Use of 8-Azidoguanosine 5′-[γ-^{32}P]Triphosphate as a Probe of the Guanosine 5′-Triphosphate Binding Protein Subunits in Bovine Rod Outer Segments†

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ABSTRACT: In an in vitro incubation, 8-azidoguanosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]-8-azido-GTP) labeled bleached rhodopsin independent of ultraviolet light. Characterization of this labeling indicated that rhodopsin was phosphorylated with [γ-^{32}P]-8-azido-GTP as a phosphate donor. At low concentrations, ATP increased this labeling activity 5-fold. In the same incubation, [γ-^{32}P]-8-azido-GTP also labeled G_α (M_r 40 000). This labeling was ultraviolet light dependent. G_β (M_r 35 000) was also labeled dependent for the most part upon ultraviolet light, but a smaller component of labeling appeared to result from phosphorylation. Differential labeling of G_α and G_β was found to vary intricately with experimental conditions, especially prebleaching of rhodopsin, tonicity of the medium, and the presence or absence of 2-mercaptoethanol. Affinity labeling of G_α and G_β by [γ-^{32}P]-8-azido-GTP in competition with ATP or GTP was kinetically complex, consistent with possible multiple binding sites for GTP on both subunits. Independent evidence for two or more binding sites on G_α has been offered by other laboratories, and recently, at least one binding site on G_β and its analogues among the N proteins of adenylate cyclases has been identified.

In retinal rod outer segments (ROS),[1] cyclic GMP phosphodiesterase (PDE)[1] has been demonstrated to undergo activation by light (Yee & Liebman, 1978) and GTP (Bitensky et al., 1978). Miki et al. (1974) showed that activation could occur if light preceded GTP treatment, but not vice versa. Experiments on activation by bleached rhodopsin further suggested a relatively stable, soluble activating agent (Pober & Bitensky, 1979).

Reconstitution studies using purified components indicated that a GTP-binding protein was required for PDE activation. This protein has been called GTP-binding protein (Godchaux & Zimmerman, 1979), transducin (Fung et al., 1981), and G protein (Kohnken et al., 1981b; Baehr et al., 1982). Here we call it G protein. G protein is composed of three nonidentical subunits with molecular weights of 40 000 (G_α), 35 000 (G_β), and 8000 (G_γ) (Kohnken et al., 1981b). This protein exhibits, in addition to PDE activation, GTPase activity and GTP binding (Godchaux & Zimmerman, 1979) as well as a light-induced binding to ROS membranes that is reversed by GTP (Kühn, 1980). It has been proposed that a relatively stable G protein/GTP complex is the soluble activating agent of PDE (Fung et al., 1981), with activation terminated by GTP hydrolysis (Bitensky et al., 1978; Liebman & Pugh, 1980).

Photoaffinity probes have proven to be valuable tools in the identification and characterization of specific macromolecular sites. Pfeuffer (1977) and Potter & Haley (1983) have used azido derivatives of GTP as photoaffinity probes to study GTP–subunit interaction in adenylate cyclase and tubulin, respectively. Uchida et al. (1981) utilized [^{32}P](4-azidoanilido)-5′-GTP to demonstrate a specific labeling of an analogous G protein in frog ROS.

In bovine ROS, Fung et al. (1981) reported that 5′-guanylyl imidodiphosphate (GMPPNP), a nonhydrolyzable GTP analogue, specifically bound to G_α, and this binding enabled separation of G_α–GMPPNP from $G_{\beta\gamma}$. They further reported that G_α–GMPPNP could activate PDE in unbleached ROS. Takemoto et al. (1981), on the other hand, reported that the photoaffinity probe and GTP analogue [γ-^{32}P]-8-azido-GTP reacted with G protein to photolabel G_β when incubated with purified, soluble G protein. We report here that both subunits appear to bind the photoaffinity probe in a complex manner.

Materials and Methods

ROS Isolation. Bovine ROS were isolated under dim red light on sucrose density gradients according to Kohnken et al. (1981b) within 12 h of slaughter. Aliquots were stored at −160 °C until use.

G Protein Isolation. G protein was purified from ROS by two different procedures, for later labeling by [γ-^{32}P]-8-azido-GTP. The first procedure employed low-salt extraction of soluble proteins from unbleached ROS. These were resolved into pure G and PDE proteins by DEAE-cellulose and gel-filtration chromatography in the presence of 2-mercaptoethanol (BME) and EDTA as previously described (Kohnken et al., 1981b).

The second procedure employed selective elution of G protein from bleached ROS by 80 μM GTP in 5 mM Tris-HCl, pH 7.4, and 1 mM dithiothreitol according to Kühn (1982). The supernatant containing G protein was further purified on a 15 × 0.7 cm hexylagarose column according to Fung et al. (1981). The column was equilibrated in 10 mM 4-morpholinepropanesulfonic acid, pH 7.5, 2 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol. After the supernatant was applied to the column, GTP was eluted by 75 mM NaCl in the equilibration buffer. G protein was eluted with 300 mM NaCl in the same buffer. $MnCl_2$

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[1] Abbreviations: BME, 2-mercaptoethanol; DEAE, diethylaminoethyl; GMPPNP, 5′-guanylyl imidodiphosphate; GTPase, 5′-guanosinetriphosphate phosphohydrolase; NTP, nucleoside triphosphate; PDE, phosphodiesterase; ROS, retinal rod outer segments; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet.

