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Guanine Nucleotide Binding Characteristics of Transducin: Essential Role of Rhodopsin for Rapid Exchange of Guanine Nucleotides†

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ABSTRACT: Transducin (G_t) is a member of a family of receptor-coupled signal-transducing guanine nucleotide (GN) binding proteins (G-proteins). Light-activated rhodopsin is known to catalyze GN exchange on G_t, resulting in the formation of the active state of the $G_{t\alpha}$–GTP complex. However, purified preparations of G_t have been shown to exchange GN in the absence of activated receptors [Wessling-Resnick, M., & Johnson, G. L. (1987) *Biochemistry 26*, 4316-4323]. To evaluate the role of rhodopsin in the activation of G_t, we studied GN-binding characteristics of different preparations of G_t. G_t preparations obtained from the supernate of GTP-treated bovine rod outer segment (ROS) disks, followed by removal of free GTP on a Sephadex G-25 column, bound GTPγS at 30 °C in the absence of added exogenous rhodopsin with an activity of 1 mol of GTPγS bound/mol of G_t (G_t-I preparations). Binding of GTPγS to G_t-I preparations closely correlated with the activation of ROS disk cGMP phosphodiesterase. GN-binding activity of G_t-I preparations was dependent on reaction temperature, and no binding was observed at 4 °C. In the presence of 10 μM bleached rhodopsin, G_t-I preparations bound GTPγS at 4 °C. However, hexylagarose chromatography of G_t-I preparations led to a preparation of G_t that showed <0.1 mol/mol binding activity following 60-min incubation at 30 °C in the absence of rhodopsin (G_t-II preparations). In the presence of as low as 0.03 μM bleached rhodopsin, G_t-II preparations rapidly bound GTPγS at 30 °C. Equimolar mixtures of G_t-I and G_t-II preparations showed a 1.0 mol/mol binding activity in the absence of added rhodopsin. While treatment of G_t-I preparations with hydroxylamine, which is known to convert rhodopsin to opsin, greatly diminished the rate of GTPγS binding to the preparation, addition of 0.03 μM bleached rhodopsin following removal of hydroxylamine restored the binding activity. These results indicate that (1) rhodopsin is essential for rapid GN exchange on G_t and (2) rapid GN exchange in the absence of exogenous rhodopsin observed in some G_t preparations is stimulated by undetected rhodopsin contamination.

Signals generated by hormones and neurotransmitters at the level of cell surface receptors are now well-known to be transmitted through a family of highly homologous GTP-binding proteins (G-proteins)[1] [for review, see Gilman (1984, 1987)]. Hormonal activation and inhibition of the enzyme adenylate cyclase and light activation of retinal rod outer segment (ROS) cGMP phosphodiesterase (PDE) are among the best characterized receptor–effector systems coupled through G-proteins. The G-proteins G_s and G_i couple stimulatory and inhibitory receptors, respectively, to the enzyme adenylate cyclase. In the homologous system of visual signal transduction, light-activated rhodopsin is coupled to retinal ROS G-protein transducin (G_t). Receptor-coupled G-proteins are heterotrimeric proteins composed of α, β, and γ-subunits.

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[1] Abbreviations: G-protein, a member of the family of signal-transducing GTP-binding regulatory proteins; G_t, vertebrate retinal G-protein (transducin); G_s and G_i, stimulatory and inhibitory GTP-binding proteins of the adenylate cyclase system, respectively; G_o, 39-kDa GTP-binding protein of high abundance in the brain; ROS, rod outer segment; PDE, phosphodiesterase; cGMP, guanosine 3′,5′-cyclic monophosphate; GN, guanine nucleotides; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole.

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Activated receptors have been shown to catalyze GTP exchange for the GDP bound to the α-subunit of G-proteins, leading to the formation of the active state of G_α–GTP. Subsequently, the intrinsic GTPase activity of the α-subunit serves as a signal terminator, causing the conversion of GTP to GDP and the formation of the inactive state of G_α–GDP.

Reconstitution of purified receptors and G-proteins has consistently shown that receptors catalyze guanine nucleotide (GN) exchange with the GDP bound to G-proteins and enhance the GTPase activity of these proteins. Such reconstitution of receptors with G-proteins has been described for the β-adrenergic receptor with G_s (Lefkowitz et al., 1985; Brandt & Ross, 1986), the muscarinic receptors with G_i (Kurose et al., 1986) and G_o (Florio & Sternweis, 1989), the α_2-adrenergic receptor with G_i and G_o (Cerione et al., 1986), the μ opioid receptors with G_i and G_o (Ueda et al., 1988), and rhodopsin with G_t (Fung & Stryer, 1980; Fung et al., 1981; Baehr et al., 1982; Wessling-Resnick & Johnson, 1987b,c; Yamazaki et al., 1987; Bennet & Dupont, 1985). These results define an essential role for receptors in the activation of G-proteins.

However, purified preparations of G-proteins have been shown to exhibit GN-binding activity in the absence of receptors with K_d's in the submicromolar range (Northup et al., 1982; Brandt & Ross, 1985; Bokoch et al., 1984; Sternweis & Robishaw, 1984; Huff & Neer, 1986; Sunyer et al., 1984). Considering that the intracellular concentration of GTP is in the near-millimolar range and much above saturation level, this phenomenon appears to contradict the central role of G-proteins as cellular signal-transducing elements. Hence, intrinsic GN-binding activity of G-proteins must either (1) play an essential physiological function or (2) be slower than the intrinsic GTPase activity of the protein, or (3) G-proteins in their native cellular environment are tonically inhibited by an as yet undescribed mechanism. Unlike other G-proteins, GN binding to purified G_t has generally been considered to be dependent on the presence of light-activated rhodopsin [for review, see Fung (1985)]. However, in most studies GN binding to purified preparations of G_t was carried out at 4 °C (Fung et al., 1981; Baehr et al., 1982; Kelleher et al., 1986; Yamazaki et al., 1987; Kanaho et al., 1988; Godchaux & Zimmerman, 1979). In a recent study, Wessling-Resnick and Johnson (1987a) have shown GTPγS binding to G_t in the absence of photolyzed rhodopsin. Likewise, in our laboratory we have consistently observed a high rate of GTPγS binding activity to G_t preparations at 30 °C without the addition of exogenous photolyzed rhodopsin to the reaction mixture. Since this phenomenon is of important functional significance, we have examined GTPγS-binding activity of different G_t preparations and studied the effect of bleached rhodopsin on this activity. Results of this study showed that GTPγS binding to highly purified preparations of G_t is very slow and that higher rates of binding in some preparations is due to the presence of undetected rhodopsin contaminating these preparations.

