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Chromium(III) β,γ-Bidentate Guanine Nucleotide Complexes as Probes of the GTP-Activated cGMP Cascade of Retinal Rod Outer Segments†

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ABSTRACT: The exchange-inert Cr(III) β,γ-bidentate guanine nucleotide complexes Cr(III)GTP and Cr(III)Gpp(NH)p were used to probe the role of transducin in activating the retinal cGMP cascade. The Cr(III) nucleotide complexes were found to have lower binding affinity for transducin as compared to the Mg^{2+} complexes. However, the rate of hydrolysis of the transducin-bound Cr(III)GTP was similar to that of Mg(II)GTP. Cr(III)Gpp(NH)p activated the cGMP phosphodiesterase of photolyzed rod outer segment membranes up to 75% of the Mg(II)Gpp(NH)p level but lacked the ability to dissociate the transducin subunits from the rod outer segment membrane. This result implies that the activation of the phosphodiesterase by transducin–GTP complex is a membrane-associated event and the formation of a soluble complex of transducin–GTP with the inhibitory peptide of the phosphodiesterase may not be an obligatory step. Both the Δ and Λ screw sense stereoisomers of Cr(III)Gpp(NH)p were capable of activating the cGMP cascade with no apparent stereoselectivity. The nature of the interaction of the metal ion and GTP at the nucleotide-binding site of transducin is discussed together with the results from previous studies using the phosphorothioate GTP analogues [Yamanaka, G., Eckstein, F., & Stryer, L. (1985) *Biochemistry 24*, 8094–8101] and is compared to the site found in homologous GTP-binding proteins such as elongation factor Tu [Jurnak, F. (1985) *Science (Washington, D.C.) 230*, 32–36; la Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385–2388]. The implications of the observed results on the molecular mechanism of visual signal transduction are discussed.

Visual excitation in vertebrate rod photoreceptor cells involves a light-activated cGMP cascade [for a review see Liebman et al. (1987), Hurley (1987), Stryer (1986), Applebury and Hargrave (1986), Chabre (1985), and Fung (1985)]. Photoexcitation of rhodopsin leads to the activation of a latent cGMP phosphodiesterase (PDE)[1] in the rod outer segments

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[1] Abbreviations: ROS, rod outer segment; T, transducin; T_α, the α-subunit of transducin; $T_{\beta\gamma}$, the β- and γ-subunit of transducin; PDE, cyclic GMP phosphodiesterase; $P_{\alpha\beta}$, the catalytic subunits of PDE; P_γ, the inhibitory peptide of PDE; Gpp(NH)p, guanosine 5′-(β,γ-imido)-triphosphate; Gpp(CH_2)p, guanosine 5′-(β,γ-methylene)triphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Cr(III)GTP, $Cr^{III}(H_2O)_4$ β,γ-bidentate GTP complex; Cr(III)Gpp(NH)p, $Cr^{III}(H_2O)_4$, β,γ-bidentate Gpp(NH)p complex; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylene imine); CD, circular dichroism; EF-Tu, elongation factor Tu.

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and results in the rapid hydrolysis of cGMP to 5′-GMP (Bintensky et al., 1978; Liebman & Pugh, 1979). The transient decrease of cGMP concentration causes the closure of the cation channels within the plasma membrane and the subsequent hyperpolarization of the photoreceptor cell (Fesenko et al., 1985; Yau & Nakatani, 1985). A GTP-binding protein called transducin, which is composed of three polypeptides (T_α, M_r 39 000, and $T_{\beta\gamma}$, M_r 36 000 and 8000), has been shown to mediate the light activation signal from photolyzed rhodopsin to the PDE (Fung et al., 1981). Transducin in its inactive form contains a tightly bound GDP on the T_α subunit. Photoexcited rhodopsin catalyzes a GTP/GDP exchange reaction in transducin. The resulting T_α–GTP complex is released from the $T_{\beta\gamma}$ subunit and in turn activates the PDE (Kuhn, 1980; Fung, 1983). After the hydrolysis of the bound GTP, T_α–GDP reassociates with $T_{\beta\gamma}$. Hence, the binding of GTP to the T_α subunit and the subsequent hydrolysis of the bound GTP represent an important control mechanism of the cGMP cascade.

Various GTP analogues, including the phosphorothioate analogues of GTP and GDP with chiral centers at the α- and β-phosphorus positions and the nonhydrolyzable GTP analogues with specific substitution on the γ-phosphate, have been used to probe the characteristics of GTP binding to T_α (Yamanaka et al., 1985, 1986). In this study, we examine the mode of binding of the divalent cation and GTP to the T_α molecule. Nucleotides in cells exist predominantly as complexes of Mg^{2+} ion. Solution studies of nucleotide divalent cation complexes indicate that they exist as a rapidly equilibrating mixture of α,β,γ-tridentate, β,γ-bidentate, and γ-monodentate isomers with rate constants for dissociation and association of 7000 s^{-1} and $\sim 10^8$ M^{-1} s^{-1}, respectively (Pecoraro et al., 1984; Huang & Tsai, 1982). The conformation of these structural isomers varies from the compact shape of the tridentate isomer to the extended structure of the monodentate isomer (Merrit et al., 1978; Haromy et al., 1984). Due to the stereospecificity of enzymes, in many cases, only one of these isomers is bound in a catalytically productive manner at the enzyme active site. However, the rapid equilibration between the Mg^{2+}-bound isomers makes it difficult to immediately identify the active species. Cleland and co-workers have prepared exchange-inert coordination complexes of Cr(III)ATP and Co(III)ATP in which the isomers are very stable with dissociation half-lives of days or weeks and can be separated and used as probes for studying the conformational preference of nucleotide-binding sites (Cleland, 1982; Gruys & Schuster, 1982). The stereoselectivity of many enzymes, including hexokinase, creatine kinase, glutamine synthetase, protein kinase, Na^+,K^+-ATPase, and F_1-ATPase, have been determined (Dunaway-Mariano & Cleland 1980a,b; Ransom et al., 1985; Schuster et al., 1975; Cleland & Mildvan, 1979; Bossard et al., 1980; Granot et al., 1979; Li et al., 1978; Frasch, 1981). In this paper, we report the use of Cr(III) β,γ-bidentate guanine nucleotide complexes as probes for GTP binding to transducin. The effects of Cr(III) guanine nucleotide complexes as cofactors in activating the retinal cGMP cascade are examined.