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was added to 4 mM, and the protein was concentrated to 1 mg/mL by positive pressure ultrafiltration. This procedure was also employed by Takemoto et al. (1981) to study G protein labeling by [γ-^{32}P]-8-azido-GTP.

Irradiation by UV and Visible Light. For UV activation of photoaffinity labeling, a Mineral Light, Model UVS-11 was directed on the sample from above at a distance of 3 cm. At this distance the lamp emitted 80–160 μW/cm^2 as measured by an International light radiometer, Model IL570, using a PT171C detector with NB254 filter. No attempt was made to exclude visible light, since UV light itself is known to bleach rhodopsin with a quantum efficiency of about 0.3 (Kropf, 1967). In control experiments, activation of the photoaffinity reaction was prevented by excluding UV but not visible light with a shield consisting of 1.5 cm of plate glass and 0.5 cm of 10% butylated hydroxytoluene between the Mineral Light and the sample. In all experiments room light accompanied illumination from the Mineral Light. Before and after timed irradiation as above, samples were uniformly handled under dim red light on ice.

UV photolysis of 20 μM 8-azido-GTP in 100 mM NaH_2PO_4, pH 7.6, and 5 mM $MgCl_2$ was carried out on ice in 1.5-mL conical polypropylene centrifuge tubes with the unshielded Mineral Light as described above. Change in UV absorption spectrum induced by photolysis was measured on a Cary 14 recording spectrophotometer using an unphotolyzed sample of 8-azido-GTP as a reference solution. Potter & Haley (1983) have shown that 8-azido-GTP exhibits an absorbance maximum at 278 nm that is diminished by UV photolysis. In our experiments, UV photolysis of 20 μM 8-azido-GTP in 100 mM NaH_2PO_4, pH 7.6, and 5 mM $MgCl_2$ produced a decrease in absorbance at 278 nm and an increase at approximately 240 nm. The decrease in absorbance was half-maximal at 4 min and was essentially complete by 30 min. The maximum change in absorbance at 278 nm was 0.176. Inclusion of 0.15 mg/mL bovine serum albumin in the buffer did not alter the time course nor the extent of the spectral change.

DE-81 Chromatography of Photolytic Products. We used Whatman DE-81 paper to chromatographically resolve photolytic products of [γ-^{32}P]-8-azido-GTP from unreacted material. Migration on DE-81 paper was also employed as an indication of [γ-^{32}P]-8-azido-GTP stability to various solvent treatments. Samples containing 1 μL of 1 mM [γ-^{32}P]-8-azido-GTP (0.2 μCi/nmol) were spotted on DE-81 paper. Ascending chromatography was performed in 50 mM sodium acetate and 50 mM citric acid, pH 3.75, and completed in 1.5 h at room temperature. Papers were air-dried and either exposed to X-ray film or sliced into 5-mm strips. Radioactivity in the strips was detected by liquid scintillation counting. Unphotolyzed [γ-^{32}P]-8-azido-GTP migration was essentially unaffected by incubation in diethyl ether or acetone for 1 h at 4 °C, in 0.1% SDS and 0.1% BME for 10 min at 90 °C, or in 5 or 100 mM NaH_2PO_4, pH 7.6, and 5 mM $MgCl_2$ for 2 h at 4 °C. A portion of the label in samples treated with 6 M urea at 4 °C for 24 h, with water at 4 °C for 36 h, with 30% ethanol and 7.5% acetic acid for 1.5 h at 25 °C, or with 10% trichloroacetic acid (TCA) and 10 mM $Na_2P_2O_7$ for 1.5 h at 4 °C exhibited a greater migration on DE-81 paper than did intact 8-azido-GTP. The remainder of the label migrated as intact 8-azido-GTP. Incubation in 10% TCA and 10 mM $Na_2P_2O_7$ at 90 °C for 10 min resulted in nearly complete loss of label from a location on DE-81 corresponding to intact 8-azido-GTP to a location corresponding most closely to free inorganic phosphate. This instability of 8-azido-GTP in hot TCA proved useful in differentiating photoaffinity labeling from TCA-stable phosphorylation. It also indicated the necessity of rapid and mild treatment of the label and labeled products.

Protein Labeling. In a typical assay, 2–5 μL of unbleached ROS (about 25 μg of protein) was incubated with 20 μM [γ-^{32}P]-8-azido-GTP (0.5 μCi/nmol) in 100 mM NaH_2PO_4, pH 7.6, 5 mM $MgCl_2$, and 200 μM ATP in a final volume of 50 μL. Reagents were mixed under dim red light on ice in 1.5-mL conical polypropylene centrifuge tubes. Both UV and room lights were turned on for 2 or 7 min. The lights were then turned off, and the samples were transferred to Beckman airfuge tubes. Soluble proteins and membrane-associated proteins were separated by centrifugation at 67000*g* for 10 min or 130000*g* for 5 min. Supernatants contained no rhodopsin detectable by visible absorbance. SDS and BME were added to both the supernatants and resuspended pellets to a final concentration of 1% each. Samples were kept at 4 °C for up to 3 h before boiling and application to 11% acrylamide gels. Autoradiography of dried gels was performed at −20 °C for 48–120 h with prefogged Kodak XAR5 film and a Cronex intensifying screen. Permutations of this procedure are described under Results. Isolated soluble proteins were labeled with [γ-^{32}P]-8-azido-GTP according to Takemoto et al. (1981) except UV photolysis was allowed to proceed for 7 min rather than 1 min.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. Gels of 11% acrylamide were prepared by the procedures of Fairbanks et al. (1971) as modified by Kohnken et al. (1981b). After completion of electrophoresis, gels were either rinsed with water and dried immediately or stained with Coomassie Brilliant Blue and destained briefly prior to drying.