Materials and Methods

Preparation of G_t. G_t was prepared from bovine retinal ROS preparations following general methods outlined by other investigators (Papermaster & Dreyer, 1974; McDowell & Kuhn, 1977; Kuhn & Hargrave, 1981; Kuhn, 1980), with some modifications. In general, we employed two methods for the preparation of bovine ROS, in laboratory light (method A) and under dim red light (method B).

Method A (Preparation of ROS Disks). In this method all steps were carried out in fluorescent laboratory light on ice and/or in a cold room at 4 °C. Retinas were dissected from 100 fresh bovine eyes obtained from a local slaughterhouse and placed into 200 mL of ice-cold solution A (65 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, and 1 mM DTT) and 45% (w/w) sucrose. Retinas in this buffer were then shaken vigorously for 1 min to shear off the ROS. Samples were then spun at 3500 rpm in a JA-14 rotor (1880*g*) for 10 min, supernatants containing the ROS were removed, and pellets were used for a second extraction with 100 mL of solution A containing 45% sucrose. Supernatants from both extractions were pooled and mixed with 3 volumes of ice-cold solution B (10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, and 1 mM DTT) to bring the sucrose concentration down to about 15%. Samples were then spun at 10000 rpm in a JA-14 rotor (15300*g*) for 30 min. Supernatants were discarded, and pellets containing ROS were suspended in 48 mL of solution B containing 24% (w/w) sucrose and layered over a step gradient of 26%, 30%, and 34% (w/w) sucrose in solution B. Samples were then spun at 27000 rpm in an SW 28 (96500*g*) for 30 min, and the bright orange layer at the 26%/30% interface containing the ROS was harvested and diluted to 2.5 times volume with solution B. ROS were then pelleted by centrifugation at 20000 rpm in a JA-20 rotor (48400*g*) for 10 min, and pellets were resuspended in solution B containing 12% sucrose (10 mL), divided into 1-mL aliquots, rapidly frozen in a dry ice–methanol bath, and stored at −80 °C.

Method A (Preparation of G_t). Frozen ROS samples were thawed and resuspended in solution C (5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM DTT) containing 100 μM PMSF (to 40 mL) with a Dounce homogenizer. Samples were spun at 20000 rpm in a JA-20 rotor (48400*g*) for 30 min, supernatants were removed and saved, and the procedure was repeated twice. All supernatants were combined and used for the preparation of retinal ROS cGMP PDE. The final pellet was resuspended in 25 mL of solution C containing 10 μM GTP to elute G_t from disk membranes. Samples were spun at 20000 rpm in a JA-20 rotor (48400*g*) for 30 min, and supernatants containing G_t were removed. G_t extraction was repeated twice; all supernatants were pooled and spun at 34000 rpm in a TY 35 rotor (90000*g*) for 1 h to remove residual contamination of ROS disks membranes. Supernatants containing the purified G_t were concentrated over Amicon PM-30 membranes down to 1–2 mL, rapidly frozen in a dry ice–methanol bath, and stored at −80 °C. Pellets of washed ROS disk membranes were resuspended in 10 mL of solution C, divided into 1-mL samples, frozen rapidly, and stored at −80 °C for further applications. G_t samples were later thawed, fortified with 100 mM EDTA to obtain a final concentration of 10 mM, and applied to a 20-mL Sephadex G-25 column preequilibrated and eluted with solution D (20 mM MOPS, pH 7.5, 1 mM EDTA, and 1 mM DTT) to remove free nucleotides. Fractions of the void volume were pooled and concentrated to 1–2 mL over Amicon PM-30 membranes. Usually about 2 mg of G_t was recovered from a preparation of 100 retinas, and samples showed >95% purity on Coomassie blue stained SDS–polyacrylamide gels. These G_t preparations consistently showed GTPγS-binding activity at 30 °C in the absence of added rhodopsin and are referred to as G_t-I.

In some experiments, ROS disk membranes remaining after the extraction described above were reused for further extraction of G_t. Frozen ROS disks were later thawed and washed with solution C, and G_t was eluted with 100 μM GTP as described above. The second extraction yielded an additional 2 mg of G_t/100 retinas, and samples showed a high

degree of purity on Coomassie blue stained SDS–polyacrylamide gels (>95%). However, these G_t preparations, following removal of free nucleotides over G-25 columns, showed very little GTPγS-binding activity at 30 °C and were referred to as G_t-II.

Method B (Preparation of ROS Disks). In this method all steps, except when indicated, were carried out under dim red light (Kodak Safelight filter no. 1A) on ice and/or in a dark cold room. Frozen retinas dissected under dim red light from dark-adapted bovine eyes were obtained from J. A. & W. L. Lawson, Co., of Lincoln, NE. Retinas (100) were thawed and placed in 200 mL of solution E (70 mM potassium phosphate, pH 7, 1 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT) containing 45% (w/w) sucrose and 100 μM PMSF. All subsequent steps were carried out as described for method A, except solution E was used throughout the procedure instead of solutions A and B and the final disk preparation was suspended in solution E containing 12% (w/w) sucrose.

Method B (Preparation of G_t). ROS disk membranes prepared under dim red light as described for method B were thawed, suspended in solution F (100 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM DTT, and 100 μM PMSF), and transferred to a motor-driven 55-mL Potter-Elvejhem homogenizer. Samples were homogenized by carrying out 3 × four passes with 1-min intervals. Samples were then transferred to a plastic beaker, and the $MgCl_2$ concentration and volume were adjusted to 3 mM and 120 mL, respectively. The homogenate was spun at 20000 rpm in a JA-20 rotor (48400*g*) for 15 min; the pellet was resuspended in solution F, homogenized, and spun as described above. The final pellet was resuspended in 1 mM $MgCl_2$–1 mM DTT, warmed to 20 °C, and bleached for 2–3 min. The sample temperature was brought back to 4 °C, the volume was adjusted to 100–120 mL with solution G (5 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM DTT), and the sample was spun at 20000 rpm in a JA-20 rotor (48400*g*) for 30 min. Supernatants were saved; pellets were resuspended in solution G and spun at 20000 rpm in a JA-20 rotor (48400*g*) for 30 min. The procedure was repeated twice. All supernatants were combined and saved for subsequent purification of ROS disk PDE. The final pellet was resuspended in 25 mL of solution G containing 80 μM GTP to elute G_t from disk membranes. The sample was spun at 20000 rpm in a JA-20 rotor (48400*g*) for 30 min, the supernatant containing G_t was saved, and the extraction was repeated twice. All supernatants were combined, spun at 34000 rpm in a TY 35 rotor (90000*g*) for 60 min to remove residual ROS disk membranes, concentrated over Amicon PM-30 membranes to 2–3 mL, rapidly frozen in a dry ice–methanol bath, and stored at −80 °C. The final pellet containing ROS disk membranes was resuspended in solution G (10 mL), divided into 1-mL samples, rapidly frozen, and stored at −80 °C for subsequent preparation of urea-washed ROS disk membranes. G_t samples were later thawed, fortified with EDTA, and applied to a 20-mL Sephadex G-25 column to remove free nucleotides as described for method A. About 2–3 mg of G_t was recovered from a preparation of 100 retinas, and the estimated purity of preparations observed on Coomassie blue stained SDS–polyacrylamide gels was >95%.