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. GTP, GDP, cGMP, ATP, ADP, guanosine 5′-(β,γ-imido)triphosphate [Gpp(NH)p], guanosine 5′-(β,γ-methylene)triphosphate [Gpp(CH_2)p], and guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) were from Sigma Chemical Co. Chromium(III) perchlorate and chromium(III) chloride were from Alfa Chemical Co. Ethanesulfonic acid was obtained from Aldrich Chemical Co. DEAE-cellulose was from Sigma, PEI–cellulose thin-layer sheets (20 × 20 cm) and Dowex 50W-X2 were from J. T. Baker Chemical Co., and (ω-aminooctyl)agarose and hexylagarose were from Miles Laboratories. [8-^{3}H]Gpp(NH)p (15 Ci/mmol), [γ-^{32}P]GTP (10 Ci/mmol), and ^{125}I-labeled protein A were from Amersham. The purity of radioactive nucleotides was confirmed by poly(ethylene imine) (PEI) paper chromatography (Randerath, 1963).

Preparation of ROS Membranes and Proteins. ROS membranes were isolated from dark-adapted retinas by the sucrose flotation method described previously (Fung et al., 1981). The isolation was carried out under dim red light (Kodak safety light with a No. 2 red filter). The ROS membranes were stored frozen in the dark at −20 °C in buffer A containing 10 mM MOPS, 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM DTT, pH 7.5. The ROS membrane contained approximately 1–3% photolyzed rhodospin, estimated spectroscopically. The rhodopsin content was determined from the absorbance at 498 nm by using a molar extinction coefficient of 42 700 cm^{-1} M^{-1} (Hong & Hubbell, 1973). Photolyzed ROS membranes were obtained by bleaching the dark-adapted membrane on ice under a fluorescent lamp for 15 min. Most of the photolyzed rhodopsin was in the meta II photointermediate state after photobleaching. The meta II photointermediate state of rhodopsin is stable at 0 °C for more than 6 h, and the photolyzed rhodopsin was used within 30 min in all experiments. Retinal PDE and transducin were extracted from photolyzed ROS membranes with low ionic strength buffer (10 mM Tris, 1 mM DTT, 1 mM $MgCl_2$, pH 7.5) in the absence and in the presence of 0.1 mM GTP, respectively. Transducin and its subunits, T_α and $T_{\beta\gamma}$ were purified by hexyl agarose and (ω-aminooctyl)agarose column chromatography as described by Fung (1983). Latent PDE, which is inactive in the absence of T_α–Gpp(NH)p, was purified by a modified procedure of Hurley and Stryer (1982) using DEAE-Sephadex, (ω-aminooctyl)agarose, and gel filtration chromatography (Fung, 1983). Antisera against T_α and PDE were obtained by immunizing rabbits with the purified proteins. Specificity of the antisera was examined by solid-phase radioimmunoassay (Tsu & Herzenberg, 1980) and Western immunoblotting technique (Burnett, 1981). In a sample of ROS membrane, the anti-T_α serum cross-reacted with a single band of 40 kDa, whereas the anti-PDE serum cross-reacted with all three polypeptides, P_α, P_β, and P_γ, of the PDE complex.

Preparation of Cr(III) Guanine Nucleotide Complexes. All Cr(III) guanine nucleotide complexes were synthesized according to the methods developed by Cleland and co-workers (DePamphilis & Cleland, 1973; Cleland, 1982) with minor modification. The tetraaqua Cr(III) β,γ-bidentate GTP [Cr(III)GTP] and tetraaqua Cr(III) β,γ-bidentate Gpp(NH)p [Cr(III)Gpp(NH)p] complexes were prepared by heating a 2-mL reaction mixture containing 10 mM $Cr^{III}(ClO_4)_3 \cdot 6H_2O$ and 10 mM GTP or Gpp(NH)p at 80 °C for 10 min in a temperature-controlled water bath (MacNeal & Purich, 1978). The reaction mixture was then cooled to 0 °C and loaded onto a Dowex 50W-X2 column (10 cm × 1 cm diameter) prewashed sequentially with concentrated NH_4OH, water, 1 N HCl, and water until the effluent was approximately at pH 4. The column was eluted at room temperature with distilled water, and the nucleotide peaks were monitored by their absorbance at 253 nm by means of a Gilson Holochrome monitor. Unreacted GTP was eluted in the void volume within the first 20 mL [first 70 mL for Gpp(NH)p]. The Cr(III)GTP com-