Miscellaneous Methods. Protein concentrations were determined according to Lowry et al. (1951). Rhodopsin concentration was determined according to Mc Connell et al. (1981). ROS cGMP PDE activation by light and GTP were performed according to Kohnken et al. (1981b). 8-Azido-GTP was substituted for GTP in the appropriate assays.

Materials. 8-Azido-GTP and [γ-^{32}P]-8-azido-GTP were purchased from Schwarz/Mann. All other reagents were obtained from Sigma.

Results

Rhodopsin Phosphorylation. Rhodopsin monomer and multimers as expected were the principal protein bands in the pellet and also accounted for the majority of the label incorporation. Phosphorylation by rhodopsin kinase is activated through conversion of rhodopsin by light to an "active" substrate (Chader et al., 1976; Frank & Buzney, 1975; Shichi & Somers, 1978). Here, phosphorylation occurred with [γ-^{32}P]-8-azido-GTP as a substrate in a time-dependent response to visible light. The product survived treatment with hot TCA as well as dialysis against 6 M urea or 4 °C water. Rhodopsin was also labeled by [γ-^{32}P]GTP but not by [α-^{32}P]GTP. Phosphorylation by [γ-^{32}P]-8-azido-GTP was prevented by preboiling or by EDTA, confirming its enzymatic basis. Phosphorylation was linearly dependent on both [γ-^{32}P]-8-azido-GTP concentration up to at least 200 μM and also rhodopsin concentration up to 92 μM. Despite the fact that rhodopsin has been shown to be a substrate for phosphorylation by either ATP (McDowell & Kühn, 1977) or GTP (Chader et al., 1976), neither ATP nor GTP competed effectively with [γ-^{32}P]-8-azido-GTP as a substrate for rhodopsin phosphorylation. Indeed, ATP at low concentrations consistently stimulated phosphorylation by [γ-^{32}P]-8-azido-GTP (Figure

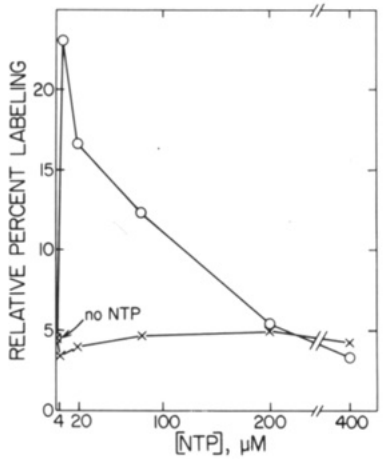

FIGURE 1: Rhodopsin labeling as a function of nucleoside triphosphate concentration. ROS were incubated for 7 min with 20 μM [γ-^{32}P]-8-azido-GTP (0.5 μCi/nmol) in the presence of varying concentrations of either ATP or GTP. Visible illumination was administered as described under Materials and Methods. ROS pellets were applied to SDS gels and subjected to electrophoresis. Autoradiograms of SDS gels were scanned with a Gelman ACD-18 scanning densitometer. Absorbance at 550 nm was automatically normalized to total absorbance within each experiment. Scanning densities here and in subsequent figures were independently shown to be linear with respect to cpm in the SDS gel bands. (○) ATP concentration was varied. (×) GTP concentration was varied.

1). However, prebleaching (in addition to bleaching during the labeling reaction) decreased rhodopsin phosphorylation unless GTP was present.

Labeling of G_α and G_β in the ROS. Photoaffinity labeling of G subunits in the ROS was almost inversely proportional to ROS concentration. At 92 μM rhodopsin, virtually no such labeling was detected. This finding was evidently due to scattering or absorbance by ROS proteins of the UV light required to activate photoaffinity labeling. To prevent UV blocking, rhodopsin concentration during photoaffinity labeling was kept at or below 6 μM.

Because it has been shown that light, tonicity, and reducing agents alter the affinity of the PDE and G proteins for the ROS membrane and for each other (Kohnken et al., 1981b; Kühn, 1980, 1982), we investigated the effects of these and related variables on labeling of G_α and G_β. G_α was labeled (Figure 2, lanes 1 and 7–10) only by UV light whereas G_β labeling contained both a visible light-dependent component [47% ± 6.7% (SEM) of total labeling; n = 5, one experiment appears in lane 4] and a UV-dependent component (lane 3, visible + UV). The visible light component was inferred to represent phosphorylation. UV-dependent photoaffinity labeling of G_α was suppressed by BME (lanes 2–6), but this agent, if anything, increased the UV-dependent labeling of G_β (compare lanes 6 and 7).

If isotonic salt was present during photoaffinity labeling, G_β labeling was suppressed (lane 5) provided the ROS had previously been bleached in hypotonic medium and then washed in hypotonic medium prior to isotonic resuspension. If the prior treatments were absent, isotonic photoaffinity labeling was not suppressed (lane 6), while the prior treatments alone did not prevent photoaffinity labeling in a hypotonic medium (lane 3).

