates between fluorescently modified G protein subunits and a lipid fluorescent probe, using resonance energy transfer. The measurement of the lateral mobilities of fluorescently labeled G protein subunits in intact cells using the fluorescence photobleaching recovery technique is currently under investigation.

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