plexes eluted shortly thereafter as a broad peak from 30 to 80 mL. Cr(III) and other cationic impurities absorbed tightly onto the anion exchanger and remained as a green band at the top of the column, which was removed from the column before the Dowex 50W column was regenerated, by acid–base washes. Under similar condition, the Cr(III)Gpp(NH)p complexes were eluted from the column between 100 and 200 mL of eluate. Fractions containing the Cr(III) guanine nucleotide complexes were pooled and concentrated by absorbing onto a DEAE-cellulose (Cl^-) column (2 cm × 2 cm diameter). The Cr(III)GTP or Cr(III)Gpp(NH)p complexes that bound onto the top of the column as a green band were quantitatively eluted with 18 mM HCl. The fractions containing Cr(III) guanine nucleotide were pooled and concentrated approximately 5-fold to 20 mM under a stream of dry nitrogen. The concentrated samples were stored at 4 °C. These Cr(III) guanine nucleotide complexes were stable in an acidic environment for at least 2 weeks. A 17% yield of the Cr(III) guanine nucleotide complexes was routinely obtained. Radioactively labeled [γ-^{32}P]Cr(III)GTP and [8-^{3}H]Cr(III)-Gpp(NH)p were prepared by using the corresponding radioactive GTP and Gpp(NH)p as starting materials.

The tetraaqua Cr(III) α,β-bidentate GDP (Cr(III)GDP) was synthesized by a similar procedure. A 20 mM solution of $Cr^{III}(ClO_4)_3 \cdot 6H_2O$ and GDP was immersed into a boiling water bath until the temperature of the reaction mixture reached 80 °C. The reaction mixture was then removed from the water bath, allowed to cool at room temperature for 1 min, placed on ice, and then loaded onto a Dowex 50W-X2 column. Free GDP was removed from the column by washing with 0.1 N HCl until the GDP content of the eluent was less than 200 μM as monitored by the UV absorbance. The column was then washed free of acid with distilled water. The bound Cr(III)GDP was then eluted with 0.1 M sodium acetate, pH 4.3. Cr(III)GDP was further purified before use by passage over the DEAE-cellulose column (1 cm × 0.5 cm diameter). Cr(III)GDP is a cation and is not retained by DEAE-cellulose, whereas anionic impurities are retained on the column.

The tetraaqua Cr(III) β,γ-bidentate ATP [Cr(III)ATP] was synthesized by the established method of Cleland (1982). A 10 mM solution of $CrCl_3$ and ATP was titrated to pH 5.5 with $KHCO_3$. Under these conditions, Cr(III) monodentate ATP was formed, and a small amount of $Cr(OH)_3$ was precipitated. Conversion of the monodentate Cr(III)ATP to the bidentate Cr(III)ATP form was achieved by incubating the solution at 25 °C for 30 min. The Cr(III)ATP complex was stored at 4 °C.

The degree of contamination by free GTP and Gpp(NH)p in the Cr(III)GTP and Cr(III)Gpp(NH)p samples was estimated by separating the free nucleotides from the Cr(III) complexes on ascending PEI–cellulose thin-layer chromatography. Two acidic solvents were used for developing the PEI–cellulose plates. Solvent I contained 250 mM Tris–acetate and 1 M NaCl, pH 4.75, and solvent II was 1.0 M LiCl in 1.0 N formic acid. The coordination of the phosphate groups with the Cr(III) ion greatly reduced the interaction of the Cr(III) nucleotide complexes with the PEI matrix. As a result, the Cr(III) nucleotide complexes migrated faster than their Mg(II) counterparts. In solvent I, the following R_f values were obtained: GTP, 0.09; Cr(III)GTP, 0.66; Gpp(NH)p, 0.06; and Cr(III)Gpp(NH)p, 0.63. The purity of the radioactive Cr(III) nucleotide complexes was estimated by cutting the developed chromatogram into 1-cm strips and counting. For nonradioactive Cr(III) nucleotide complexes, the chromatogram was examined under UV light to locate the Cr(III) nucleotide and the contaminating materials.

The UV–vis absorption spectra of the Cr(III) nucleotide complexes were recorded with a Perkin-Elmer Lamda 4B or Cary 219 spectrophotometer. The stoichiometry of the guanine nucleoside, chromium ion, and phosphate in the complexes was analyzed. Guanine content was determined from the UV absorbance at 257 nm by using a molar extinction coefficient of 13 700 cm^{-1} M^{-1}. Chromium determinations were made from the absorbance at 370 nm after the Cr(III) was oxidized by H_2O_2 to dichromate (Postmus & King, 1955). Phosphate content was determined colorimetrically as a phosphomolybdate complex following the hydrolysis of the nucleotides in 70% perchloric acid for 90 min in a boiling water bath (Chen et al., 1956).

Separation of the Diastereomers of Cr(III)GTP and Cr(III)Gpp(NH)p. The exchange-inert Cr(III) β,γ-bidentate GTP complexes are a mixture of four diastereomers. The stereochemical configuration is generally defined in terms of the screw sense of the coordination of the metal ion. An axis is passed through the metal perpendicular to the coordination plane and the bond from the β-phosphorus to the rest of the nucleotide molecule [the guanyl group of Cr(III)GTP] forms the skew line that defines the screw sense (Cleland, 1982). The four diastereomers of Cr(III)GTP include two left-handed screw sense isomers defined as the Λ isomers and two right-handed isomers as the Δ isomers. The separation of the four diastereomers was achieved by using the ion-pairing reverse-phase HPLC method developed by Gruys and Schuster (1982). An Altex Model 110A isocratic HPLC system equipped with a UV–vis detector and a 8-μL flow cell was used. The reverse-phase column was composed of a 3-cm guard column (Brownlee Labs Aquapore RP-300, 10 μm) and two Altex Ultrasphere ODS C_{18} reverse-phase columns (5 μm, 25 cm × 4.6 mm i.d.) connected in series. Ethanesulfonic acid (2 mM) adjusted to pH 2.5 with 5 N NaOH was used as the isocratic buffer and ion-pairing agent. The optimal separation of the four diastereomers of Cr(III)GTP and Cr(III)Gpp-(NH)p was achieved at 4 °C with a flow rate of 1 mL/min. The separation was monitored by the absorbance at either 254 or 280 nm.

