Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094-11099.
Kühn, H. (1985) *Prog. Retinal Res. 3*, 123-156.
Liebman, P. A., & Evanczuk, A. T. (1981) *Methods Enzymol. 81*, 532-542.
Liebman, P. A., & Sitaramayya, A. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 215-225.
Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320-6327.
Navon, S. E., & Fung, B. K.-K. (1987) *J. Biol. Chem. 262*, 15746-15751.
Penn, R. D., & Hagins, W. A. (1972) *Biophys. J. 12*, 1073-1094.
Pugh, E. N., Jr., & Cobbs, W. H. (1986) *Vision Res. 26*, 1613-1643.
Sitaramayya, A., Harkness, J., Parkes, J. H., Gonzalez-Oliva, C., & Liebman, P. A. (1986) *Biochemistry 25*, 651-656.
Wensel, T. G., & Stryer, L. (1986) *Proteins 1*, 90-99.
Yamanaka, G., Eckstein, F., & Stryer, L. (1986) *Biochemistry 25*, 6149-6153.
Yamazaki, A., Stein, P. J., Chernoff, N., & Bitensky, M. W. (1983) *J. Biol. Chem. 258*, 8188-8194.

Chemical Modification of Bovine Transducin: Probing the GTP-Binding Site with Affinity Analogues†

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ABSTRACT: The structure of the GTP-binding site of transducin, a signal-transducing G-protein involved in the visual excitation process, was studied by affinity labeling. Radioactive GTP analogues with reactive groups attached to different moieties of the GTP molecule were obtained and include 8-azido-GTP, P^3-(4-azidoanilino)-P^1-5′-GTP (AA-GTP), 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG), 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-GTP (ANPAP-GTP), the 2′,3′-dialdehyde derivative of GTP (oGTP), and a bifunctional cross-linking analogue, 8-azido-P^3-(4-azidoanilino)-P^1-5′-GTP (8-azido-AA-GTP). With the exception of FSBG, all of the analogues were found to bind to transducin specifically and serve as a cofactor to activate the retinal cGMP cascade or act as a competitive inhibitor for the GTPase activity of transducin. The labeling sites of these analogues were localized by tryptic peptide mapping. ANPAP-GTP and oGTP were unable to covalently modify transducin, suggesting that the 2′- and 3′-hydroxy groups on the ribose ring of GTP are not in direct contact with the protein. AA-GTP only labeled the T_α subunit of transducin and was localized on the 21-kDa tryptic fragment of T_α. This indicates that the phosphate moiety of the bound GTP is in direct contact with this peptide. On the other hand, 8-azido-GTP labeled both the T_α and $T_{\beta\gamma}$ subunits of transducin. The labeling on T_α was on the 12-kDa tryptic fragment, suggesting that the guanine ring binding site is composed of a different peptide fragment than the phosphate binding region. Treatment with the bifunctional analogue 8-azido-AA-GTP generated the cross-linked products of T_α and $T_{\beta\gamma}$. This observation implies that the guanine ring of the bound GTP on T_α could be in close proximity with $T_{\beta\gamma}$. The overall result of mapping the nucleotide binding site of transducin with affinity labeling is in complete agreement with the proposed model of T_α [Hingorani, V. N., & Ho, Y.-K. (1987) *FEBS Lett. 220*, 15-22] based on the crystal structure of the GTP-binding site of elongation factor Tu [Jurnak, F. (1985) *Science 230*, 32-36; la Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385-2388].

Transducin (T),[1] a signal-transducing GTP-binding protein (Gilman, 1987; Stryer & Bourne, 1986), plays a pivotal role in the visual excitation process that involves a light-activated cGMP enzyme cascade [for reviews, see Liebman et al. (1987), Applebury and Hargrave (1986), Stryer (1986), and Chabre (1985)]. Transducin contains three polypeptide chains that can be functionally separated into two subunits, T_α (40 kDa) and $T_{\beta\gamma}$ (37 and 8 kDa) (Fung, 1983). In the rod outer segment (ROS) of the photoreceptor cell, photoexcited rhodopsin catalyzes the activation of hundreds of transducin molecules that via a GTP/GDP-exchange reaction lead to the formation of T_α–GTP complexes. The T_α–GTP complex dissociates from rhodopsin and in turn activates the latent cGMP phosphodiesterase (PDE), which rapidly hydrolyzes intracellular cGMP. The transient decrease in cGMP concentration causes closure of the cation channels on the plasma membrane and results in hyperpolarization of the rod photoreceptor cell (Fesenko et al., 1985; Yau & Nakatani, 1985). After the hydrolysis of the bound GTP, the transducin is deactivated and the T_α–GDP recombines with $T_{\beta\gamma}$ and can

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[1] Abbreviations: ROS, rod outer segment; T, transducin; T_α, α subunit of transducin; $T_{\beta\gamma}$, β and γ subunits of transducin; PDE, cyclic GMP phosphodiesterase; AA-GTP, P^3-(4-azidoanilino)-P^1-5′-GTP; oGTP, 2′,3′-dialdehyde derivative of GTP; ANPAP-GTP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-GTP; FSBG, 5′-[*p*-(fluorosulfonyl)-benzoyl]guanosine; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; kDa, kilodalton; EF-Tu, elongation factor Tu; TLC, thin-layer chromatography.

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again be activated by photolyzed rhodopsin. The coupling action of transducin constitutes a switching mechanism for the cGMP cascade in which flow of information relies on a subunit association/dissociation cycle of transducin regulated by the guanine nucleotide exchange/hydrolysis reaction.

The nature of GTP binding to transducin has been investigated with various GTP analogues. In early studies, 8-azido-GTP was used to aid in the identification of the guanine nucleotide binding subunit of transducin (Takemoto et al., 1981; Takemoto & Takemoto, 1985; Kohnken & McConnell, 1985). Phosphorothioate analogues have been used to study the stereochemistry of the site (Yamanaka et al., 1985). GTP analogues with specific substitution on the γ-phosphate and the guanine ring have been used to probe the stereoselectivity of nucleotide binding to transducin (Yamanaka et al., 1986; Kelleher et al., 1986). The exchange-inert Cr(III) β,γ-bidentate–guanine nucleotide complexes have been employed to probe the interaction of the divalent metal ions with the guanine nucleotide (Frey et al., 1988). In this paper, a battery of affinity analogues with reactive groups attached to different positions of the GTP molecule was used to map the structure of the GTP-binding site of T_α. The results are integrated and compared with the proposed structure of the GTP-binding site deduced from protein modeling (Hingorani & Ho, 1987).

Materials and Methods

Materials. Dark-adapted bovine eyes were obtained fresh from Brown Packing Co., South Holland, IL. The retinas were dissected in the dark and stored at −70 °C. Hexylagarose was purchased from Miles Laboratories. [γ-^{32}P]GTP (10 Ci/mmol), [γ-^{32}P]-8-azido-GTP (32.1 Ci/mmol), and [α-^{32}P]-8-azido-GTP (17.3 Ci/mmol) were from ICN Radiochemicals. Guanine nucleotides were from P-L Biochemicals. TPCK–trypsin was from Boehringer Mannheim and soybean trypsin inhibitor was from Cooper Biomedical. [^{32}P]-P^3-(4-Azidoanilino)-P^1-5′-GTP and *p*-azidoaniline were generous gifts from Dr. Mark Rasenick. All other reagents were of the highest purity available.