If 500 μM GTP and 4 mM EDTA—which might be expected to release additional G protein from the bleached ROS—were added after completion of labeling, no effect was seen on relative intensity of subunit labeling (compare lanes 8 and 1). However, if the same *amounts* of these agents were added in an 8-fold hypotonic dilution, the relative intensity

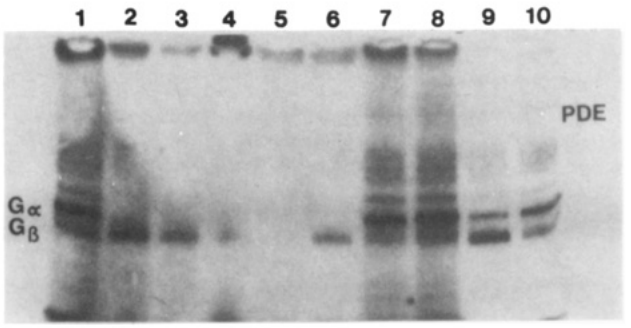

FIGURE 2: Autoradiogram following SDS–polyacrylamide gel electrophoresis of supernatant proteins labeled by [γ-^{32}P]-8-azido-GTP under conditions indicated. The locations of G_α ($M_r \simeq 40\,000$), G_β ($M_r \simeq 35\,000$), and PDE ($M_r \simeq 88\,000$) are indicated at the margins. (Lane 1) Isotonic ROS + high Mg – BME + UV. ROS were diluted to 5 mg/mL in 100 mM $NaPO_4$, pH 7.6, and 5 mM $MgCl_2$. A total of 5 μL of this ROS was next incubated in 20 μM [γ-^{32}P]-8-azido-GTP, 200 μM ATP, 100 mM $NaPO_4$, pH 7.6, and 5 mM $MgCl_2$ in a 50-μL volume for 7 min under UV/visible light. The light was turned off and the suspension centrifuged in a Beckman airfuge centrifuge for 10 min at 67000g. After addition of 1% SDS and BME, samples of the clear supernatant were boiled and applied to SDS–polyacrylamide (11%) gels. Autoradiography was performed on dried gels. (Lane 2) Hypotonic ROS + BME + UV. ROS were diluted to 5 mg/mL in 5 mM Tris-HCl, pH 7.8, 10 mM BME, and 1 mM $MgCl_2$. A total of 5 μL of this ROS was next incubated in the same medium plus 20 μM [γ-^{32}P]-8-azido-GTP and 200 μM ATP for 7 min under UV/visible light. Subsequent treatment was the same as for the sample in lane 1. (Lane 3) Hypotonic ROS + BME prebleached, washed, and resuspended in hypotonic medium + UV. ROS diluted in hypotonic Tris-HCl, BME, and $MgCl_2$ as in lane 2 were prebleached with visible light on ice for 5 min and then centrifuged at 30000g for 30 min in a Sorvall centrifuge. After resuspension of the sedimented ROS to 5 mg/mL in hypotonic medium, 5 μL was incubated in the same medium plus [γ-^{32}P]-8-azido-GTP and ATP for 7 min under UV/visible light. Subsequent treatment here and in remaining lanes was the same as before. (Lane 4) Hypotonic ROS + BME prebleached, washed, and resuspended in hypotonic medium – UV. Treatment of ROS was the same as for lane 3, except UV light was excluded. (Lane 5) Hypotonic ROS + BME prebleached and washed but resuspended in isotonic medium + UV. Treatment was the same as for lane 3 except the washed ROS were resuspended in isotonic phosphate buffer + [γ-^{32}P]-8-azido-GTP + ATP before UV/visible irradiation. BME concentration during irradiation was 1 mM. (Lane 6) Isotonic ROS + low Mg + BME + UV. ROS were diluted to 5 mg/mL in 100 mM $NaPO_4$, pH 7.6. A total of 5 μL of this ROS was next incubated in 5 mM Tris-HCl, 10 mM BME, 1 mM $MgCl_2$, and 90 mM $NaPO_4$, pH 7.6, + [γ-^{32}P]-8-azido-GTP + ATP with UV/visible light. (Lane 7) Isotonic ROS + low Mg – BME + UV. Treatment was the same as for lane 6 except Tris-HCl and BME were omitted from incubation. Comparison should also be made (low vs. high Mg) with lane 1. (Lane 8) Isotonic ROS – BME + UV + GTP and EDTA added later. Treatment was identical with that of lane 1, except that after incubation and labeling 500 μM GTP and 4 mM EDTA were added before centrifugation. (Lane 9) Isotonic ROS – BME + UV + GTP and EDTA added later in hypotonic dilution. Treatment was the same as lane 8 except that before centrifugation the same *amounts* (as in lane 8) of GTP and EDTA were added in an 8-fold volume of H_2O. (Lane 10) Isotonic ROS – BME + UV + later hypotonic dilution. Treatment was the same as lane 9 except for omission of GTP and EDTA.

was altered in favor of G_β (lane 9)—an effect not attributable to the dilution alone (lane 10). A difference of 5 mM vs. 1 mM Mg^{2+} had no effect on either G_α or G_β labeling (lane 1 vs. lane 7).