Assays. Protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976) using γ-globulin from Bio-Rad Laboratories as a standard. SDS–polyacrylamide gel electrophoresis (13%) was performed according to the method of Laemmli (1970). The photolyzed-rhodopsin-catalyzed [^{3}H]Gpp(NH)p binding activity of transducin was assayed by filtration onto nitrocellulose filters (Millipore HA, 0.45 μm), and the GTPase activity was measured by the release of [^{32}P]P_i from [γ-^{32}P]GTP as described previously (Fung & Stryer, 1980). Binding of purified [^{3}H]Cr(III)Gpp(NH)p stereoisomers to transducin in the presence of photolyzed rhodopsin was assayed by filtration onto nitrocellulose filters (Millipore HA, 0.45-μm pore) (Fung & Stryer, 1980). The PDE activity of ROS membrane was monitored by the decrease of medium pH due to the hydrolysis of cGMP (Yee & Liebman, 1978). The change of pH was recorded by a system composed of a pH microelectrode (Microelectrodes Inc., Londonderry, NH), a Radiometer PHM 82 pH meter, and a Soltex strip chart recorder.

The release of transducin from photolyzed ROS membrane in the presence of GTP analogues or Cr(III) guanine nucleotide complexes was estimated by the centrifugation method. Guanine nucleotides were incubated with photolyzed ROS membrane for 5 min at 25 °C. The solubilized transducin was separated from the ROS membrane by centrifu-

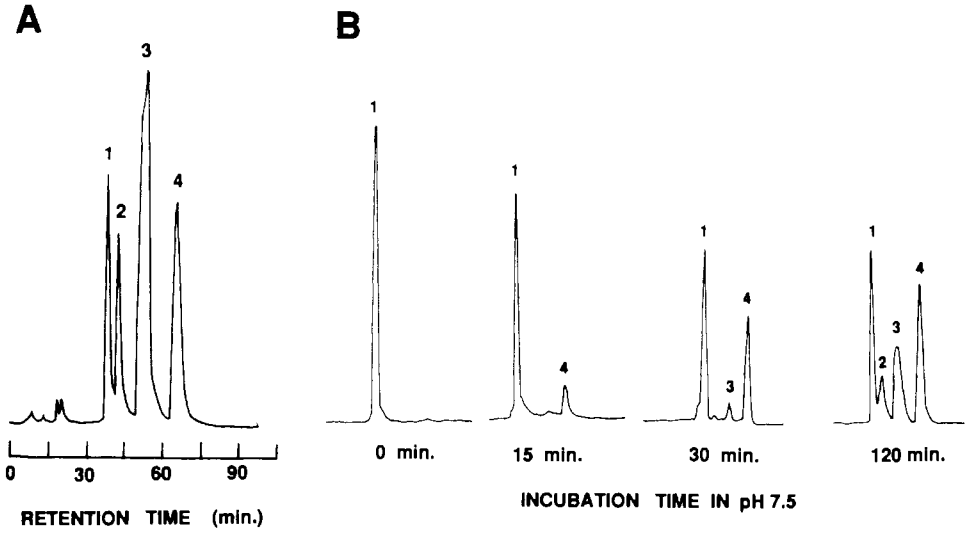

FIGURE 1: (A) Separation of the stereoisomers of Cr(III) β,γ-bidentate Gpp(NH)p by reverse-phase HPLC. The isocratic HPLC system with a reverse-phase C_{18} column (5 μm, 50 cm × 4.6 mm i.d.) is described under Materials and Methods. Ethanesulfonic acid, 2 mM, pH 2.5, was used as solvent with a flow rate of 1.0 mL/min at a pressure of 900 psi at 4 °C. The elution was monitored with a Altex UV detector at 254 nm. The retention times for the four stereoisomers of Cr(III)Gpp(NH)p were 38, 42, 53, and 65 min. According to the elution order, they were assigned as peaks 1–4. The stereoisomers of Cr(III)GTP were also separated by the same methods, and the four isomers of Cr(III)GTP showed retention times of 10.9, 12.8, 15.1, and 18 min (data not shown). CD spectra of the purified isomers indicated that peaks 1 and 4 are the Δ screw sense isomers and peaks 2 and 3 are the Λ isomers as described by Gruys and Schuster (1982). (B) Time course of epimerization of the stereoisomers of Cr(III)Gpp(NH)p at pH 7.5. Purified Cr(III)Gpp(NH)p isomer 1 (a Δ isomer) was incubated in PDE assay buffer (200 mM MOPS, pH 7.5, 2 mM $MgCl_2$, and 200 mM NaCl) at room temperature. The interversion of the stereoisomers was monitored by HPLC. The samples were analyzed by HPLC at the times indicated.

gation in a Beckman Airfuge (5 min at 20 psi). Proteins in the supernatant were separated by SDS–polyacrylamide gel electrophoresis, and the amount of transducin released was quantitated by densitometric analysis of the Coomassie blue stained bands of the transducin subunits using a Biomed Instrument laser scanning densitometer.