Rod Outer Segment Membranes and Protein Isolation. Rod outer segment (ROS) disk membrane was isolated from bovine retinas by the sucrose flotation method. Rhodopsin in reconstituted membrane was prepared by the detergent dialysis method (Hong & Hubbell, 1973). Stripped ROS membrane was prepared by washing the purified ROS membranes four times with buffer containing 2 mM MOPS, 1 mM DTT, and 1 mM EDTA, pH 7.5. Transducin was extracted from ROS membrane with low ionic strength buffer (10 mM Tris, 1 mM DTT, 1 mM $MgCl_2$, pH 7.5) in the presence of 0.1 mM GTP and was purified by hexylagarose column chromatography (Fung et al., 1981). The purified proteins were stored in 40% glycerol at −20 °C. SDS–polyacrylamide gel electrophoresis with subsequent Coomassie blue staining revealed that the purified transducin contained three polypeptides of 40, 37, and 8 kDa.

Synthesis of GTP Affinity Analogues. (A) 2′,3′-Dialdehyde Derivative of GTP (oGTP). The oGTP was prepared according to a modification of the procedure of Easterbrook-Smith et al. (1976). GTP (0.1 mmol) and periodic acid (0.11 mmol) were dissolved in 1.1 mL of water, and the solution pH was adjusted to 4.0 with 1 N NaOH. The solution was allowed to stir in the dark at room temperature for 90 min. To the reaction vessel was added a mixture of 0.8 mL of 1 M NaI in ethanol and 0.2 mL of 4 N HCl. The resulting purple-brown suspension was poured onto 22 mL of ice-cold ethanol and centrifuged at 14000*g* for 15 min. The dark supernatant solution was discarded and the precipitate dissolved in 0.8 mL of water. This solution was neutralized with 1 N NaOH to pH ~7.2 and added to 10 mL of ice-cold ethanol. The suspension was centrifuged again and the supernatant solution was discarded. The resulting white pellet was dried in a vacuum desiccator overnight, yielding a quantitative recovery of oGTP. The purity of oGTP was confirmed by thin-layer chromatography on poly(ethylenimine) sheets using 1 N LiCl and 1 N formic acid (1:1 by volume) as the developing solvent and ultraviolet light to locate the position of the nucleotide. Chromatography of the purified sample revealed only one spot near the origin. No GTP (R_f = 0.22) was detectable. Although 2′,3′-dialdehyde nucleotides undergo β-elimination (Khym & Cohn, 1961), the product of this reaction, an α–β-unsaturated aldehyde that has an ultraviolet absorption peak at 220–230 nm, was not detected in the purified preparation. Radioactive oGTP was synthesized from [α-^{32}P]GTP as starting material.

(B) 3′-O-{3-[N-(4-Azido-2-nitrophenyl)amino]-propionyl}-GTP (ANPAP-GTP). The procedure used is a modified procedure for synthesizing the ATP analogue (Jeng & Guillory, 1975) by coupling GTP to *N*-(4-azido-2-nitrophenyl)-β-alanine. To obtain *N*-(4-azido-2-nitrophenyl)-β-alanine, 4-fluoro-3-nitrophenyl azide was first prepared as an intermediate. 4-Fluoro-3-nitroaniline (4.38 g, 28 mmol) was dissolved in 30 mL of concentrated HCl and 5 mL of water at 45 °C. The solution was placed in an acetone/dry ice bath (−15 to −20 °C), and sodium nitrite (2.4 g, 34 mmol), dissolved in 5 mL of water, was added slowly with stirring. The mixture was stirred for 10 min and filtered into a flask at −20 °C. To the filtrate, under dim red light, was added sodium azide (2.2 g, 32 mmol) in 8 mL of water dropwise with stirring. Some bubbling and foaming occurred during this addition. The resulting yellowish brown solid was filtered, washed with ice water, and then dried in a vaccum desiccator. 4-Fluoro-3-nitrophenyl azide (4.9 g, 26.9 mmol) was recovered in a 96% yield. All subsequent steps were carried out in dim red light. 4-Fluoro-3-nitrophenyl azide (900 mg, 4.9 mmol) was added to a solution containing β-alanine (534 mg, 6 mmol) and sodium carbonate (1080 mg, 10 mmol) dissolved in 5.4 mL of water. Ethanol (6.75 mL), water (5.4 mL), and additional ethanol (13.5 mL) were added in sequence to improve the homogeneity of the mixture. The resulting suspension was stirred at 50 °C overnight with an attached cooling condenser. The resulting dark red mixture was concentrated with a rotary evaporator to less than one-third the original volume and diluted with 18 mL of water. Two extractions with 45 mL ether were carried out to remove the unreacted starting azide. The aqueous layer was acidified to pH 2 with 3 N HCl and was then extracted with three 90-mL portions of ether. The combined ether layers were washed with three 50-mL portions of saturated NaCl, dried over sodium sulfate, and evaporated to dryness. The *N*-(4-azido-2-nitrophenyl)-β-alanine was recrystallized from hot ethanol to yield 300 mg (1.19 mmol, 24% yield) of an orange solid.

The final coupling reaction was carried out with carbodiimidazole as a coupling reagent. Carbodiimidazole (135 mg, 835 μmol) and *N*-(4-azido-2-nitrophenyl)-β-alanine (63 mg, 250 μmol) were dissolved in 250 μL of dimethylformamide (dried over molecular seives) and stirred for 15 min at room temperature. GTP (61 mg, 100 μmol) dissolved in 1.25 mL of water was then added, and the reaction mixture was allowed to stir at room temperature overnight. The solvent was evaporated to dryness, and the resulting residue was washed three times with cold acetone and centrifugedd at 18000*g* for 20 min. The acetone-washed residue was redissolved in about

2 mL of water, applied to Whatman 3MM paper, and eluted with 1-butanol–water–acetic acid (5:3:2). The unreacted *N*-(4-azido-2-nitrophenyl)-β-alanine was seen as an orange band at the solvent front. Unreacted GTP was visualized by UV quenching at $R_f = 0.15$. 3′-*O*-{3-[*N*-(4-Azido-2-nitrophenyl)amino]propionyl}-GTP (ANPAP-GTP) was seen as a broad orange band at $R_f = 0.34$. This band was cut out and eluted with about 80 mL of water. The eluted solution was centrifuged at 18000*g* for 20 min to remove cellulose fibers and then filtered through a sintered glass funnel. The filtrate was lyophilized to give 9 mg, 10.7 μmol (10.7% yield), of an orange powder. Radioactive ANPAP-GTP was synthesized from [α-^{32}P]GTP as the starting material.

(*C*) *5′-[p-(Fluorosulfonyl)benzoyl]guanosine* (*FSBG*). The procedure of Colman and colleagues was used (Tomich et al., 1981). To a suspension of guanosine (5 g, 17.6 mmol) in 50 mL of dimethylformamide was added slowly 2 mL of concentrated hydrochloric acid. To the resulting solution was added gradually 65 mL of tetrahydrofuran to precipitate the product, followed by an additional 87 mL of tetrahydrofuran. Gaunosine hydrochloride, which formed a white precipitate, was filtered and dried in a vaccum desiccator. Guanosine hydrochloride (1 g, 3.2 mmol) was dissolved in 8 mL of hexamethylphosphoric triamide with slight warming. After the solid had dissolved, the flask was placed in an ice bath and *p*-(fluorosulfonyl)benzoyl chloride (1.72 g, 7.7 mmol) was added in small portions while stirring. The solution turned yellow during the addition and small bubbles formed. After all the solid has been added and dissolved, the viscous yellow solution was allowed to stir at room temperature for about 4 h. The reaction mixture was then extracted with two 50-mL portions of petroleum ether, and the upper layers were discarded. The lower yellow layer was placed in a 250-mL beaker to which 100 mL of ethyl acetate–ether (1:1) mixture was added. This resulted in an oily yellow precipitate which was washed two more times with 100 mL of ethyl acetate–ether (1:1). The 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG) was recrystallized with hot acetone, filtered, and dried under vacuum. The compound was identified by its absorption spectrum and its purity determined on TLC plates. The TLC analysis was carried out on EM silica gel 60 F-254 plates developed with methyl ethyl ketone–acetone–water (60:20:15). FSBG exhibits an R_f of 0.50, and guanosine shows an R_f of 0.35.