Time Course of G_α and G_β Labeling in the ROS. Figure 3 shows the time course of labeling in one experiment. The rate of labeling appeared to be linear for the first 2 min, with extent of labeling approaching a maximum by 10 min. In a number of other experiments, we chose a 2-min labeling time because it appeared from Figure 3 to offer the maximum labeling consistent with a linear labeling rate, so that we could compare experimental conditions with respect to their effects

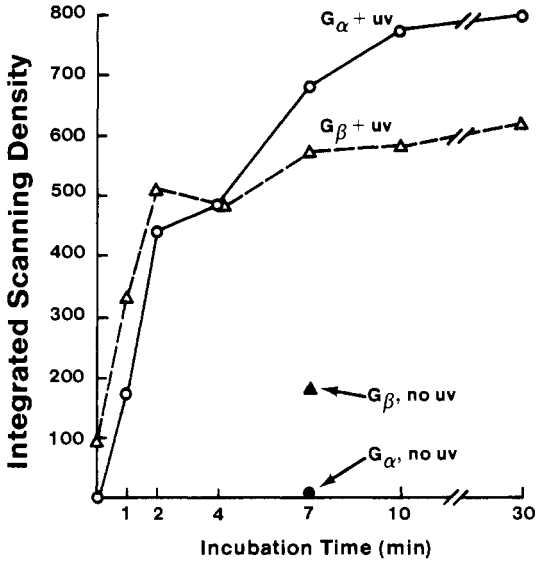

FIGURE 3: Time course of G_α and G_β labeling. ROS were incubated for various times under UV/visible light with [γ-^{32}P]-8-azido-GTP (0.5 μCi/nmol) as described under Materials and Methods. Supernatants were collected by centrifugation at 67000g for 10 min in a Beckman airfuge and applied to SDS gels. Autoradiograms of the gels were analyzed on a Helena scanning densitometer. During labeling, 200 μM ATP was present.

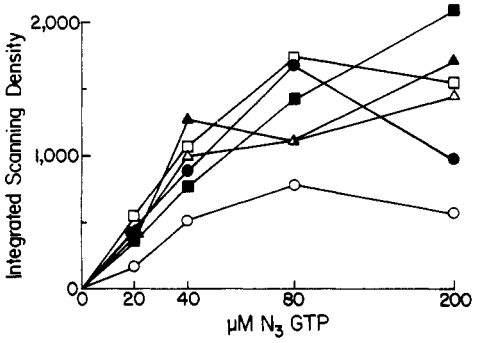

FIGURE 4: Labeling of G_α and G_β as a function of [γ-^{32}P]-8-azido-GTP concentration. ROS were incubated with [γ-^{32}P]-8-azido-GTP under UV/visible light as described under Materials and Methods. Except as indicated, 200 μM ATP was present. Incubation times were 2 or 7 min as indicated. Autoradiograms of dried gels were analyzed with a Helena densitometer. (○) G_α at 2 min + ATP; (●) G_β at 2 min + ATP; (△) G_α at 7 min + ATP; (▲) G_β at 7 min + ATP; (□) G_α at 7 min, no ATP; (■) G_β at 7 min, no ATP. The gain settings on the scanning densitometer were the same for each curve, and background density was numerically subtracted from each point before plotting.

on rate. Note that in this time-course experiment, 200 μM ATP was present in order to minimize loss of label to phosphorylation, principally of rhodopsin.

Dependence of G_α and G_β Labeling Rate on [γ-^{32}P]-8-Azido-GTP Concentration. Figure 4 shows the dependence of labeling of these two subunits on affinity label concentration up to 200 μM. In some instances, 2-min incubation times were employed, and in others 7 min. At 7 min, the effect of omitting ATP was also examined. All of the labeling curves except G_β, at 7 min with no ATP, appeared to saturate at 80 μM affinity label. Under these conditions, G_β did not saturate even at 500 μM azido-GTP (data not shown).

Nucleoside Triphosphate (NTP) Competition with G_α and G_β Labeling in the ROS. Both inhibition and stimulation of G_α and G_β labeling by NTPs were seen, depending on the subunit, the NTP employed, its concentration, and the incubation time. Figure 5 reveals these complexities. Only at NTP concentrations $\geq$200 μM were the effects of GTP and ATP

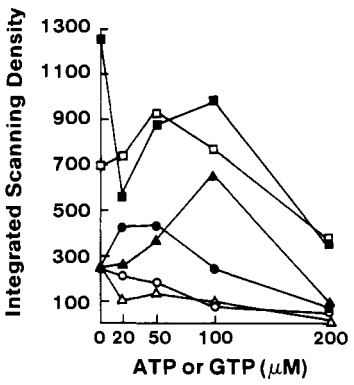

FIGURE 5: Effects of nucleoside triphosphate competition on G_α and G_β labeling. ROS were incubated with [γ-^{32}P]-8-azido-GTP under UV/visible light as described under Materials and Methods. Incubation times were 2 or 7 min as indicated. Autoradiograms were analyzed with a Helena densitometer. (○) G_α at 2 min + ATP; (●) G_β at 2 min + ATP; (△) G_α at 2 min + GTP; (▲) G_β at 2 min + GTP; (□) G_α at 7 min + GTP; (■) G_β at 7 min + GTP. The 7-min curves represent different gain settings from 2-min curves.

(also GMPPNP—not shown) uniformly inhibitory. No attempt was made in this experiment to differentiate UV-dependent from non-UV-dependent labeling. At 2 min in this and other experiments the concentrations of GTP and ATP giving 50% inhibition of G_α labeling were 20–25 μM and 80–160 μM, respectively. At 7-min incubation, GTP at 50 μM stimulated G_α labeling up to 2-fold in some experiments. G_β labeling exhibited a peak at 100 μM GTP at both 2 and 7 min and at 50 μM ATP at 2 min. In other competition experiments it was possible to select conditions in which stimulation of labeling was limited to a smaller range of relatively low NTP concentrations, but the outcome described in Figure 5 appeared more representative of the general case.