RESULTS

Characterization of the Cr(III)GTP and Cr(III)Gpp(NH)p Complexes. The Cr(III)GTP and Cr(III)Gpp(NH)p complexes were characterized by their UV–vis absorption spectra. The guanine ring structure has a strong UV absorption at 257 nm, and the chelation of the chromium ion by the β,γ-phosphates generates two absorption peaks in the visible region at 426 and 610 nm. By use of an extinction coefficient of 13 700 M^{-1} cm^{-1} for the guanine ring absorption at 257 nm, the extinction coefficients at 426 and 610 nm were estimated to be 22.6 and 21.4 M^{-1} cm^{-1} for Cr(III)GTP and 25.5 and 21.6 M^{-1} cm^{-1} for Cr(III)Gpp(NH)p, respectively. The characteristics of the UV–vis spectra of Cr(III) guanine nucleotides are similar to those reported for Cr(III)ATP (Cleland & Mildvan, 1979). The stoichiometry of chromium and phosphate in the complexes was quantitated by chemical methods. The results indicated that both Cr(III)GTP and Cr(III)-Gpp(NH)p complexes were found to have a guanine:Cr:P_i ratio of 1.0 (±0.05):1.0 (±0.1):3.0 (±0.2).

Only highly purified Cr(III) nucleotide complexes may be used in studying the activation of the cGMP cascade; the presence of a minute quantity of free guanine nucleotide in the sample could lead to a significant activation of the PDE via the cascade cycle and mask the effect of Cr(III) nucleotide. We have examined the purity of the Cr(III) nucleotide complexes by PEI–cellulose thin-layer chromatography. The purity of the radioactive [γ-^{32}P]Cr(III)GTP and [^{3}H]Cr(III)Gpp-(NH)p samples was found to be above 99.6% with a minor contaminant that interacted strongly with the PEI support and remained at the origin of the chromatogram. This impurity is definitely not free nucleotides which migrate on the PEI–cellulose plate with R_f values of 0.09 and 0.06 for GTP and Gpp(NH)p, respectively, and is likely to be polymers of the guanine nucleotides generated during the heating step of the synthesis. This byproduct was totally removed by the reverse-phase HPLC method used to separate the stereoisomers of the Cr(III) nucleotide complexes.

The identification of the Δ and Λ isomers separated with reverse-phase HPLC was based on the CD spectra of the separated isomers[2] (Dunaway-Mariano & Cleland, 1980; Gruys & Schuster, 1982). The exchange-inert Cr(III) complexes are stable in an acidic environment. However, above pH 6.0, the interconversion of the β,γ-bidentate complexes from one isomer to an equilibrium of all four isomers can occur in minutes (Gruys & Schuster, 1982; Gruys et al., 1983). Since the study of the cGMP cascade is carried at pH 7.5, it is essential to show that the time required for assaying the PDE activation is faster than the rate of epimerization of the Cr(III) nucleotide isomers. We have examined the rate of isomerization of the Cr(III)Gpp(NH)p at pH 7.5 under the PDE assay conditions. A sample of HPLC-purified Cr(III)Gpp-(NH)p isomer was incubated in PDE assay buffer at room temperature, and aliquots of the sample were removed at various times. The epimerization of the isomer was analyzed by the same HPLC method. Figure 1A shows the separation of the isomers by reverse-phase HPLC. Figure 1B shows that the interconversion among the *d* and *l* conformations of the same screw sense isomers occurred initially at 4 min and reached equilibrium in approximately 35 min. However, the interconversion between two screw sense isomers proceeded relatively slowly. Complete equilibration of all four isomers required more than 2 h. Since the initial rate of PDE activation can be measured within 2 min, at which time no significant epimerization between the Δ and Λ isomers occurs, accurate information concerning the stereoselectivity of the screw sense isomers can be derived from the PDE activation study. The rate of interconversion of the Cr(III)Gpp(NH)p isomers is slower than the reported rate for Cr(III)ATP

[2] The CD spectra of the Cr(III)GTP isomers were obtained from Dr. Thomas Nowak, University of Notre Dame.

complex (Gruys & Schuster, 1982). The instability of the Cr(III) nucleotide complexes in neutral or slightly alkaline solution is thought to be due to the deprotonation of a water ligand and should not be affected by the nucleotide base. The observed slower rate of isomerization for Cr(III)Gpp(NH)p could be entirely due to the imido linkage of the β,γ phosphates of the Gpp(NH)p molecule.

Activation of the cGMP Cascade by Cr(III)Gpp(NH)p and Cr(III)GTP. The interaction of Cr(III) guanine nucleotide complexes with transducin was examined by the ability of the Cr(III) complex to act as a cofactor in the activation of the ROS cGMP cascade and to dissociate the transducin subunits from the photolyzed ROS membranes. The efficiency of the Cr(III)GTP and Cr(III)Gpp(NH)p in activating PDE was estimated in a concentration-dependent study. The apparent K_m values were deduced from double-reciprocal plot analysis (data not shown) and found to be 50 μM for Cr(III)GTP, 2.9 μM for Mg(II)GTP, 30 μM for Cr(III)Gpp(NH)p, and 0.76 μM for Mg(II)Gpp(NH)p. The apparent V_{max} for Cr(III)-GTP and Cr(III)Gpp(NH)p in activating PDE is approximately 75% that of the Mg(II)Gpp(NH)p. At a saturating concentration of 3 mM, Mg(II)GTPγS and Mg(II)Gpp-(NH)p fully activated the cGMP PDE as well as dissociated the transducin subunits from the ROS membrane, whereas Mg(II)Gpp(CH_2)p exhibited a maximum of ~17% activation of the PDE and very little release of the transducin subunits. In contrast, Cr(III)GTP and Cr(III)Gpp(NH)p were totally unable to release the transducin subunits from photolyzed ROS membranes in spite of the 75% activation of the PDE. The results are summarized in Figure 2. The stable β,γ-bidentate coordination of the guanine nucleotide seems to partially alter the coupling function of transducin. Cr(III)GDP, Cr(III)ATP, and Cr(III)ADP were unable to activate the PDE or dissociate the transducin subunits.