(*D*) *[α-^{32}P]-8-Azido-P^3-(4-azidoanilino)-P^1-5′-GTP* (*8-Azido-AA-GTP*). The bifunctional cross-linking GTP analogue was prepared by coupling [α-^{32}P]-8-azido-GTP with *p*-azidoaniline. The nonradioactive compound was not synthesized since nonradioactive 8-azido-GTP is not available commercially. All steps were carried out in dim red light. Dicyclohexylcarbodiimide (3 mg) was dissolved in 400 μL of 0.1 M triethanolamine at pH 7.4, and the resulting solution was added to [α-^{32}P]-8-azido-GTP (17.3 Ci/mmol, 500 μCi) which had been evaporated to dryness under a stream of nitrogen. To this solution was added *p*-azidoaniline (6 mg) dissolved in 200 μL of distilled dioxane. The reaction was allowed to proceed at room temperature for about 20 h. The reaction mixture was then extracted with three 500-μL portions of ether to remove the unreacted *p*-azidoaniline. The residual ether was removed with a stream of nitrogen. The [α-^{32}P]-8-azido-P^3-(4-azidoanilino)-P^1-5′-GTP was used without further purification. The purity of the sample was determined by TLC on poly(ethylenimine) paper to be approximately 80% pure with the unreacted 8-azido-GTP as the major contaminant.

Affinity Labeling of Transducin with GTP Analogues. Prior to modification transducin was passed through a Bio-Rad P-6 desalting column equilibrated with 50 mM MOPS, 200 mM NaCl, and 2 mM $MgCl_2$, pH 7.5, in order to remove the glycerol in the storage solution. The reaction mixture generally contained transducin at 1–2 mg/mL and excess radioactive GTP affinity analogues (~100 μM). The binding affinity of various GTP analogues to transducin was estimated from their inhibition of the GTPase activity of transducin or their ability to activate PDE in ROS membrane. The binding of GTP analogues to transducin was catalyzed by the addition of 6 μM photolyzed rhodopsin reconstituted in phospholipid vesicles. After 20-min incubation at room temperature, the rhodopsin membrane was removed by centrifugation (Beckman Airfuge, 5 min at 20 psi) and the excess GTP analogue was removed by passing the supernatant through a Bio-Rad P-6 gel filtration column. Transducin, with the tightly bound GTP analogue, was collected from the void volume. The sample containing the photoaffinity analogues was then irradiated under short-wavelength ultraviolet light (UVP, Inc., Model R-52G) for 5 min at a distance of 5 cm to activate the azido group for covalent labeling. The transducin sample was then digested with TPCK–trypsin for peptide mapping analysis. Identical results were obtained when the ultraviolet light irradiation was carried out directly on the reaction mixture in the presence of rhodopsin or prior to P-6 gel filtration chromatography after the removal of the rhodopsin membrane. For the dialdehyde analogue oGTP, the transducin sample was treated with 1 mM $NaBH_4$ in order to reduce any Schiff base formed between the oGTP and transducin.

Assays. Protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976) using γ-globulin from Bio-Rad Laboratories as a standard. Rhodopsin concentration was determined by the absorbance at 498 nm with a molar extinction coefficient of 42 700 cm^{-1} M^{-1} (Hong & Hubbell, 1973). Guanine nucleotide concentration was determined by the absorbance at 257 nm with a molar extinction coefficient of 13 700 cm^{-1} M^{-1}. SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). The following molecular weight standards from Sigma, lysozyme (14 400), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 200), and phosphorylase *b* (92 500), were used for molecular weight calibration of stained gels.

The GTPase assays were performed by measuring the release of [^{32}P]P_i from [γ-^{32}P]GTP (Fung & Stryer, 1980). The reaction mixture contained 30 μL of 0.05–0.2 mg/mL transducin and 6–10 μM rhodopsin in reconstituted membrane. Reactions were initiated by the addition of 30 μL of 60 μM [γ-^{32}P]GTP with or without the added GTP analogues. The reaction was stopped after 10 min by the addition of 0.2 M perchloric acid and followed by ammonium molybdate precipitation. The inorganic precipitates were filtered onto Whatman glass fiber filters, and the radioactivity was counted. Under the experimental conditions, the GTP hydrolysis rate was linear up to 20 min. Therefore, the assayed activity at the 10-min time point represents the initial rate of GTP hydrolysis. In the absence of photolyzed rhodopsin, the purified transducin does not hydrolyze GTP.

The PDE activity was monitored by the decrease of medium pH due to the hydrolysis of cGMP (Yee & Liebman, 1978). The reaction mixture contained ROS membrane containing rhodopsin, transducin, and PDE at a concentration equivalent to 25 μM photolyzed rhodopsin and 5 mM cGMP, in 10 mM MOPS, 200 mM NaCl, and 2 mM $MgCl_2$, pH 7.5. Reactions

were initiated by the addition of the GTP analogue. The concentration of the GTP analogues used in each set of experiments was varied from 1 to 500 μM. The change of pH in the reaction medium was monitored by a pH microelectrode (Microelectrodes Inc., Londonderry, NH) and a Radiometer PHM 82 pH meter. The results were recorded on a Soltex strip chart recorder. The change in pH was then converted to the amount of cGMP hydrolyzed.

Limited Tryptic Digestion and Peptide Mapping. Limited tryptic proteolysis of the affinity-labeled transducin was carried out according to the procedure of Fung and Nash (1983). A transducin to trypsin ratio of 25:1 (w/w) was used. The proteolysis was carried out at 0 °C with a transducin concentration of approximately 0.6 mg/mL. The cleavage reaction was stopped by the addition of a 10-fold excess of soybean trypsin inhibitor. Upon treatment with trypsin, T_α is first cleaved at Lys_{18} and a 2-kDa peptide is removed from the amino terminus, resulting in a 38-kDa peptide. A second cleavage at Arg_{310} removes a 5-kDa fragment from the carboxy terminus to generate a transient 33-kDa fragment, which is finally cleaved at Arg_{204} into a 21- and a 12-kDa fragment. T_β has a single tryptic cleavage site at Arg_{129} and results in an amino-terminal 15-kDa and a carboxy-terminal 23-kDa peptide. T_γ is not cleaved by trypsin (Fung & Nash, 1983; Hurley et al., 1984; Medynski et al., 1985; Lochrie et al., 1985). The tryptic fragments were analyzed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining. The radioactivity associated with each tryptic fragment due to affinity labeling was revealed by autoradiography on Kodak XAR-5 X-ray film with a Du Pont–New England Nuclear Lightning Plus intensifying screen.