In a separate experiment, G_t was eluted from disk membranes with solution G containing 10 μM GTP followed by solution G containing 100 μM GTP. These G_t samples were pooled and treated separately, as described above. This procedure yielded about 2 and 4 mg of G_t from each elution step, respectively. All G_t preparations, obtained following method B showed GTPγS-binding activity at 30 °C in the absence of added exogenous rhodopsin to the reaction mixture and were referred to as G_t-I.

Hexylagarose Chromatography of G_t. Samples of G_t preparations were subjected to hexylagarose chromatography for further purification as described by Fung et al. (1981). Briefly, in a typical experiment, the G_t sample purified according to method B containing 0.8 mg of protein was diluted with solution D containing 20 mM NaCl to 5 mL and loaded onto a 9-mL column (14 × 0.9 cm diameter) of hexylagarose (capacity = 10–12 mg of BSA/mL) preequilibrated with solution D containing 20 mM NaCl. The column was washed with 5 mL of the same solution, and G_t was eluted with a linear gradient (80 mL) of 20–200 mM NaCl in solution D, at a flow rate of 25 mL/h. One-milliliter fractions were collected, and 25-μL aliquots of fractions with absorbance at 280 nm were used for the [^{35}S]GTPγS-binding assay in the presence (4 °C, 1 h) and absence (30 °C, 1 h) of added rhodopsin (0.5 mg/mL). Fractions with peak GTPγS-binding activity (25–50 mM NaCl) were pooled, concentrated over Amicon PM-30 to 1–2 mL, rapidly frozen, and stored at −80 °C. About 50% of the applied G_t was recovered in the final step, and this sample showed the highest degree of purity compared to all previous preparations of G_t. On Coomassie blue stained SDS–polyacrylamide gels, only the bands corresponding to the α-, β-, and γ-subunits of G_t were visible. G_t samples eluted from the hexylagarose column showed very little GTPγS-binding activity after 1-h incubation at 30 °C in the absence of exogenous rhodopsin. The ratio of control to rhodopsin-activated GTPγS-binding activity after 1-h incubation was <0.1. These samples were referred to as G_t-II.

Urea-Washed ROS Disks. ROS disk membranes were treated with 5 M urea to strip all peripherally bound membrane proteins as described by Yamazaki et al. (1982). Frozen ROS disk membranes remaining after elution of G_t by methods A and B were thawed and suspended in 3 volumes of a solution containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, and 5 M urea. Suspensions were passed through a 21-gauge needle three times, stored on ice for 60 min, and spun at 34000 rpm in a TY 35 rotor (90000*g*) for 30 min. The supernatants were discarded, and the pellets were further treated with urea solution twice. Pellets were then washed three times with solution H (100 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT). The final pellet of the urea-washed ROS disk membranes was suspended in a minimum volume of solution H containing 12% (w/v) sucrose, divided into 1-mL samples, rapidly frozen, and stored at −80 °C. On Coomassie blue stained SDS–polyacrylamide gels, rhodopsin was found to account for about 95% of the protein content in these preparations. With ROS disk membrane preparations obtained by methods A and B, preparations were carried out in laboratory light and under dim red light, respectively.

Hydroxylamine Treatment of ROS Disks. Urea-washed preparations of bleached ROS disk membranes were thawed and suspended in 3 volumes of solution H containing 100 mM hydroxylamine hydrochloride. Samples were spun at 20000 rpm in a JA-21 rotor (48400*g*) for 30 min; the pellets were resuspended in the same solution and kept on ice in a cold room at 4 °C under direct and continuous illumination of fluorescent light for 12 h. Suspensions were spun at 20000 rpm in a JA-21 rotor (48400*g*) for 30 min, the supernatants discarded, and the pellets treated as before twice for 4 h each. Pellets were washed three times with solution H. The final pellet was suspended in solution H containing 12% (w/v) sucrose, divided into 1-mL samples, rapidly frozen, and stored at −80 °C.

GN Binding to G_t. The GN-binding assay was based on a method described by Northup et al. (1982). [^{35}S]GTPγS and [^{3}H]Gpp(NH)p binding to G_t (0.3–3 μM) was carried out in solution containing 20 mM MOPS (pH 7.5), 3 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 3 mg/mL BSA, 100 mM NaCl, and 10 μM GN ([^{35}S]GTPγS at about 5000 cpm/pmol and [^{3}H]Gpp(NH)p at the specific activity obtained, about 6000 cpm/pmol) at 30 °C or at the indicated temperature. Reactions were terminated after 1 or 2 h, or at selected time intervals, by direct dilution of reaction mixtures (5–50 μL) with 2 mL of ice-cold solution I containing 20 mM Tris-HCl (pH 8), 100 mM NaCl, and 25 mM $MgCl_2$. Samples were then rapidly filtered, under a constant vacuum of about 600 mmHg, over 25-mm 0.45-μm nitrocellulose membranes (Millipore type HA), and membranes were washed seven times with 2 mL of ice-cold solution I. Membranes were dried, and radioactivity associated with membranes was counted in a cocktail of toluene and 0.5% PPO.

Rhodopsin-catalyzed binding of GN to G_t was based on a method described by Fung et al. (1981). Binding assays were carried out in the presence of urea-washed ROS disk membranes at the indicated concentrations of rhodopsin on ice, or at 30 °C. In the initial studies, rhodopsin-catalyzed GTPγS binding to G_t, used as a measure of total binding activity of the preparation, was determined in assays carried out in the presence of 0.5 mg/mL dark-adapted urea-washed ROS disk membranes (containing about 12 μg of rhodopsin) on ice in a dark room, with repeated (five to six times) bleachings of 10–15 s, for 60 min. Later experiments were carried out with bleached preparations of urea-washed ROS disk membranes containing 1.0 μM rhodopsin, and reactions were carried out in laboratory light at 30 °C, for 1 h, or as indicated. Results obtained from both of these methods were identical. In all experiments, nonspecific binding was determined in the absence of G_t in the reaction mixture.