Labeling of Subunits in Isolated G Protein. G protein purified from ROS by our method (Kohnken et al., 1981b) in darkness in the presence of 10 mM BME did not label with [γ-^{32}P]-8-azido-GTP unless the BME was removed by dialysis. Then, both G_α and G_β were labeled, but not in a UV-dependent manner. G protein isolated by Kühn's (1982) procedure using GTP to pull the G protein away from bleached ROS exhibited some UV-dependent affinity labeling, more for G_α than for G_β.

8-Azido-GTP as a GTP Analogue. In view of the lack of uniform inhibition of [γ-^{32}P]-8-azido-GTP labeling by GTP (Figure 5), we sought to demonstrate that 8-azido-GTP behaves as a functional analogue of GTP in the ROS, particularly with respect to G protein. Kühn (1980) showed that GTP reverses a visible light-induced binding of G protein to the ROS. Following Kühn, we bound G protein to ROS membranes by visible light in 5 mM Tris-HCl, pH 7.8, 10 mM BME, and 1 mM $MgCl_2$ These bleached ROS were subjected to centrifugation, and the supernatant was applied to an SDS gel (Figure 6, lane 1). The predominant protein present in this supernatant was PDE; some G protein was also eluted. The ROS pellet was resuspended hypotonically and aliquoted, and various nucleotides were added to the aliquots. Supernatants from a second centrifugation of these resuspended ROS were examined by SDS gel electrophoresis for G protein elution. In Figure 6, lane 2, is the second supernatant with no nucleotides added. Supernatants in lanes 3 and 4 arose from the second centrifugation of ROS with 20 and 200 μM 8-azido-GTP added. Lanes 5 and 6 are the supernatants from the second centrifugation of ROS with 20 and 200 μM GTP added. As can be seen, both 8-azido-GTP and GTP at 20 and 200 μM eluted a significant amount of the residual G protein

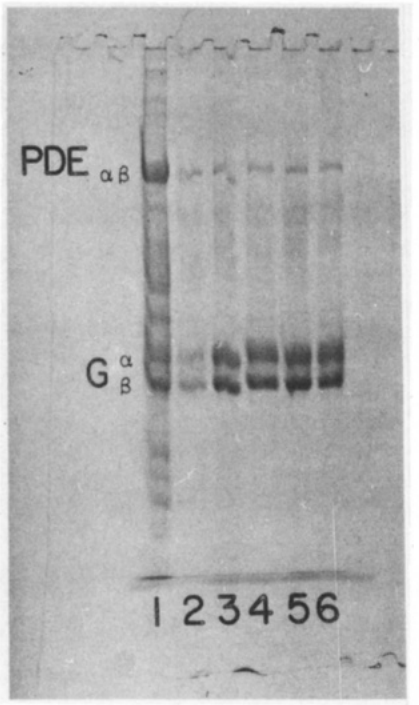

FIGURE 6: G protein elution by 8-azido-GTP. ROS were bleached and washed hypotonically as described previously, resuspended, aliquoted, and incubated in 5 mM Tris-HCl, pH 7.8, and 10 mM BME, with no additions, 20 or 200 μM 8-azido-GTP, or 20 or 200 μM GTP. Incubated suspensions were bleached with visible light, and supernatants were collected by centrifugation. Supernatants were analyzed on SDS gels and stained with Coomassie blue. (Lane 1) First hypotonic supernatant from bleached ROS. (Lane 2) Second hypotonic supernatant from bleached ROS washed with no added nucleotides. (Lanes 3 and 4) Second hypotonic supernatants from bleached ROS washed with 20 and 200 μM 8-azido-GTP, respectively. (Lanes 5 and 6) Second hypotonic supernatants from bleached ROS washed with 20 and 200 μM GTP, respectively. $PDE_{\alpha\beta}$, G_α, and G_β bands are identified.

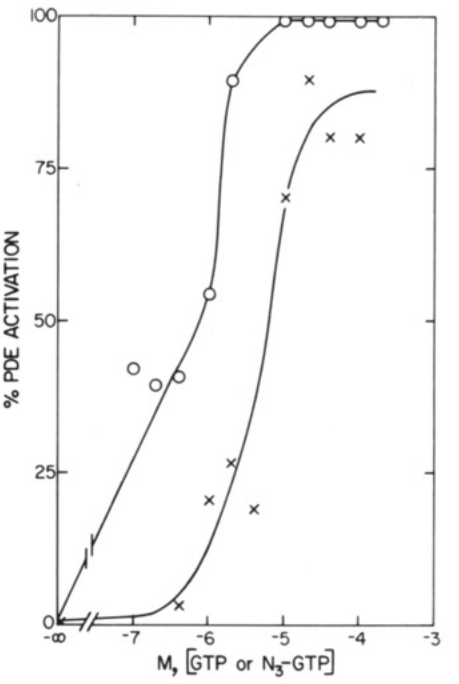

FIGURE 7: ROS cyclic GMP PDE activation. ROS were assayed for cyclic GMP PDE activation according to Kohnken et al. (1981b). The activation reaction was initiated by addition of substrate and assayed in light in the presence of varying concentrations of GTP or 8-azido-GTP. Zero percent activation was defined as PDE activity in the absence of added nucleoside triphosphates; 100% activation was defined as phosphodiesterase activity in the presence of 40 μM GTP. (O) GTP added to the reaction. (×) 8-Azido-GTP added to the reaction.

from the ROS membranes. By this criterion then, 8-azido-GTP behaved as did GTP in the ROS.