The observed K_m values deduced from the PDE activation assay reflect the involvement of guanine nucleotide in two consecutive steps of the cascade, namely, the binding of guanine nucleotide to transducin and the subsequent interaction of the transducin–guanine nucleotide complex with PDE. Since at saturating concentrations the Cr(III) guanine nucleotide complexes still can activate 75% of the PDE activity, the observed 20–30-fold increase of the K_m values for the Cr(III) complexes is probably due to the reduced binding affinity to transducin caused by the bidentate chelation of Cr(III) to the β,γ phosphates of the nucleotides. It has been shown that the binding of guanine nucleotide to soluble transducin in the absence of photolyzed rhodopsin is very tight, with a dissociation constant of ~10^{-7}–10^{-8} M (Baehr et al., 1982). However, as transducin associates with photolyzed rhodopsin, the guanine nucleotide binding site of transducin opens up to allow the exchange reaction to occur. As a result, the dissociation constant of guanine nucleotide from the photolyzed rhodopsin–transducin complex could increase to 10^{-4}–10^{-5} M (Bennett & Dupont, 1985). Since Cr(III)GTP and Cr(III)Gpp(NH)p do not dissociate transducin from ROS membrane, the high K_m values for the Cr(III) complexes may reflect the continuous interaction of the transducin–Cr(III)-Gpp(NH)p complex with photolyzed rhodopsin.

We have also examined the stereoselectivity of the individual stereoisomers of Cr(III)Gpp(NH)p in activating the retinal PDE. [^{3}H]Cr(III)Gpp(NH)p was prepared and used for monitoring the separation and purity of the isolated Δ and Λ isomers by reverse-phase HPLC. The purified isomers were stored at 4 °C in 2 mM ethanesulfonic acid at pH 2.5. Prior to the PDE assay, the pH of the sample was adjusted to pH

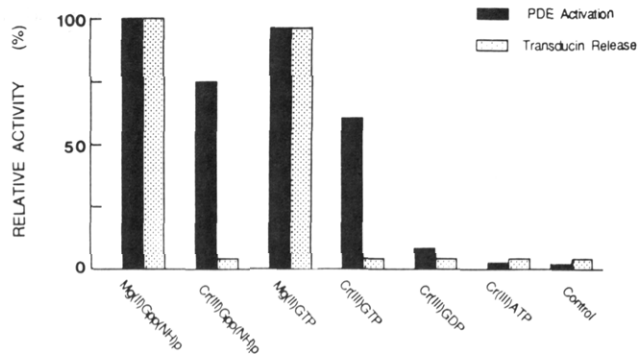

FIGURE 2: Effect of Cr(III)GTP and Cr(III)Gpp(NH)p on the coupling actions of transducin. Relative effect of Cr(III) and Mg(II) nucleotides in dissociating transducin subunits from ROS membrane and activating the cGMP cascade. PDE activation (solid bar) was assayed as described under Materials and Methods except a saturating concentration (3 mM) of Cr(III) and Mg(II) nucleotides was used. The dissociation of transducin from ROS membrane (dotted bar) was assayed by the centrifugation method as described under Materials and Methods. The reaction mixture contained photolyzed ROS membrane (20 μM rhodopsin), 10 mM MOPS, 2 mM $MgCl_2$, 200 mM NaCl, and 1 mM DTT, pH 7.5. After 5 min of incubation with 3 mM Cr(III) or Mg(II) nucleotides, the ROS membrane was removed by centrifugation, and the amount of transducin released into the supernatant solution was analyzed by SDS–polyacrylamide gel electrophoresis and quantitated by densitometric scanning. Since Gpp(NH)p has been shown to be the most potent activator among the nucleotide analogues, the activities of the Mg(II)Gpp(NH)p sample are presented as 100% for comparison. Under the reaction conditions, the 100% PDE activation corresponds to 98 μmol of cGMP hydrolyzed/s. The control sample contained buffer in place of nucleotide. In the presence of 3 mM Gpp(NH)p, 90% of the total transducin in the system was released into solution with approximately 10% remaining tightly bound. This value was used as 100% for the Gpp-(NH)p sample.

7.5 by the addition of an aliquot of 100 mM Tris buffer and then diluted to the final concentrations of Cr(III) nucleotide used in the assay. Up to a concentration of 40 μM Cr(III)-Gpp(NH)p, the activation of the PDE showed a linear concentration dependence. The assay for PDE activation was carried out within 10 s after the pH adjustment to avoid the interconversion between the different stereoisomers of the Cr(III)Gpp(NH)p. As shown in Figure 3, both screw sense isomers activated the retinal PDE equally well. The incorporation of the purified stereoisomers of [^{3}H]Cr(III)Gpp-(NH)p to transducin in the presence of photolyzed rhodopsin was assayed, and the initial rates of binding were found to be identical for both screw sense isomers (data not shown). This result indicates that the nucleotide-binding site of T_α is either quite exposed to the surface of the molecule or contains sufficient flexibility to accommodate the different configurations of the Δ and Λ isomers.