Assay of cGMP PDE. Assay of PDE activity was based on a method described by Butcher and Sutherland (1962) and modifications described by Sharma and Wang (1979). Briefly, ROS disk cGMP PDE activity was determined in a reaction mixture (600 μL) containing 20 mM MOPS (pH 7.5), 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mg/mL snake venom (*Ophiophagus hannah*), 2 mM cGMP, and 2–10 μg of ROS disk membranes. Reactions were carried out at 30 °C for 15 min and were terminated by addition of 100 μL of 55% TCA. Samples were spun at 4000 rpm in an HS-4 rotor (3079*g*) for 30 min, and 500 μL of clear supernatants was used for the determination of inorganic phosphate released by the method of Fiske and Subbarow (1925). ROS disk membranes used in these studies were prepared by method A, prior to the elution of PDE and G_t, and were washed four times with 10 volumes of a solution containing 20 mM MOPS (pH 7.5), 20 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and 0.25 M sucrose to remove all traces of sodium phosphate from the preparation. These preparations of disk membranes showed very little basal PDE activity. However, enzyme activity was stimulated by micromolar concentrations of GTPγS and Gpp(NH)p (3–300 μM) and nanomolar concentrations (3–15 nM) of G_t preactivated with GTPγS and Gpp(NH)p. Basal enzyme activity was linear as a function of time, studied for up to 60 min, while GTPγS- and G_t–GTPγS-activated enzyme activities were linear for up to 20 min.

Other Methods and Reagents. Protein concentration was determined by the amido black binding assay described by Schaffner and Weissmann (1973), with BSA as a standard. SDS–polyacrylamide gels were carried out in Laemmli's (1970) buffer system. The acrylamide concentration [30% acrylamide/0.8% bis(acrylamide)] of stacking and running gels (11 cm) was 5% and 11%, respectively. Gels were run at a constant current of 25 mA/slab with constant cooling (8–12 °C) and were stained with a 0.25% Coomassie blue solution in 30% 2-propanol and 10% acetic acid.

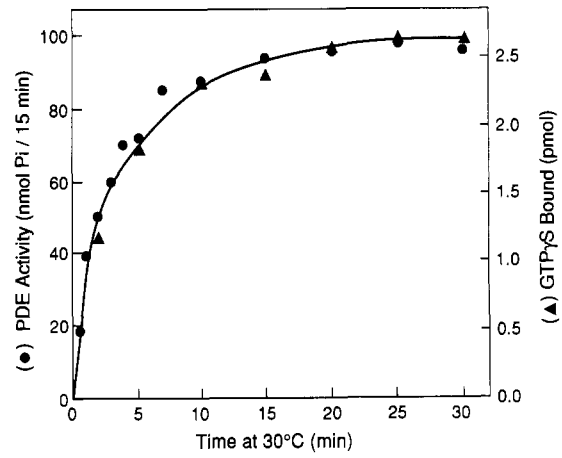

FIGURE 1: Time courses of GTPγS binding to G_t-I and activation of ROS disk cGMP PDE. G_t-I (0.5 μM) prepared according to method A was incubated with 10 μM [^{35}S]GTPγS at 30 °C, and 5-μL aliquots were taken at different times for the determination of amount of GTPγS bound to G_t (▲). In an identical experiment on the same preparation of G_t-I, aliquots of 5 μL were drawn at different time intervals and pipeted directly into reaction mixtures containing ROS disk membranes (reaction volume = 600 μL, 2.5 μg of ROS disks) for the assay of cGMP PDE activity as described under Materials and Methods. PDE assays were carried out for 15 min at 30 °C (●).

Purified bovine opsin (>99%) reconstituted into phospholipid vesicles (phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine at a molar ratio of 5:2:3, respectively) was a generous gift from Dr. James W. Clack (Department of Ophthalmology, Yale University School of Medicine, New Haven, CT). This preparation was obtained from stripped bovine ROS membranes solubilized in 18 mM CHAPS followed by purification of solubilized opsin over Con A–Sepharose and Fractogel TSK-DEAE columns (Clack & Stein, 1988).

Radiolabeled [^{35}S]GTPγS (1200 Ci/mmol) was obtained from New England Nuclear, and [^{3}H]Gpp(NH)p (12 Ci/mmol) was obtained from ICN Biomedicals, Inc. Highly purified samples of GTPγS and Gpp(NH)p were obtained from Boehringer Mannheim and Sigma Chemical Co. Snake venom (*O. hannah*), cGMP (Na salt), and hexylagarose were obtained from Sigma. 9-*cis*-Retinal (98%) was obtained from Aldrich. All other chemicals and reagents were of the highest grade of purity commercially available.

Results

In order to simplify the method of purification of G_t, we have adopted a modified method for its preparation, method A. This method was based on the fundamental principles outlined by Kuhn (1980). However, since G_t was known to remain bound to bleached ROS disk membranes (Kuhn, 1980), all steps of purification were carried out in laboratory light. G_t preparations obtained by this method consistently showed full binding activity to GN, 1 mol of GTPγS or Gpp(NH)p was bound per mol of G_t in the preparation, at 30 °C in the absence of added exogenous rhodopsin to the reaction mixture. Figure 1 shows the time course of GTPγS binding to such a preparation of G_t (G_t-I). As shown in this figure, GTPγS binding to this preparation of G_t, at 30 °C and in the absence of added rhodopsin, was rapid and reached

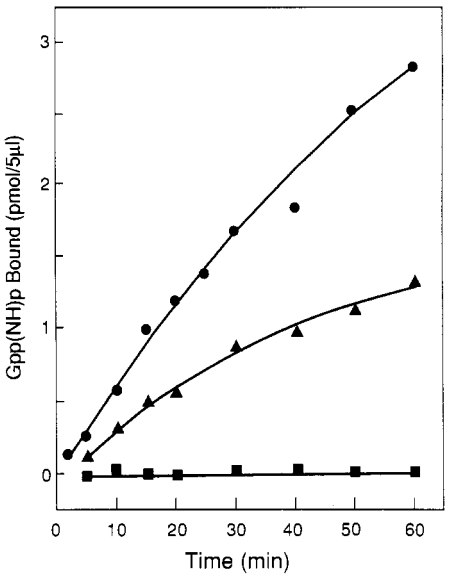

FIGURE 2: Effect of reaction temperature on the rate of Gpp(NH)p binding to G_t-I. Samples of G_t-I (1 μM) prepared according to method A were incubated with 10 μM [^{3}H]Gpp(NH)p at 4 (on ice) (■), 25 (▲), and 30 °C (●). Aliquots of 5 μL were withdrawn at the indicated times for determination of the amount of Gpp(NH)p bound to the preparation as described under Materials and Methods.