ROS cGMP PDE is activated by light and GTP (Yee & Liebman, 1978; Bitensky et al., 1978), requiring G protein (Kohnken et al., 1981a; Pober & Bitensky, 1979). It has been theorized that activation takes place through the interaction of G protein/GTP complex with the PDE (Fung et al., 1981; Liebman & Pugh, 1980). As shown in Figure 7, GTP activated ROS PDE, reaching half-maximal activation at less than 1 μM. 8-Azido-GTP also activated ROS PDE though not as well as GTP. Half-maximal activation, relative to maximal activation by GTP, required approximately 10 μM 8-azido-GTP. These results are consistent with those of Uchida et al. (1981), who reported that 8-azido-GTP required a 10-fold higher concentration than GTP to attain half-maximal activation of frog ROS PDE. These characteristics indicate that 8-azido-GTP is a functional if less effective analogue of GTP in the ROS.

8-Azido-GTP Concentration. Even though it did not maximize ^{32}P incorporation (Figure 4), 20 μM 8-azido-GTP was routinely used because G protein was maximally eluted from bleached ROS membranes (as in Figure 6) and PDE was almost fully activated (Figure 7) at that concentration. In addition, a low concentration should have minimized ^{32}P incorporation into SDS gel bands from nonphotoaffinity, non-GTP-specific reactions. At 200 μM 8-azido-GTP, at least eight SDS gel bands in the ROS supernatant were labeled, although neither UV dependence nor the identity of these bands was explored. Potter & Haley (1983) reported that nonspecific labeling increases greatly at 8-azidopurine concentrations greater than 50–60 μM.

DISCUSSION

We have demonstrated that [γ-^{32}P]-8-azido-GTP labeled both rhodopsin by phosphorylation and G protein subunits by a photoaffinity reaction. We observed here that rhodopsin phosphorylation by [γ-^{32}P]-8-azido-GTP was markedly stimulated by low levels of ATP (Figure 1). This finding appears to complement that of Swarup & Garbers (1983), who observed a marked stimulation of [γ-^{32}P]ATP phosphorylation of rhodopsin by low levels of GTP, GDP, and GMP. However, the failure of either ATP or GTP to compete effectively with azido-GTP as phosphorylation substrates argues that this photoaffinity label has limitations as an analogue compound for phosphorylation. In fact, it appears to be a better substrate for phosphorylation than either of the native substrates.

G protein subunit labeling in the presence of the ROS was found to vary intricately with experimental conditions. Photoaffinity labeling required UV photolysis and in contrast to phosphorylation was unstable to hot TCA treatment. In the presence of the ROS, G_α was labeled only by the photoaffinity reaction, whereas G_β labeling also included a component of labeling seen in the absence of UV light and therefore attributed to phosphorylation. Prebleaching and tonicity of the medium appeared to be critical although unexplained determinants of whether G_β was labeled. In some experiments (not shown), we found it necessary to prebleach and hypotonically wash the ROS in order to see G_β labeling. This argues that the G_β environment near or on the membrane must be altered before G_β can be affinity labeled or phosphorylated. One possibility is that of a shared affinity site between G_α and G_β that can be experimentally exposed to labeling in both, one, or neither subunit according to their relative orientations.

Prevention of G_α labeling or apparent enhancement of G_β labeling by BME is unexplained. While rapid reduction of azido groups by dithiols has been reported (Staros et al., 1978), reduction by monothiols is much too slow ($t_{1/2}$ = ca. 24 h; Staros et al., 1978) to account for blocking of G_α labeling here. If the effects of BME are exerted on the peptides themselves, very careful documentation would be required. It was recently reported by Ho & Fung (1984) and by Reichert & Hofmann (1984) that sulfhydryl modification of the G protein alters its interaction with rhodopsin. These observations appear to be consistent with a direct effect of BME on G protein peptide conformation.

G_α and G_β subunits were also labeled by [γ-^{32}P]-8-azido-GTP when G protein was exposed to the photoaffinity label in the absence of the ROS. However, the extent and UV dependence of labeling were less than when the ROS were present and clearly dependent on the method of isolation of the G protein. We believe our observations are consistent with those reported by Takemoto et al. (1981) because it now becomes apparent that differential photoaffinity labeling of G_α and G_β depends on ostensibly minor differences in treatment of the ROS or of the peripheral proteins. A recent communication from Takemoto's laboratory confirms this (D. J. Takemoto, personal communication).

The complex kinetics of G_α and G_β labeling under competition by GTP or ATP present interpretive difficulties. For example, 50 μM GTP stimulated G_α labeling by [γ-^{32}P]-8-azido-GTP in a 7-min incubation but not in a 2-min incubation (Figure 5). One possible explanation is that in the absence of GTP the photoaffinity reaction rate is more rapid but levels off upon substrate depletion. In the presence of GTP the initial photoaffinity reaction rate is slower due to GTP competition, but the extent of the reaction is ultimately greater because GTP competes preferentially for degradative reactions, leaving more N_3-GTP to react with G_α. Therefore, at 2 min we may be observing relative reaction rates but at 7 min a difference in extent of reaction.

Apparent failure of G_β labeling to saturate in the absence of ATP [Figure 4 (■)] within the concentration range of N_3-GTP studied may be related to the evident presence of both a UV-dependent and a UV-independent labeling site. At least one of these may have a high K_m for N_3-GTP, and ATP competes for this site, allowing detectable saturation of the low K_m site [Figure 4 (▲)]. These complex labeling behaviors suggest the possibility of more than one GTP affinity site on G_β.