Turnover of Cr(III)GTP by Transducin. Transducin possesses a slow GTPase activity that hydrolyzes bound GTP with a turnover rate of approximately once or twice per minute. The turnover of the bound Cr(III)GTP was examined by using [γ-^{32}P]Cr(III)GTP. The turnover of Cr(III)GTP was found to be approximately 40-fold slower than that of Mg(II)GTP at 1 mM. Since the binding of Cr(III)GTP to transducin is approximately 30-fold less than that of the Mg(II)GTP as suggested from the apparent K_m values, the estimated intrinsic turnover rates of the bound Cr(III)GTP and Mg(II)GTP are similar. However, since the product of the hydrolysis reaction is a complex of Cr(III)$(H_2O)_4$(GDP)(P_i) and the rate of breakdown of Cr(III)$(H_2O)_4$(GDP)(P_i) to release the [^{32}P]P_i may affect the measured rate of GTP hydrolysis, we have developed a second method, based on PDE activation, to es-

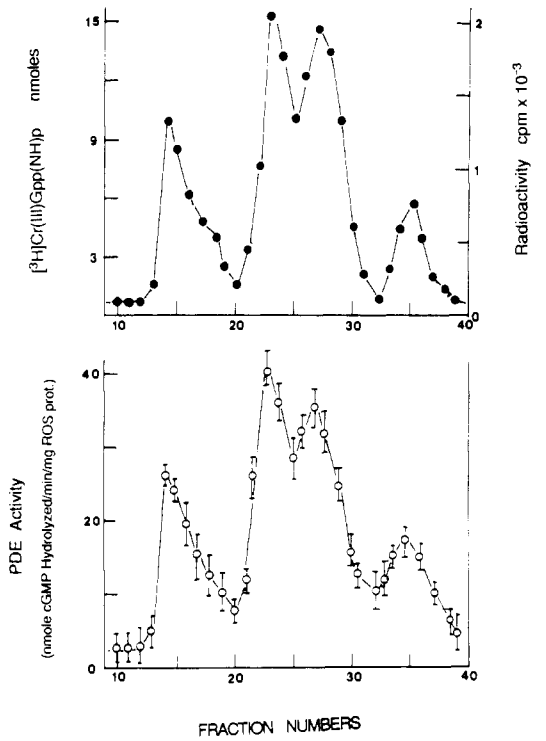

FIGURE 3: Activation of the retinal cGMP cascade by the stereoisomers of Cr(III)Gpp(NH)p. Stereoisomers of [^{3}H]Cr(III)Gpp(NH)p complexes were separated by HPLC as described, and 20-μL fractions (3 drops) were collected. The separation and relative concentration of the isomers was assayed by the radioactivity associated with each fraction (top). The fractions were neutralized to pH 7.5 by the addition of an aliquot of 100 mM Tris buffer, pH 7.5, and the ability of each fraction to activate the PDE in photolyzed ROS membrane was assayed immediately (bottom). The conditions for PDE assay were the same as described in Figure 2. The error bars represent the average of four sets of measurements.

timate the turnover rate of the activated transducin. Figure 4A shows the change of the PDE activity when a limited amount of GTP is added to a photolyzed ROS membrane in the presence of excess cGMP. The PDE activity was maximal upon the addition of GTP but slowly diminished to the basal level after hydrolysis of the GTP. When an additional aliquot of GTP was added, another cycle of PDE activation was induced. The rate of turning off the PDE activity can be used as an indirect measure of the rate of intrinsic GTP hydrolysis. From the experimental trace, the value of GTP_t/GTP_0 was determined at different time points for kinetic analysis. GTP_0 represents the total GTP in the reaction mixture at time 0, whereas GTP_t represents the amount of GTP remaining at time t. Therefore, GTP_t/GTP_0 represents the fraction of GTP remaining at time t. Figure 4B shows that the turning-off rate of the PDE activity due to the hydrolysis of the transducin-bound GTP follows first-order kinetics. A linear plot of ln (GTP_t/GTP_0) vs time was obtained. The slope of the linear plot gave the first-order rate constant of the GTP hydrolysis reaction. The rate constants for the hydrolysis of Cr(III)GTP and Mg(II)GTP were both 1.85×10^{-2} s^{-1}, suggesting that Cr(III)GTP is hydrolyzed in a similar fashion to Mg(II)GTP by transducin. Furthermore, as shown in Figure 4A the first and second additions of Cr(III)GTP to the sample generated the same activation cycle of the PDE, which suggests that the hydrolyzed product of the Cr(III)GTP can be easily released from the transducin binding site, which allows the second activation cycle to occur.