Identification of the Cross-Linked Products. Identification of the cross-linked products of transducin generated by the bifunctional GTP analogue 8-azido-AA-GTP was based on molecular weight determination and the Western immunoblotting method (Burnett, 1981) using rabbit antisera against purified T_α and $T_{\beta\gamma}$. The titer and specificity of the antisera were examined in a previous cross-linking study (Hingorani et al., 1988). There was no significant cross-reactivity among these antisera which were used directly in Western immunoblotting. At a dilution of 1:100 for the anti-T_α antiserum and 1:500 for the anti-$T_{\beta\gamma}$, the detection limit in Western immunoblotting was found to be approximately 1 ng of antigen. The cross-linked samples were first separated on SDS–polyacrylamide gels in duplicate and then electrophoretically transferred onto nitrocellulose paper (0.45-μm pore size) in a Hoeffer Transphor apparatus for a 12-h period at 0.3 A at 4 °C. Under this condition, the transfer of the high molecular weight cross-linked products onto the nitrocellulose blot is sufficient, but the retention of the 8-kDa T_γ peptide of transducin on the nitrocellulose paper was poor. The nitrocellulose blots were placed in 5% BSA overnight to block the nonspecific binding sites and then incubated with specific antisera against T_α and $T_{\beta\gamma}$. The nitrocellulose blots were then treated with a second biotinylated goat anti-rabbit IgG antibody (Vectastain ABC kit from Vector Laboratories, Inc.). The cross-reactive bands were revealed by developing the blots in a 50-mL solution containing 4-chloro-1-naphthol (0.6 mg/mL) and 25 μL of hydrogen peroxide (30%). The relative molecular weight of the bands was determined by using prestained molecular weight standards from Sigma, triosephosphate isomerase (26 600), lactic acid dehydrogenase (36 500), fumarase (48 500), pyruvate kinase (58 000), fructose-6-phosphate kinase (84 000), β-galactosidase (116 000), and α_2-macroglobulin (180 000), which were also transferred to the nitrocellulose paper for calibration.

Results

The Proposed GTP-Binding Site of Transducin. A hypothetical model of the GTP-binding site of transducin has been constructed on the basis of the structural homology that exists among a family of GTP-binding proteins including elongation factor Tu (EF-Tu) and the *ras* oncogene p21 protein (Hingorani & Ho, 1987), for which the three-dimensional structure has been solved by X-ray crystallography (Jurnak, 1985; la Cour et al., 1985; deVos et al., 1988). On a linear peptide map, the GTP-binding domain of these homologous proteins is composed of two separate peptide regions (Halliday, 1984). A segment near the amino terminus forms the site of interaction for the γ-P_i of GTP, and a longer segment closer to the carboxyl terminus forms the guanine ring binding structure. These two regions fold together in three-dimensional space to form the GTP-binding site. Figure 1A shows the location of these two regions on the linear peptide map of T_α obtained from tryptic digestion. The γ-P_i binding region is associated with the 21-kDa tryptic fragment of T_α, whereas the guanine ring binding region is on the 12-kDa fragment. Assuming that the folding patterns of the peptides in the GTP-binding domains are the same for T_α and EF-Tu, the molecular structure of the nucleotide binding site of T_α can be deduced (Figure 1B) by substituting the corresponding amino acid residues of T_α to the EF-Tu structure obtained from X-ray crystallography (la Cour et al., 1985). As shown in Figure 1B, the proposed GTP-binding site of T_α is situated on the surface of the protein. The guanine ring binding region is associated with the 12-kDa tryptic fragment. The specificity for the guanine ring may be regulated via interaction with -Asp_{265}-Lys_{266}-. The phosphate-binding site is associated with residues -Gly_{36}-Ala-Gly-Glu-Ser-Gly-Lys-Ser_{43}-, which are located in the amino-terminal side of the 21-kDa tryptic fragment. The 2′- and 3′-hydroxy groups of the ribose are exposed to the solvent. Mg^{2+} ion binding occurs in a loop situated diametrically opposite the β-phosphate of GDP, and a salt bridge may be formed between the Mg^{2+} and the carboxyl group of Asp_{196}. The Mg^{2+} ion is likely to form a monodentate ligand with the terminal phosphate of the guanine nucleotide.

How accurate is the proposed model representing the GTP-binding site of T_α? The characteristics of the proposed structure allow one to design experiments to test the model. Since the guanine ring, the ribose, and the phosphates of the GTP molecule are associated with different parts of the T_α molecule, it is possible to experimentally map the site by using a battery of affinity analogues of GTP with reactive groups attached to different parts of the GTP molecule, as shown in Figure 2. Regions associated with the guanine ring can been probed with 8-azido-GTP, and covalent labeling should occur with the 12-kDa tryptic fragment of T_α. The sites that interact with the phosphate moiety of GTP can be probed with P^3-(4-azidoanilino)-P^1-5′-GTP (AA-GTP) and 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG). The labeling site of these analogues should be associated with the 21-kDa tryptic fragment. The ribose binding regions can be examined with 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-GTP (ANPAP-GTP) and the 2′,3′-dialdehyde derivative of GTP (oGTP). Since the proposed structure of T_α suggests that both the 2′- and 3′-hydroxyl groups of the ribose are exposed to the solvent, it is likely that the labeling with AA-GTP and oGTP would be poor due to the lack of interacting residues in the vicinity. The proposed relationship between T_α and the bound GTP deduced from model can be experimentally verified by using the affinity labeling approach.

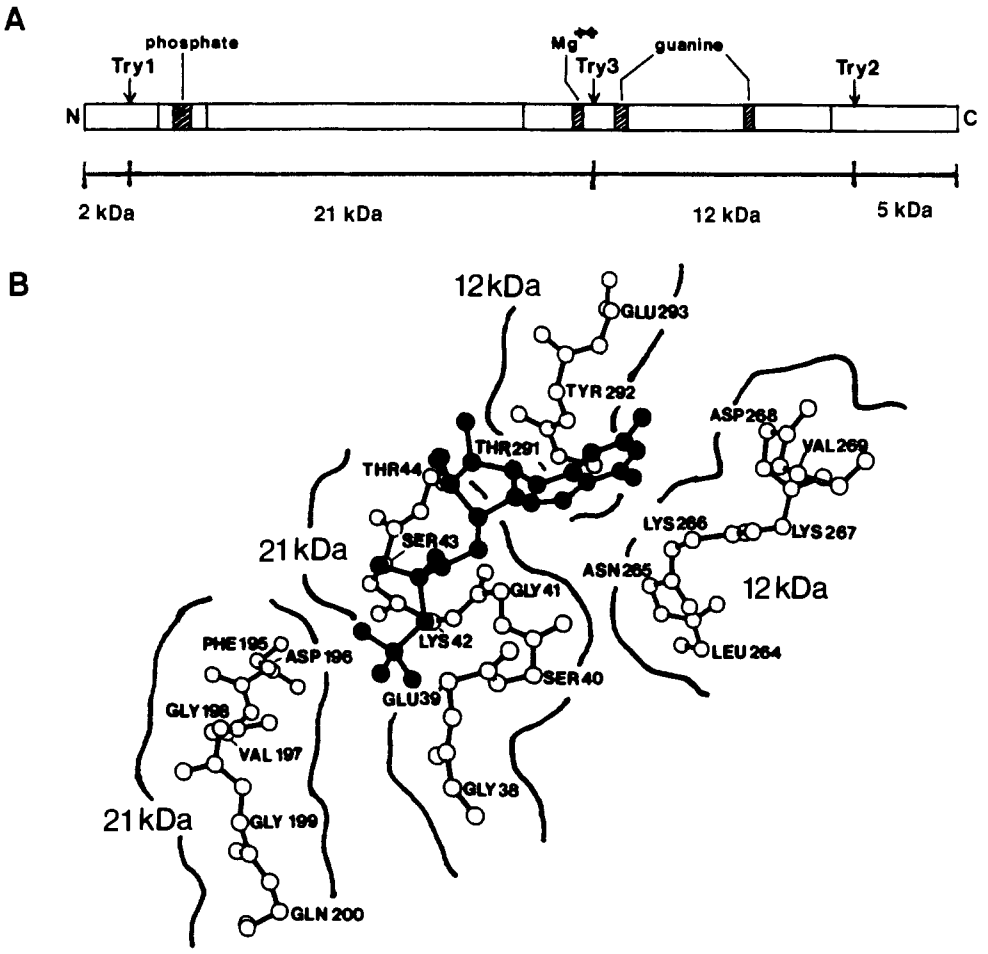

FIGURE 1: Proposed structure of the GTP-binding site of T_α. (A) Linear tryptic peptide map of T_α. The hatched areas represent the location of the GTP-binding domain that shares homology with EF-Tu and *ras* p21 protein. (B) Proposed structure of the GTP-binding site of T_α. The tryptic peptides that contribute to the GTP-binding site are indicated. The binding site structure was deduced from a three-dimensional model of T_α (Hingorani & Ho, 1987) constructed with the aid of the three-dimensional structure of EF-Tu (la Cour et al., 1985). Corresponding amino acid residues have been substituted for the T_α model. A GDP molecule shown in black is incorporated in this model. Mg^{2+} is believed to associate with Asp_{196}, and a monodentate ligand is formed between the Mg^{2+} and the terminal phosphate of the bound guanine nucleotide.