Table I: GTPγS-Binding Characteristics of Different Transducin Preparations

transducin (G_t) prepn[a]	sp act. (mol of GTPγS bound/mol of G_t) control[b]	sp act. (mol of GTPγS bound/mol of G_t) rhodopsin[c]	ratio of control/rhodopsin
(1) method A/10 μM	0.965	1.101	0.876
(2) method B/80 μM	1.267	1.204	1.052
(3) method A/100 μM[d]	0.152	1.002	0.152
(4) method B/10 μM[e]	1.165	1.216	0.958
(5) method B/100 μM[e]	1.410	1.489	0.947
(6) hexylagarose[f]	0.195	1.387	0.141
(7) 6 + 5[g]	1.390	1.454	0.956
(8) 6 + 4[g]	1.255	1.315	0.954
(9) 3 + 1[g]	0.944	1.052	0.897

[a] Preparation designation corresponds to the procedures described under Materials and Methods with the variation in GTP concentration used to elute G_t. [b] GTPγS binding was carried out at 30 °C for 2 h with 0.5 μM G_t and 10 μM [^{35}S]GTPγS as described under Materials and Methods. [c] Rhodopsin-catalyzed GTPγS binding to G_t was carried out at 0.5 μM G_t (4 °C for 2 h) with 0.5 mg/mL dark-adapted urea-washed ROS disk membranes (about 12 μM rhodopsin) as described under Materials and Methods. [d] This sample of G_t was eluted with 100 μM GTP from bleached ROS disk membranes previously used for the preparation of G_t (eluted with 10 μM GTP) according to method A, frozen, and stored at −80 °C. [e] Samples 4 and 5 of G_t were obtained by sequential elution of the same ROS disk membrane preparation with 10 and 100 μM GTP, respectively, according to method B. [f] The hexylagarose preparation of G_t is a sample of method B purified G_t chromatographed over a hexylagarose column as described under Materials and Methods. [g] This sample is an equimolar mixture of the two indicated samples.

completion within 15–20 min of incubation with GTPγS. To examine the biological activity of these preparations of G_t, we studied their effect on the activity of retinal ROS disk cGMP PDE. G_t preparations incubated with GTPγS or Gpp(NH)p, followed by removal of free nucleotides on Sephadex G-25 columns, showed direct activation of the enzyme cGMP PDE in a concentration-dependent manner (1–10 pmol/600-μL reaction volume) when reconstituted with retinal ROS disk membranes (data not shown). Figure 1 also shows the kinetics of GTPγS activation of G_t as assessed by activation of ROS disk PDE. As shown in this figure, the rate of GTPγS binding to G_t directly correlated with activation of the target enzyme cGMP PDE.

However, GN binding to G_t in the absence of rhodopsin added to the reaction mixture exhibited by these preparations does not agree with observations made by other investigators, who inferred an essential role of rhodopsin for such an exchange reaction (Fung, 1985). A review of the methods employed by other investigators revealed three important differences between our methods and those of other laboratories: (1) temperature of GN-binding assay to G_t, (2) preparation of G_t from ROS disk membranes in the dark or light, and (3) hexylagarose chromatography of G_t. Hence, we have examined the effects of these variables on the GN-binding properties of G_t.

The effect of reaction temperature on GN binding to G_t-I preparations is shown in Figure 2. Results of these studies showed that G_t-I does not bind GN at 4 °C, and the rate of GN binding to G_t-I was directly related to reaction temperature (see Figure 2). These results appeared to explain the discrepancy between our observations and those of other laboratories. We have also carried out GTPγS-binding assays to G_t on ice and in the presence of rhodopsin, as described by other investigators. These experiments showed rhodopsin catalyzed GTPγS binding to G_t-I at 4 °C (Table I), thereby indicating a direct interaction between G_t-I and rhodopsin. As shown in Table I, these characteristics of GTPγS binding to G_t-I were not unique to preparations of G_t obtained from bleached retinas and exposed to light throughout the preparation process of method A. G_t preparations obtained according to Kuhn's (1980) method in the dark, method B, employed by most investigators in the field, displayed identical GN-binding characteristics with G_t-I prepared according to method A (Table I, second row).

Additionally, we have also noticed that the amount of G_t obtained in method A was about half of that obtained by other investigators (Baehr et al., 1982). A second extraction of bleached ROS disk membranes that have been used for the preparation of G_t according to method A, frozen and stored at −80 °C, with 100 μM GTP resulted in the elution of substantial amounts of G_t (about 2 mg/100 retinas). However, these preparations of G_t did not exhibit the well-characterized rapid kinetics of GN binding to G_t-I at 30 °C. These preparations were not inactive, since they showed full binding activity in the presence of rhodopsin (Table I, third row). Hence, this preparation of G_t was named G_t-II. Initially, these results led us to believe that there were two types of G_t in ROS disk membranes and that sequential treatment of these membranes with 10 and 100 μM GTP resulted in the selective elution of the two populations of G_t. However, we failed to detect any structural differences between the type I and type II preparations of G_t with V8 protease maps, nor did epitope analysis of the reactivity of 14 polyclonal antisera to α- and $\beta\gamma$-subunits reveal any difference. Neither did we detect any incorporation of ^{32}P from labeled ATP into α- or $\beta\gamma$-subunits in dark- or light-prepared ROS disks. Furthermore, sequential treatment of fresh ROS disk membranes with 10 and 100 μM GTP resulted in the elution of similar G_t preparations, both of which showed full binding activity at 30 °C in the absence of added rhodopsin (Table I, rows 4 and 5). Thus, we concluded that the distinction(s) between G_t-I and G_t-II involved some aspect of the preparation which was not well controlled in our modified Kuhn procedures.