Multiple GTP-Binding Sites. Godchaux & Zimmerman (1979) described two distinct binding sites for GTP on G protein (without designation of the subunit), one of which could also accept GDP. Two distinct possible GTP-binding sites at different peptide locations on G_α have been suggested by ADP ribosylation—one in the presence of cholera toxin (Van Dop et al., 1984a) and the other in the presence of pertussis toxin (islet-activating protein or IAP) (Manning et al., 1984). Earlier, it had been shown that the former site was accessible only if G protein were still associated with disc membranes when ADP ribosylation was attempted (Abood et al., 1982). On the other hand, the IAP site has been reported to be accessible either in the dark ROS (Van Dop et al., 1984b) or after removal of G protein from the discs (Manning et al., 1984). These contrasting findings may relate to our observation here that G_α failed to bind the affinity label in a UV-dependent manner if G protein had been removed from ROS in darkness (Kohnken et al., 1981b) and then dialyzed before labeling. One interpretation is that binding of the label by G_α was prevented by interaction of G_α in that instance with G_β, which may be favored by removal of G protein from the membrane.

Independent evidence of an affinity site on adenylate cyclase G_β for (4-azidoanilido)-GTP has recently been reported by Rasenick et al. (1984), as well as an affinity site for the same compound on ROS G_β (M. M. Rasenick, personal communication). If GTP binds ROS G_β physiologically, it may complicate the PDE-activation scheme advanced by Fung et al. (1981) in which GTP binding is restricted to G_α. Similar reflections apply to the analogous β subunit well characterized in the GTP-binding (N) protein of adenylate cyclases (Bokoch et al., 1984; Katada et al., 1984a–c).

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Registry No. ATP, 56-65-5; GTP, 86-01-1; 8-azido-GTP, 65114-35-4; cGMP phosphodiesterase, 9068-52-4.

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Effects of Detergent on Substrate Binding and Spin State of Purified Liver Microsomal Cytochrome P-450$_{LM2}$ from Phenobarbital-Treated Rabbits†

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ABSTRACT: Spectral changes accompanying the binding of the nonionic detergent *n*-octyl β-D-glucopyranoside (*n*-octyl glucoside) to cytochrome P-450$_{LM2}$ purified from liver microsomes of phenobarbital-treated rabbits have been compared to changes in catalytic activity obtained in a reconstituted system consisting of various levels of detergent, P-450$_{LM2}$, and NADPH-cytochrome P-450 reductase. In the absence of substrate and reductase, addition of *n*-octyl glucoside to 2–3 mM resulted in a difference spectrum (detergent-bound minus detergent-free cytochrome) characterized by a small maximum at 390 nm and a minimum at 410 nm, suggestive of a slight stabilization of the high-spin ($S = {}^5/_2$) state of the cytochrome. As the detergent concentration was increased to 4–8 mM (corresponding to maximal activity and pentameric or hexameric P-450), a new peak appeared at 427 nm while the minimum remained at 410 nm. Between 10 and 30 mM *n*-octyl glucoside (conditions which produced catalytically inactive and monomeric P-450) the minimum in the difference spectrum shifted to 390 nm and the maximum to 425 nm, characteristic of a shift in spin equilibrium toward low-spin ($S = {}^1/_2$) cytochrome. At low and high detergent concentrations, substrate [*d*-benzphetamine with *n*-octyl glucoside or cyclohexane with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)] was bound to P-450$_{LM2}$ with formation of high-spin P-450, although the increase in high-spin cytochrome was less at high detergent levels than at low. The affinity of P-450 for substrate decreased by 2–3-fold at high detergent. The decreased substrate affinity is not, however, sufficient to explain the depletion of catalytic activity observed with high detergent. Furthermore, a correlation between spin state and catalytic activity at high detergent levels was not evident. The data suggest the presence of at least two classes of binding sites for amphiphiles: high-affinity site(s) that may correspond to a site essential for catalytic activity and low-affinity site(s) that become accessible as P-450 disaggregates.

Cytochrome P-450$_{LM2}$,[1] the major P-450 isozyme induced in rabbit liver by phenobarbital, catalyzes xenobiotic hydroxylation when reconstituted in solution at a 1:1 molar ratio with NADPH-cytochrome P-450 reductase in the presence of an amphiphile (Coon et al., 1976; Miwa et al., 1979; Guengerich & Holladay, 1979; French et al., 1980; Dean & Gray, 1982; Wagner et al., 1984). The reaction requires NADPH, molecular oxygen, and substrate, and the rates achieved are similar to those observed with intact microsomes (White & Coon, 1980). We recently demonstrated that the nonionic detergent *n*-octyl glucoside (Baron & Thompson, 1975) or the zwitterionic detergent CHAPS (Hjelmeland, 1980) substituted for phospholipid in reconstituted catalytic activity (Dean & Gray, 1982; Wagner et al., 1984). Hydrodynamic studies showed that maximum catalytic activity with either detergent occurred in the absence of formation of a stable complex between P-450 and NADPH-cytochrome P-450 reductase. In the case of *n*-octyl glucoside, maximum rates of *d*-benzphetamine demethylation occurred at 4–8 mM detergent (depending on the preparation used); activity de-

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[1] Abbreviations: P-450$_{LM2}$, the cytochrome P-450 isozyme induced in rabbit liver by phenobarbital; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; *n*-octyl glucoside, *n*-octyl β-D-glucopyranoside; di-12-GPC, dilauroylglyceryl-3-phosphocholine; reductase, NADPH-cytochrome P-450 reductase.

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