Biochemical Efficacy of the GTP Analogues. Before carrying out the affinity labeling experiments, it was necessary to confirm that the synthetic analogous bind specifically to the GTP-binding site of T_α. The efficacy of binding of these analogues to transducin was determined by the ability of the analogues to inhibit the transducin GTPase activity in ROS membrane as well as the ability to serve as cofactors in the activation of PDE. The results are summarized in Figure 3A. With the exception of FSBG, which had no effect on the GTPase activity of transducin, all the analogues showed inhibition. The relative binding affinities to transducin deduced from these results are as follows: AA-GTP > oGTP > ANPAP-GTP = Gpp(NH)p = 8-azido-GTP > GDP. Modification of the ribose ring of the GTP molecule by either adding a bulky group on the 3′-position or opening up the ring structure did not affect the binding to transducin. This observation is consistent with the proposed structure, which shows that the ribose is relatively exposed to solvent. The lack of binding of FSBG to transducin is not too surprising. Nonhydrolyzable GTP analogues with specific substitution on the γ-phosphate have been used to probe the stereoselectivity of the GTP-binding site at this region (Yamanaka et al., 1986). It has been shown that the more hydrophobic the substitution on the γ-phosphate of GTP, the less effective the analogues are in binding to transducin. The hydrophobic nature of FSBG could limit its binding to transducin.

Figure 3B shows that the GTP affinity analogues could serve as cofactors to activate the ROS membrane PDE via binding to transducin. FSBG was again found to be completely ineffective. The relative efficacy of PDE activation follows the order of AA-GTP = GTP > ANPAP-GTP = 8-azido-GTP > oGTP. oGTP, which inhibited the transducin GTPase activity effectively, was a poor activator of the cGMP PDE and saturated at about 50–60% of the maximal level exhibited with GTP. It is possible that the opening up of the ribose ring of GTP makes the molecule flexible enough to bind well to the GTP site and effectively block the GTPase activity. However, the oGTP molecule is not rigid enough to fully induce the necessary conformational change in T_α that leads to PDE activation. ANPAP-GTP, with the intact ribose ring structure and a large hydrophobic group incorporated in the 3′-hydroxy group, inhibited the GTPase and activated the PDE equivalent to Gpp(NH)p and GTP, respectively. These results imply that the 2′- and 3′-hydroxy groups of ribose are quite exposed to solvent. Therefore, even a large substituting group such as a 3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl group at these positions did not affect the binding or alter the biological activity.

Tryptic Peptide Mapping of the Labeled Sites. Radioactive analogues with ^{32}P at the α-phosphate position of GTP were prepared in order to identify the labeling location. It is essential to use the α-^{32}P-labeled nucleotide in this study, since most of the GTP analogues can be hydrolyzed at the γ-phosphate position by the intrinsic GTPase activity of trans-

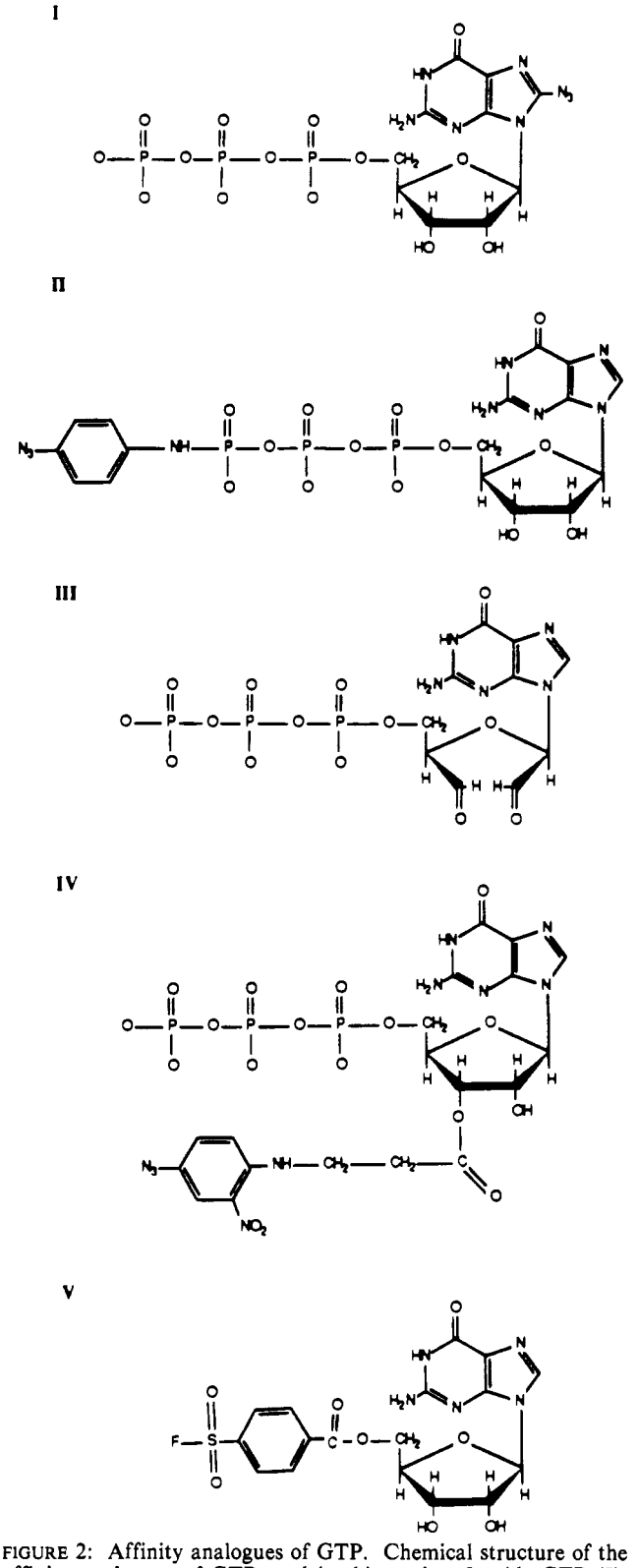

FIGURE 2: Affinity analogues of GTP. Chemical structure of the affinity analogues of GTP used in this study: 8-azido-GTP (I), P^3-(4-azidoanilino)-P^1-5′-GTP (AA-GTP) (II), 2′,3′-dialdehyde derivative of GTP (oGTP) (III), 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)-amino]propionyl]-GTP (ANPAP-GTP) (IV), and 5′-[*p*-(fluorosulfonylbenzoyl]guanosine (FSBG) (V).

ducin. The analogues were first incorporated into transducin in the presence of photolyzed rhodopsin, covalently labeled, and then subjected to limited tryptic digestion. The tryptic fragments were separated by SDS–polyacrylamide gel electrophoresis, and the labeled peptide was identified by autoradiography. FSBG was omitted in the labeling experiment