Most investigators have not used any additional steps for the purification of G_t other than selective elution with GN followed by removal of free nucleotides. However, Fung et al. (1981) employed an additional step of purification of G_t over a hexylagarose column. In this process, G_t was retained on the hexylagarose column, free nucleotides were eluted with 75 mM NaCl solution, and G_t was subsequently eluted with

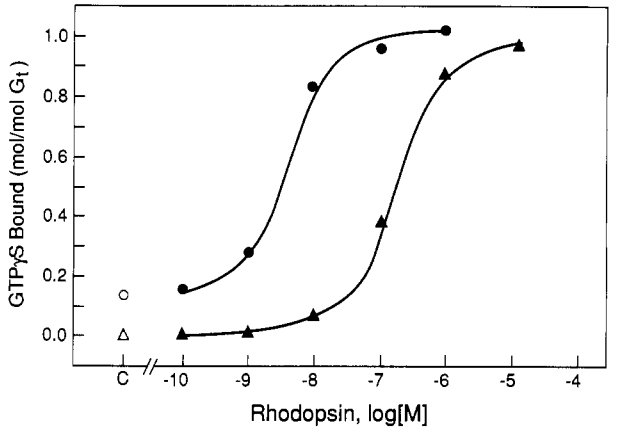

FIGURE 3: Rhodopsin concentration dependence of GTPγS binding to type II G_t. Dark-adapted urea-washed ROS disk membranes were illuminated with laboratory light for 5 h before use. GTPγS-binding assays (50 μL) were carried out in laboratory light for 2 h at 4 (△, ▲) and 30 °C (○, ●) as described under Materials and Methods. Open symbols are control values (shown under C) in the absence of rhodopsin. G_t-II (0.5 μM) used in this study was a sample of G_t-I prepared according to method B and subsequently subjected to hexylagarose chromatography. Data points are an average of duplicates.

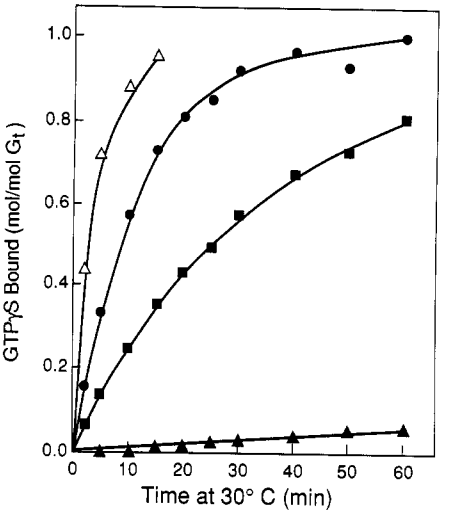

FIGURE 4: Kinetics of rhodopsin-catalyzed GTPγS binding to type II G_t. GTPγS binding to 0.5 μM G_t-II in the absence (▲) and presence of 0.01 (■), 0.03 (●), and 0.1 μM (△) bleached rhodopsin was carried out at 30 °C with continuous exposure to laboratory light. Bleached rhodopsin was prepared as described in Figure 3, and the binding assay was performed as described under Materials and Methods.

300 mM NaCl solution. In our laboratory, initial attempts of hexylagarose chromatography on G_t-I did not alter the GN-binding characteristics of the preparation. However, there was a significant difference between our preparation of hexylagarose and that employed by Fung et al. (1981), since in our hands G_t was eluted from our preparation of hexylagarose at about 25–50 mM NaCl. Subsequent application of hexylagarose chromatography on G_t-I preparations, controlling for the binding capacity of the resin, resulted in a preparation of G_t that showed very little binding activity at 30 °C in the absence of added rhodopsin. Similar to G_t-II preparations, this preparation of G_t showed full binding activity in the presence of rhodopsin (Table I, sixth row). This result indicated that our G_t-I preparations contained a factor that was removed by hexylagarose chromatography. Further, this experiment excluded the possibility that G_t-I preparations bound GN in the absence of rhodopsin due to the lack of bound GDP.

Since the difference between the degree of purity of G_t-I applied to the column and that of G_t-II eluted from the column, visualized on SDS–polyacrylamide gels, was insignificant, these results pointed out that G_t-I must have contained a minute quantity of a catalyst that was removed over the column. To test this possibility, we examined GTPγS binding to equimolar mixtures of G_t-I and G_t-II preparations at 30 °C and in the absence of added rhodopsin. Results of these experiments (Table I, rows 7–9) showed full binding activity of G_t-II at 30 °C in a mixture with G_t-I, thereby indicating that G_t-I preparations must contain a catalytic substance. Hence, we have undertaken to obtain functional evidence for the presence of rhodopsin in G_t-I preparations.

Figure 3 shows the concentration-dependent effect of bleached rhodopsin on GTPγS binding to G_t-II. In this experiment, and in that of Figure 4, rhodopsin of dark-adapted urea-washed ROS disk preparations was bleached for 5 h with fluorescent laboratory light to obtain preparations that would resemble those that may be present in G_t-I preparations obtained according to method A. Furthermore, the binding assay was carried out in laboratory light for 2 h at 4 and 30 °C. It is evident from the results shown in Figure 3 that rhodopsin caused a concentration-dependent activation of GTPγS binding to G_t-II at both temperatures. Figure 3 also shows that, in the presence of as low as 0.01 μM bleached rhodopsin, GTPγS binding to G_t-II following 2-h incubation at 4 °C was insignificant, 7.2% of total, while that achieved at 30 °C was about 84% of total binding activity. These results suggest that the presence of about 0.01 μM bleached rhodopsin in 0.5 μM G_t-I could account for the GN-binding characteristics of these preparations at 4 and 30 °C in the absence of added rhodopsin. Figure 4 shows the time course of GTPγS binding to 0.5 μM G_t-II at 30 °C in the absence and presence of 0.01, 0.03, and 0.1 μM bleached rhodopsin. It is evident from the rate of GTPγS binding to G_t-II shown in Figure 4 that our G_t-I preparations must contain about 0.01–0.03 μM rhodopsin at a G_t concentration of 0.5 μM. This represents about 2–6% of the G_t concentration. The data of Figure 3 also show that as little as 0.2% contamination of G_t with rhodopsin would lead to a preparation showing significant GN exchange in our assays.