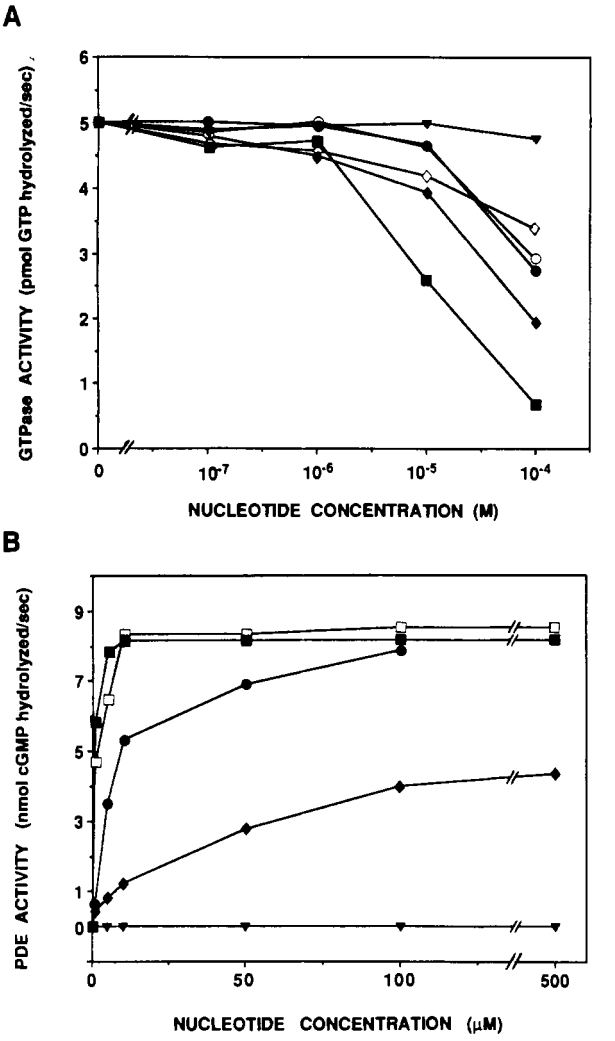

FIGURE 3: Biochemical efficacy of GTP analogues. The interaction of the GTP analogues with the transducin nucleotide binding site was examined for the ability of the analogues to inhibit the GTPase activity of transducin or to serve as a cofactor to activate the latent PDE. (A) Inhibition of the GTPase activity of transducin by GTP analogues at various concentrations. Conditions for the GTPase assay are described under Materials and Methods. Each assay was carried out in a reaction mixture containing 30 μL of 0.2 mg/mL transducin, 6 μM rhodopsin in reconstituted membrane, and 0.1 μM [γ-^{32}P]GTP. (B) Activation of cGMP PDE by GTP analogues at various concentrations. Each assay was carried out with 5 μM ROS in the presence of 2 mM cGMP. Activation with ANPAP-GTP was not measured at higher concentration due to its limited solubility. ○, □, ◇, ▼, ■, ●, and ♦ represent Gpp(NH)p, GTP, GDP, FSBG, AA-GTP, ANPAP-GTP, and oGTP, respectively.

since it was unable to serve as a cofactor in the activation of PDE.

Although the biochemical assays indicated that [α-^{32}P]-ANPAP-GTP and [α-^{32}P]oGTP bound to T_α and exhibited biological activity, they were unable to covalently modify transducin. The lack of labeling by oGTP can be explained in terms of the proposed structure of T_α (Figure 1). Since there is no lysine residue near the 2′- or 3′-hydroxy position of the ribose that could form a Schiff base with the dialdehyde derivative, covalent labeling is not expected. This negative result of the ANPAP-GTP labeling is also consistent with the proposed structure, which indicates that this region of the ribose is exposed to the solvent and is away from the protein side chains.

Radioactive 8-azido-GTP and AA-GTP were able to covalently label transducin. The labeling of transducin by these two analogues was blocked in the presence of GTPγS. The

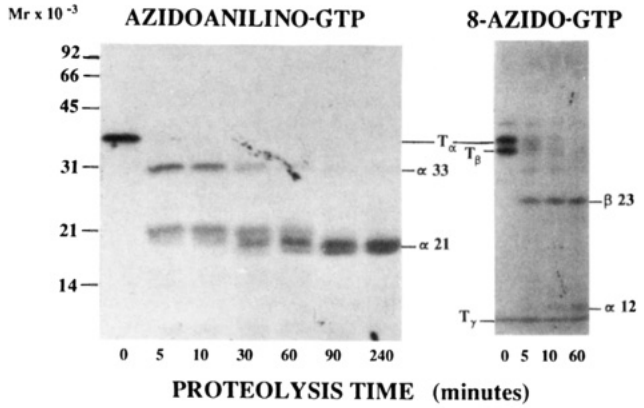

FIGURE 4: Tryptic peptide mapping of covalently labeled transducin. Covalent labeling of transducin with [α-^{32}P]-8-azido-GTP and [α-^{32}P]AA-GTP was carried out as described under Materials and Methods. The labeled transducin was subjected to limited tryptic proteolysis to generate fragments for peptide mapping. The labeled peptide was separated on SDS–polyacrylamide gel and identified by autoradiography. [α-^{32}P]AA-TGP (left panel) labeled the 21-kDa tryptic fragment of T_α. [α-^{32}P]-8-Azido-GTP (right panel) labeled the 12-kDa fragment of T_α and the 23-kDa fragment of T_β. A small amount of labeling was noticed on T_γ.

labeling efficiency was estimated to be approximately 4–6% on the basis of the specificity of the radioactivity. Figure 4 shows the results of the tryptic peptide mapping of the labeling site of these two analogues. [α-^{32}P]AA-GTP labeled the T_α subunit alone. Tryptic peptide mapping showed that covalent modification occurred on the 21-kDa tryptic fragment of T_α as suggested by the proposed model. [α-^{32}P]-8-Azido-GTP also labeled transducin; however, covalent modification occurred on T_α as well as on the T_β and T_γ peptides. An equal amount of label was observed on the T_α and T_β peptides whereas T_γ picked up only a small amount. Tryptic digestion indicated that the labeling of the T_α was on the 12-kDa fragment and the modification of T_β was associated with the carboxyl-terminal 23-kDa fragment. The guanine ring binding site of T_α could be located on the 12-kDa fragment as suggested by the model (Hingorani & Ho, 1987).

Cross-Linking of Transducin Subunits with a Bifunctional GTP Analogue. A comparison of the labeling pattern of AA-GTP and 8-azido-GTP raised an interesting question. Since the two analogues presumably bind to the same site of transducin, why does one only label T_α and the other label T_α as well as $T_{\beta\gamma}$? There are two possible explanations for this observation. First, $T_{\beta\gamma}$ may have a separate nucleotide binding site distinct from the one on T_α. The observed labeling result could be due to a difference in the specificity of each site; the $T_{\beta\gamma}$ binding site may interact strongly with 8-azido-GTP but not with the AA-GTP, whereas the T_α site may bind both analogues. The possibility that T_α and $T_{\beta\gamma}$ contain separate GTP-binding sites is small. An inspection of the primary sequence of $T_{\beta\gamma}$ failed to identify any potential nucleotide binding site containing the consensus sequence of -Gly-X-Gly-X-X-Gly- (Halliday, 1984). Also the 8-azido-GTP labeling on $T_{\beta\gamma}$ may be due to nonspecific binding. Second, the GTP binding site of T_α is in close proximity to the interacting $T_{\beta\gamma}$ subunit. Since the guanine ring structure is sufficiently exposed, it may be partially in contact with $T_{\beta\gamma}$. As a result, the 8-azido-GTP would be capable of labeling both the T_α and $T_{\beta\gamma}$ subunits. However, the phosphate binding region of the GTP molecule on T_α is buried inside the T_α molecule in such a way that the affinity group attached to the phosphate position does not interact with $T_{\beta\gamma}$. Therefore, AA-GTP would be expected to only label T_α, but not $T_{\beta\gamma}$.