Therefore, we devised experiments to test for the presence of minute quantities of rhodopsin in G_t-I preparations. We have examined the effect of hydroxylamine on the kinetics of GTPγS binding to G_t-I. Hydroxylamine is known to convert rhodopsin to opsin plus retinal oxime (Hubbard et al., 1971). Figure 5A shows the time course of GTPγS binding to G_t-I and G_t-I plus hydroxylamine. As shown in this figure, hydroxylamine greatly diminished the rate of GTPγS binding to G_t-I. To determine if this effect of hydroxylamine was a direct effect on G_t, we have treated G_t-I with 10 mM hydroxylamine for 7 h and later removed hydroxylamine on a Sephadex G-25 column. Results of this experiment are shown in Figure 5B and demonstrate that the rate of GTPγS binding to hydroxylamine-treated G_t-I was very slow and that addition of hydroxylamine to the reaction mixture did not cause any additional effect on the rate of GTPγS binding to the preparation. Addition of 0.03 μM bleached rhodopsin to the preparation restored full binding activity, thereby indicating that hydroxylamine treatment had no direct effect on G_t. However, despite a long period of treatment with hydroxylamine and inclusion of hydroxylamine in the assay mixture, the rate of GTPγS binding to G_t-I at 30 °C was never reduced to that of G_t-II. Data shown in Figure 5B reveal that after 60 min of incubation at 30 °C GTPγS binding to hydroxylamine-treated G_t-I reached about 30% of full binding activity, while G_t-II showed only 6% of total binding activity under identical conditions (Figure 4). These results suggest either

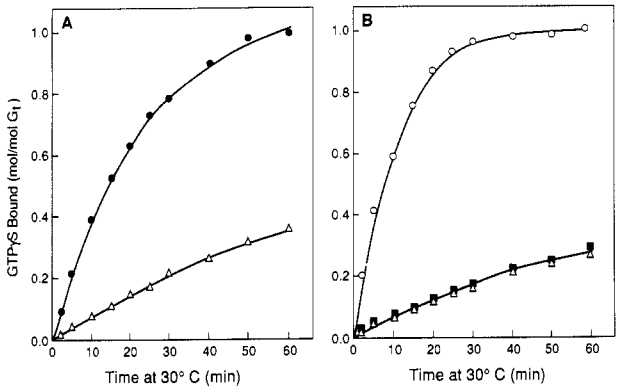

FIGURE 5: Effect of hydroxylamine on the kinetics of GTPγS binding to G_t-I. Panel A shows the time course of GTPγS binding to G_t-I (0.5 μM; G_t sample was prepared according to method B) at 30 °C (●) and the time course of GTPγS binding to the same preparation of G_t-I (0.5 μM) following 2-h incubation with 10 mM hydroxylamine with continuous illumination and in the presence of the same concentration of hydroxylamine in the binding assay (△). Panel B shows the time course of GTPγS binding to the same preparation of G_t-I, as in panel A, following 7-h incubation with 10 mM hydroxylamine and continuous illumination with fluorescent light at 4 °C, followed by removal of hydroxylamine over a 1-mL Sephadex G-25 column. Binding assays were carried out in the absence of hydroxylamine or rhodopsin (■), in the presence of 10 mM hydroxylamine added to the final assay (△), and in the presence (○) of 0.03 μM bleached rhodopsin (urea-washed ROS disk membranes).

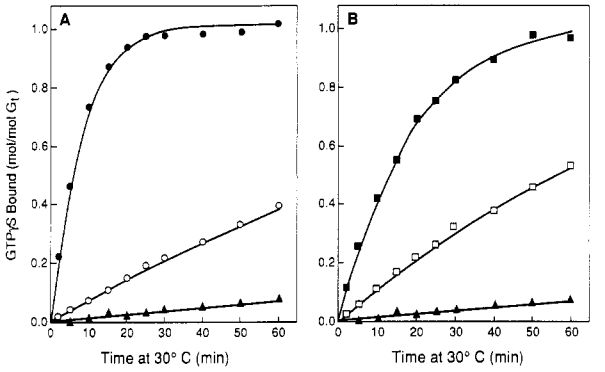

FIGURE 6: Effect of opsin preparations on the kinetics of GTPγS binding to type II G_t. (Panel A) ROS disk membranes were treated with hydroxylamine as described under Materials and Methods. Opsin was reconstituted with 9-*cis*-retinal by combining 10 μM opsin with 100 μM 9-*cis*-retinal at 4 °C in the dark for 7 h in a solution containing 20 mM MOPS (pH 7.5), 1 mM DTT, 1 mM EDTA, 100 mM NaCl, and 3 mg/mL BSA. Dilutions of the reconstituted opsin were carried out in this same solution. Reconstituted opsin was bleached as described under Materials and Methods. Binding of 10 μM [^{35}S]GTPγS to 0.5 μM hexylagarose-purified G_t was performed at 30 °C under continuous exposure to light in the absence of other additions (▲), with 0.03 μM opsin (○), or with 0.03 μM 9-*cis*-retinal-reconstituted opsin (●). The reaction was terminated at the indicated times, and bound GTPγS was determined as described under Materials and Methods. (Panel B) An identical experiment as described for panel A was carried out with purified opsin reconstituted in phospholipid vesicles. GTPγS binding to G_t alone (▲), G_t with 0.03 μM purified opsin (□), or G_t with 0.03 μM purified opsin reconstituted with 9-*cis*-retinal (■) was determined as described under Materials and Methods.

that opsin has a partial catalytic effect on GTPγS binding to G_t or that treatment with hydroxylamine did not cause a 100% conversion of rhodopsin to opsin. Panels A and B of Figure 6 show the effect of hydroxylamine-treated ROS disk membranes and purified opsin on the time course of GTPγS binding to G_t-II. Bleached ROS disk membranes were treated three times with 100 mM hydroxylamine for a total of 24 h and continuous illumination to ensure complete conversion of rhodopsin to opsin (see Materials and Methods). As shown in Figure 6A, this preparation of hydroxylamine-treated ROS disk membranes had a significant effect on the rate of GTPγS binding to G_t-II. After 60 min of incubation at 30 °C, a concentration of 0.03 μM opsin of hydroxylamine-treated ROS disk membranes enhanced GTPγS binding to 0.5 μM G_t-II to about 39.6% of total binding activity (Figure 6A). This effect was not unique to opsin of hydroxylamine-treated ROS disks. Purified opsin reconstituted into phospholipid vesicles (Clack & Stein, 1988) at a concentration of 0.03 μM increased GTPγS binding to the same preparation of G_t-II to about 53.5% of total binding activity after 60-min incubation at 30 °C (Figure 6B). Figure 6 also shows that both opsin preparations when reconstituted with 9-*cis*-retinal caused a rapid increase in the rate of GTPγS binding to G_t-II, thereby indicating that preparations of opsin obtained after extensive hydroxylamine treatment retained full activity.

Discussion

Unlike other G-proteins, GN binding to G_t has generally been considered to be dependent on the presence of activated receptor, light-activated rhodopsin. In general, GN-binding assays to G_t have been carried out on ice, and GN exchange was catalyzed by micromolar concentrations of photolyzed rhodopsin. Despite extensive studies regarding rhodopsin activation of GN exchange on G_t (Wessling-Resnick & Johnson, 1987b,c; Bennet & Dupont, 1985), for the most part, the literature lacks a clear description of GN-binding characteristics of purified preparations of G_t in the absence of added rhodopsin. A recent report by Wessling-Resnick and Johnson (1987a) shows GN binding to G_t in the absence of added photolyzed rhodopsin. These investigators, however, emphasized that G_t shows GN binding in the absence of added rhodopsin similar to that of other G-proteins in the absence of activated hormone receptors.

In this study we have carried out GN binding to purified preparations of G_t in the absence and presence of added photolyzed rhodopsin. We have attempted to reconcile data obtained by different laboratories using a number of preparations of G_t. It is quite evident from our study that preparations of G_t obtained by Kuhn's procedure in the absence of further purification would bind GN in the absence of added rhodopsin, if the binding assay was carried out at 30 °C and the reaction was followed for at least 1–2 h. Our results clearly demonstrate that this is a common characteristic of these G_t preparations regardless of the degree of light exposure of ROS disk membranes and the concentration of GTP used for the elution of G_t from disk membranes. Moreover, these results clearly demonstrate that the inability of some investigators to observe GN binding to their preparations of G_t could very likely be due to the fact that their GN-binding assays were carried out on ice. Consequently, it appears that one reason for Wessling-Resnick and Johnson's (1987a) varied observation is due to the fact that their binding assay was carried out at room temperature rather than on ice.

However, our results also show that rapid binding of GN to G_t preparations at 30 °C is mainly due to the fact that these preparations contain rhodopsin. This conclusion is well supported by the fact that hydroxylamine treatment, which converts bleached rhodopsin to opsin and retinal oxime, greatly diminished the rate of GN binding to such preparations of G_t, and purification of G_t preparations over hexylagarose columns resulted in a preparation of G_t that lacked such rapid GN binding to G_t in the absence of light-activated rhodopsin. Our results show that as low as a 1 nM concentration of completely

bleached rhodopsin is sufficient to catalyze GTPγS binding to G_t at 30 °C. This amount represents about 0.2% of total G_t concentration at 0.5 μM G_t in the assay. Our results also show that micromolar concentrations of bleached rhodopsin are required only when the binding assays are carried out on ice.

Surprisingly, our data show that continuous bleaching of rhodopsin for several hours does not lead to complete inactivation of its catalytic activity. Light activation of rhodopsin is known to result in the formation of the active state of metarhodopsin II, which in turn either decays to metarhodopsin III or directly converts to *all-trans*-retinal and opsin (Fung, 1985). Bovine metarhodopsin III is stable for hours (Fung, 1985), and the half-life of conversion of metarhodopsin II to III is about 68 min (Ostroy, 1977). Although the effect of metarhodopsin III on GN exchange of G_t is not known, these data are consistent with our results. Moreover, G_t has been shown to stabilize the active state of metarhodopsin II (Emeis et al., 1982). This would suggest that the presence of minute quantities of metarhodopsin II in G_t preparations would be stabilized in this state, and its conversion to an inactive state would be extremely slow. Our results also clearly demonstrate that opsin possesses catalytic activity. To our knowledge, this is the first report to show an effect of opsin on GN exchange on G_t. This observation, in fact, is in contrast to an earlier report by Fukada et al. (1981). These investigators reported a lack of effect of 1 μM opsin on cGMP PDE activity of dark-adapted ROS disks in the presence of GTP. Their failure to observe an action by opsin may stem from the fact that their PDE assays were carried out for a short period of 1–5 min at 30 °C, and the use of GTP instead of one of its nonhydrolyzable analogues. In addition, endogenous G_t of the ROS disk membranes is in the presence of a 10-fold rhodopsin concentration in the disk membranes, which might have blocked the effect of added opsin to the reaction mixture.

Our observation regarding the very low rate of GTPγS binding to G_t preparations obtained from previously extracted and frozen ROS disk membranes was fortuitous. One explanation of this observation is that freezing and thawing have lead to the fusion of very fine membrane particles to much larger ones, thereby ensuring their sedimentation upon centrifugation at 100000*g*. This assumption suggests that centrifugation of G_t-I preparations at 100000*g* did not lead to a complete removal of very fine particles of ROS disk membranes from the preparations. Such fine low-density particles of ROS disk membranes must have been generated by certain steps of the preparation. The most likely steps are those leading to the opening of ROS disk membranes by mechanical disruption or osmotic shock. As a result, the degree of contamination of G_t preparations with rhodopsin would be directly dependent on the method of preparation employed. In our hands, this appeared to lead to variations in the rate of GN binding to different G_t-I preparations at 30 °C. A comparison of data shown in Figures 1, 2, and 5 clearly demonstrates that these preparations of G_t-I contained different quantities of bleached rhodopsin. The differences in the kinetics of GN binding to our G_t preparations suggest that these preparations varied from 0.2 to 6% contamination with rhodopsin. Only those preparations with greater than 1% contamination bound stoichiometric amounts of GN in 2 h at 30 °C.

Our data clearly show that hexylagarose chromatography is an essential step for the removal of minute quantities of ROS disk membranes from G_t preparations. Nevertheless, our hexylagarose-purified preparations of G_t showed a detectable rate of 0.001 mol of GTPγS binding (mol of G_t)$^{-1}$ min^{-1} at 30 °C. This very slow rate of exchange is very likely to be an intrinsic characteristic of G_t. It should be noted that this rate of exchange is much slower than the estimated rate of GTPase for G_t at 1 mol of GTP (mol of G_t)$^{-1}$ min^{-1} at 30 °C (Baehr et al., 1982). Therefore these data suggest that, in the ROS, G_t activation is directly coupled to light-activated rhodopsin.

In conclusion, our results emphasize the essential role of rhodopsin for the activation of GN exchange on G_t and argue against the significance of spontaneous exchange of GN on this protein at physiological conditions. However, it is not clear if these characteristics are unique to G_t. A recent report by Higashijima et al. (1988) showing that mastoparan, a peptide toxin, mimics receptors and catalyzes GN binding to several purified G-proteins and a report by Rubenstein et al. (1987) showing that tryptic fragments of the β-adrenergic receptor activate G_s suggest that the presence of small peptide fragments of protease digests of receptors in purified preparations of G-proteins could account for their high rate of GN binding in the absence of added active receptors. However, the fact that all other G-proteins, unlike G_t, were purified by several steps of chromatography argues against the possibility of such contaminations with receptors, or receptor fragments. Nevertheless, further examination of GN-binding characteristics of highly purified preparations of G-proteins is essential for a better understanding of their functional state under physiological conditions.

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