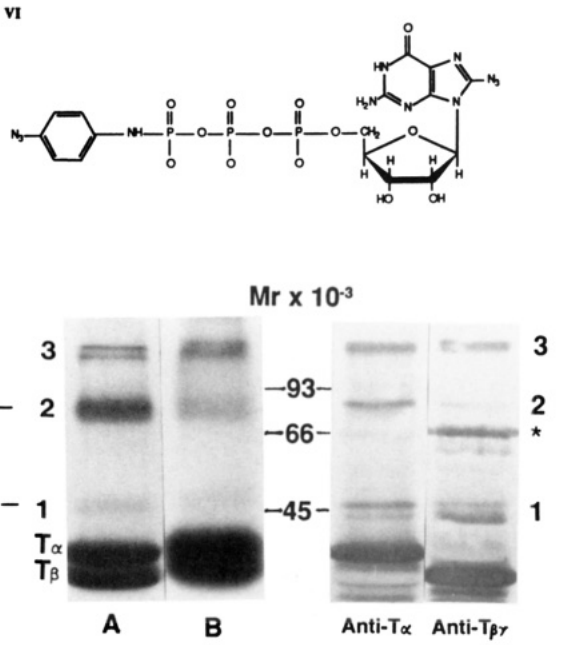

FIGURE 5: Cross-linking of transducin subunits with a bifunctional photoaffinity analogue. The chemical structure of the bifunctional photoaffinity analogue [α-^{32}P]-8-azido-P^3-(4-azidoanilino)-P^1-5′-GTP (8-azido-AA-GTP) (VI) is shown on top. The incorporation of 8-azido-AA-GTP to T_α was described under Materials and Methods. Subunits of the labeled transducin were separated on SDS–polyacrylamide gel. On the left panel, lane A shows the Coomassie blue stained pattern of the cross-linked transducin. Lane B is the autoradiogram of the same gel. Three high molecular weight cross-linked products were identified. Band 1 is probably a T_α–T_γ cross-linked product, band 2 is a T_α–T_β product, and band 3 is higher molecular weight aggregates. The cross-linking efficiency was estimated to be ~1–3%. The predicted molecular weights of T_α–T_γ (M_r 48 000) and T_α–T_β (M_r 76 000) are shown for comparison. The peptide composition of the cross-linked products was confirmed by Western immunoblotting analysis using antisera against T_α and $T_{\beta\gamma}$. The procedure used for the Western immunoblotting analysis was previously described (Hingorani et al., 1988) as outlined under Materials and Methods. The nitrocellulose blots were first reacted with the anti-T_α and anti-$T_{\beta\gamma}$ sera and then treated with a second biotinylated goat anti-rabbit IgG antibody. The cross-reactive bands were revealed by treatment with a solution containing 4-chloro-1-naphthol and hydrogen peroxide. The results are shown in the right panel. The asterisk indicates a nonspecific cross-reactive band in the $T_{\beta\gamma}$ blot.

A bifunctional cross-linking analogue, 8-azido-P^3-(4-azidoanilino)-P^1-5′-GTP (8-azido-AA-GTP) with reactive groups on both positions of the GTP molecule, was synthesized to probe the possibility that the interacting $T_{\beta\gamma}$ is near the GTP-binding site of T_α. If the second possibility is correct, treatment of transducin with 8-azido-AA-GTP would result in cross-linking of the T_α and $T_{\beta\gamma}$ subunits. On the other hand, if the first possibility is correct, the labeling pattern of 8-azido-AA-GTP should be similar to that of 8-azido-GTP without the formation of cross-linked products between T_α and $T_{\beta\gamma}$. Figure 5 shows that three additional radioactive labeled cross-linked products of transducin subunits were obtained with 8-azido-AA-GTP. The molecular weights of these cross-linked products suggest that they include covalently linked T_α–T_β and T_α–T_γ. Furthermore, the amount of radioactivity per milligram of protein associated with the cross-linked products was found to be half of that with the individual subunits. The labeling of the transducin subunits as well as the generation of the cross-linked products by 8-azido-AA-GTP was diminished in the presence of GTPγS. The peptide origin of the cross-linked products was confirmed by the Western immunoblotting technique (Hingorani et al., 1988). All three bands reacted with anti-T_α and anti-$T_{\beta\gamma}$ sera as shown in Figure 5,

indicating that they are cross-linked products of T_α–T_β and T_α–T_γ. This implies that each cross-linked product contains two different protein components bridged by a radioactive 8-azido-AA-GTP molecule. These results strongly support the notion that the guanine ring binding region of T_α is in close proximity with the interacting $T_{\beta\gamma}$ subunit.

Discussion

In early studies radioactive 8-azido-GTP has been used to identify the nucleotide binding subunit of transducin (Takemoto et al., 1981; Takemoto & Takemoto, 1985; Kohnken & McConnell, 1985). The results were similar to our current study in that both T_α and $T_{\beta\gamma}$ subunits were labeled. However, due to the difference in the reaction condition, the T_β was the major component that carried the radioactive label. This observation had led to the conclusion that T_β is the activator containing the nucleotide binding site. The lack of labeling on the T_α in the previous studies could be due to the following reasons. First, [γ-^{32}P]-8-azido-GTP was used instead of the α-^{32}P-labeled compound. As a result, the γ-^{32}P label could be removed by the intrinsic GTPase activity of T_α. Second, the early experiments were carried out in the absence of photolyzed rhodopsin, which catalyzes the incorporation of the GTP analogue to T_α. If the radioactive analogue did not fully exchange into T_α, then labeling would be diminished. In light of the current results on the cross-linking of T_α and $T_{\beta\gamma}$ with the bifunctional 8-azido-AA-GTP, the suggestion that $T_{\beta\gamma}$ contains a separate nucleotide site and possibly functions as the activator of the cGMP cascade should be reconsidered.

One interesting aspect of this work is that the results from affinity labeling are in complete agreement with the proposed structure of the GTP-binding site of T_α developed from protein modeling according to EF-Tu. If the proposed structure of the GTP-binding site is correct, it should explain the results obtained in studies using other structural analogues of GTP. As discussed below, the proposed model is indeed strengthened by those results.

(1) Phosphorothioate analogues of GTP have been used to study the stereochemistry of the GTP-binding site (Yamanaka et al., 1985). The binding affinities of these chiral phosphorothioate GTP compounds extend over a large range with GTPγS = (S_p)-GTPαS > (R_p)-GTPαS > (S_p)-GTPβS $\gg$ (R_p)-GtPβS, and the order is the same in the presence of Mg^{2+} or Cd^{2+}. Since Cd^{2+} has a much higher affinity for sulfur ligands whereas Mg^{2+} has a higher affinity for oxygen, the lack of preference in using Mg^{2+} and Cd^{2+} by the β-substituted analogues would imply that the β-phosphate of GTP is not in direct contact with Mg^{2+} ion. In other words, Mg^{2+} may form a monodentate ligand with the terminal phosphate of the bound GTP as suggested in the model. This explanation is in agreement with the proposed structure of T_α.

(2) The exchange-inert Cr(III) β,γ-bidentate–guanine nucleotide complexes Cr^{III}GTP and Cr^{III}Gpp(NH)p have been used as probes for GTP binding to transducin (Frey et al., 1988). Both the Δ and Λ screw-sense stereoisomers of Cr^{III}Gpp(NH)p are capable of activating transducin with no apparent stereoselectivity. The proposed model suggests that the GTP-binding site is located on the surface of the protein and has sufficient room to accommodate the different configurations of the Cr(III) bidentate–GTP stereoisomers. Moreover, Cr^{III}GTP and Cr^{III}Gpp(NH)p activate the PDE but lack the ability to dissociate the transducin subunits from ROS membrane. This observation may imply that Mg^{2+} and GTP do not form a β,γ-bidentate complex at the binding site of T_α. Instead, Mg^{2+} and GTP bind independently to the site and interact in a monodentate manner as suggested in the proposed structure. Removal of the Mg^{2+} interaction with the β,γ-phosphate of GTP by Cr(III) chelation interrupts the dissociation of the transducin subunits. However, the PDE activation could be triggered by the steric effect of the GTP binding that still exists in the Cr(III) complex. As a result, Cr^{III}GTP is able to dissect these two switching events that occur on T_α.

(3) Nonhydrolyzable GTP analogues with specific substitution on the γ-phosphate have been used to probe the stereoselectivity of the GTP-binding site at this region (Yamanaka et al., 1986). The order of selectivity in activating transducin is GTPγS > GTPγF > GTPγMe > GTPγPh. Substitution on the 2-amino group of the guanine ring with the *p-n*-butylphenyl group has little effect on the binding affinity relative to GTP; however, substitution of a (6-aminohexyl)amino group on the γ-phosphate of GTP dramatically interferes with binding (Kelleher et al., 1986). The 2-amino group of GTP is probably located at the periphery of the binding site facing outward and only weakly interacting with the binding pocket. The proposed model indicates that the phosphate binding pocket of T_α contains charged amino acid residues such as Glu_{39} and Lys_{42}. Therefore, the more hydrophobic the substitution at the γ-phosphate position, the weaker the observed binding of the GTP analogues to T_α.

It is noteworthy to point out that the current model of the T_α molecule was constructed from the crystal structure of EF-Tu. Recently, the structure of another homologous GTP-binding protein, *ras* p21 protein, was available (deVos et al., 1988). A similar model for G-proteins can be deduced from the structure of the p21 protein (Holbrook & Kim, 1989). There are some discrepancies between the models deduced from EF-Tu and *ras* p21, mainly on the topology of the β-strands in the amino-terminal half of the molecule and the attachment sites of Mg^{2+} involved in the phosphate binding regions. The overall structure of the GTP-binding site based on these two models is similar. The proximity of the guanine ring structure with the 12-kDa tryptic fragment as well as the phosphate interacting site on the 21-kDa fragment remains unchanged in both models.

The long-term goal of the affinity labeling and the protein structure modeling studies is not just to characterize the GTP-binding site of T_α but to gather information for the elucidation of the signal coupling mechanism of transducin. T_α can be viewed as a multi-active-site enzyme. Besides the guanine nucleotide binding site, it also contains binding sites for rhodopsin, $T_{\beta\gamma}$, and PDE. The communication between these sites coordinates the coupling events of transducin. Binding to photolyzed rhodopsin switches on the guanine nucleotide binding site, which allows GTP to exchange in. The incorporation of GTP generates two signals. One feeds back to the rhodopsin/$T_{\beta\gamma}$ interacting site and triggers the dissociation of T_α from the ROS membrane and $T_{\beta\gamma}$. The other is conveyed to the PDE interacting site, which turns on the activation mechanism for PDE. To understand the structural and functional basis for the interaction between functional domains with various ligands requires information obtained from different approaches such as biochemical characterization, molecular cloning, and X-ray crystallography. In this paper, we provide a small but interesting piece of a rather large and complicated puzzle.

Acknowledgments

We are indebted to Dr. Mark Rasenick for his gift of AA-GTP and illuminating discussion of our results. We thank John Sainet for obtaining bovine retinas and Diane Tobias for her assistance.

Registry No. I, 65114-35-4; II, 60869-76-3; III, 58045-02-6; [α-^{32}P]-III, 121071-24-7; IV, 121919-35-5; V, 68267-13-0; [α-^{32}P]GTP, 5087-49-0; 5′-GDP, 146-91-8; 5′-GTP, 86-01-1; GTPase, 9059-32-9; Gpp(NH)p, 34273-04-6; [α-^{32}P]-8-azido-AA-GTP, 121919-36-6; 8-azido-AA-GTP, 121919-37-7; *p*-azidoaniline, 14860-64-1; *N*-(4-azido-2-nitrophenyl)-β-alanine, 58775-35-2; 4-fluoro-3-nitrophenyl azide, 28166-06-5; 4-fluoro-3-nitroaniline, 364-76-1; β-alanine, 107-95-9; guanosine, 118-00-3; guanosine hydrochloride, 68267-14-1; *p*-(fluorosulfonyl)benzoyl chloride, 402-55-1; phosphodiesterase, 9025-82-5; cGMP phosphodiesterase, 9068-52-4.

References

Applebury, M. L., & Hargrave, P. A. (1986) *Vision Res. 26*, 1881–1895.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burnett, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Chabre, M. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 331–360.

deVos, A. M., Tong. L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S.-H. (1988) *Science 239*, 837–952.

Easterbrook-Smith, S. B., Wallace, J. C., & Keech, D. B. (1976) *Eur. J. Biochem. 62*, 125–130.

Fesenko, E. E., Kolesnikov, S. S., & Lynbarsky, A. L. (1985) *Nature (London) 313*, 310–313.

Frey, S. E., Hingorani, V. N., Su-Tsai, S.-M., & Ho, Y.-K. (1988) *Biochemistry 27*, 8209–8218.

Fung, B. K.-K. (1983) *J. Biol. Chem. 258*, 10495–10502.

Fung, B. K.-K., & Nash, C. R. (1983) *J. Biol. Chem. 258*, 10503–10510.

Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615–649.

Halliday, K. R. (1984) *J. Cyclic Nucleotide Protein Phosphorylation Res. 9*, 435–448.

Hingorani, V. N., & Ho, Y.-K. (1987) *FEBS Lett. 220*, 15–22.

Holbrook, S. R., & Kim, S.-H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1751–1755.

Hong, K., & Hubbell, W. L. (1973) *Biochemistry 12*, 4517–4527.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., & Gilman, G. (1984) *Science 230*, 32–36.

Jeng, S. J., & Guillory, R. J. (1975) *J. Supramol. Struct. 3*, 448–468.

Jurnak, F. (1985) *Science 230*, 32–36.

Kelleher, D. J., Dudycz, L. W., Wright, G. E., & Johnson, G. L. (1986) *Mol. Pharmacol. 30*, 603–608.

Khym, J. X., & Cohn, W. F. (1961) *J. Biol. Chem. 236*, PC9–PC10.

Kohnken, R. E., & McConnell, D. G. (1985) *Biochemistry 24*, 3803–3809.

la Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385–2388.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Liebman, P. A., Parker, K. R., & Dratz, E. A. (1987) *Annu. Rev. Physiol. 49*, 765–791.

Lochrie, M. A., Hurley, J. B., & Simon, M. I. (1985) *Science 228*, 96–99.

Medynski, D. C., Sullivan, K., Smith, D., Van Dop, C., Chang, F. H., Fung, B. K.-K., Seeburg, P. H., & Bourne, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4311–4315.

Stryer, L. (1986) *Annu. Rev. Neurosci. 9*, 87–119.

Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391–419.

Takemoto, D. J., & Takemoto, L. J. (1985) *Biochem. J. 225*, 227–232.

Takemoto, D. J., Haley, B. E., Hansen, J., Pinkett, D., & Takemoto, L. J. (1981) *Biochem. Biophys. Res. Commun. 102*, 341–347.

Tomich, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.

Yamanaka, G., Eckstein, F., & Stryer, L. (1985) *Biochemistry 24*, 8094–8101.

Yamanaka, G., Eckstein, F., & Stryer, L. (1986) *Biochemistry 25*, 6149–6153.

Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 313*, 579–582.

Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902–8909.