Effect of Cr(III)Gpp(NH)p on the Onset of the cGMP Cascade. It has been well demonstrated that a single photolyzed rhodopsin can activate ~500 transducin molecules in the first stage of amplification of the cGMP cascade (Liebman & Pugh, 1982; Fung & Stryer, 1983). The dissociation of transducin–GTP complexes from the ROS membrane allows the photolyzed rhodopsin to continuously activate the transducin molecules. Since Cr(III)GTP and Cr(III)Gpp(NH)p do not release transducin from the ROS membrane, the catalytic use of the photolyzed rhodopsin for transducin ac-

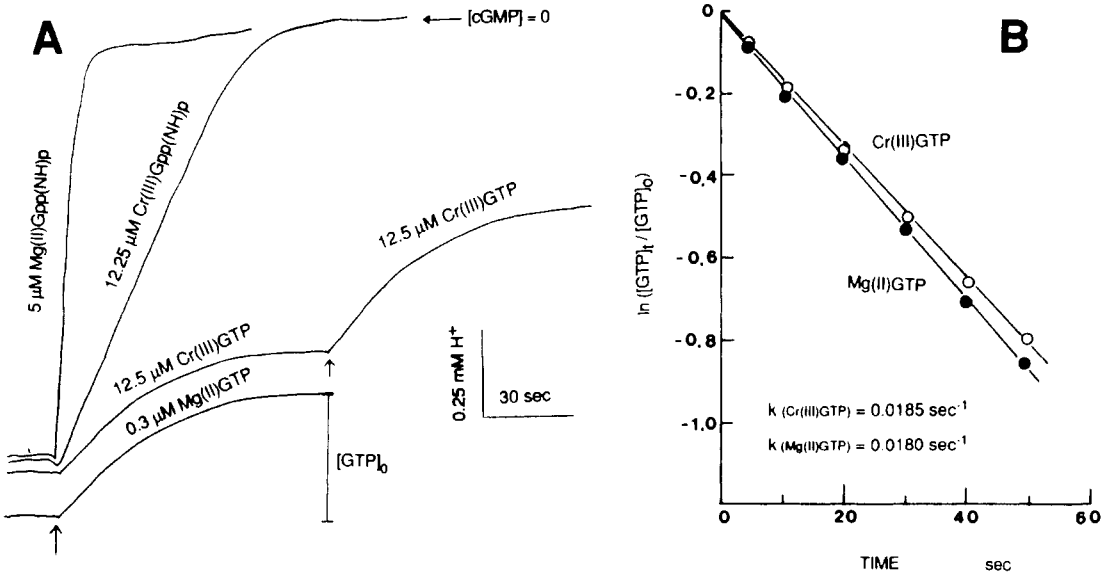

FIGURE 4: Hydrolysis of transducin-bound Cr(III)GTP and Mg(II)GTP in ROS membranes. The hydrolysis of transducin-bound GTP was assayed indirectly by the decrease in PDE activation in the presence of limited amounts of Cr(III)GTP (25 μM) and Mg(II)GTP (0.625 μM). The conditions for the PDE activity measurement were the same as described. (A) Results of phosphodiesterase activation assay monitored by the rate of proton release due to cGMP hydrolysis using a pH microelectrode. Arrows indicate the addition of Cr(III)GTP and Mg(II)GTP. The value of GTP_t/GTP_0 was determined from the experimental trace of the PDE assay as indicated in (A) and was used for the kinetic analysis of the deactivation of PDE activity due to the hydrolysis of the bound GTP. GTP_0 represents the total GTP content in the reaction mixture at time 0, whereas GTP_t represents the amount of GTP remaining at time t. Thereafter, GTP_t/GTP_0 represents the fraction of GTP remaining at time t. Total hydrolysis of the cGMP in the reaction mixture was shown by activating the cascade with nonhydrolyzable Gpp(NH)p. (B) First-order kinetic plot of the rate of PDE deactivation due to the hydrolysis of Cr(III)GTP (○) and Mg(II)GTP (●). A linear plot of ln (GTP_t/GTP_0) vs time was obtained, and the first-order rate constant was calculated from the slope.

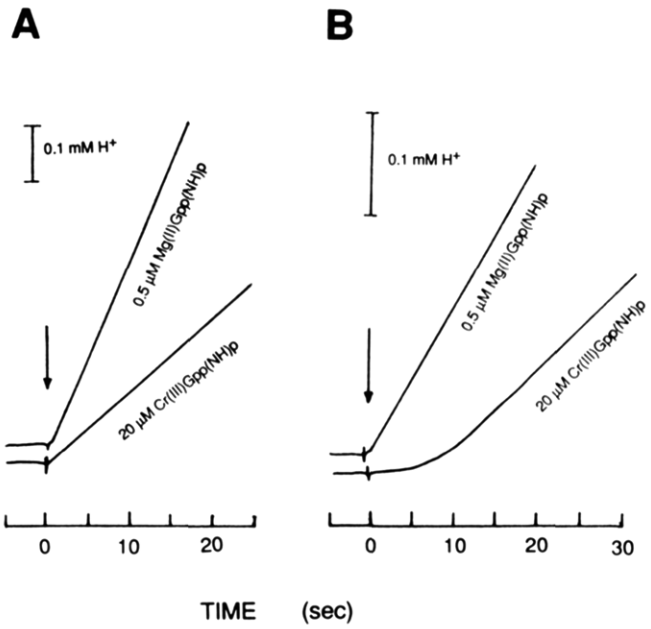

FIGURE 5: Onset of the ROS cGMP cascade mediated by Cr(III)-Gpp(NH)p and Mg(II)Gpp(NH)p under high and low photobleaching levels. The conditions for the PDE assay were the same as described in Figure 2. cGMP hydrolysis was monitored by measuring the change of medium pH. ROS membranes of two different photobleaching levels were prepared as described under Materials and Methods. High-bleached sample contained all meta-II rhodopsin (panel A), whereas the low-bleached sample contained approximately 3% photolyzed rhodopsin (panel B). The arrows indicate the addition of either 0.5 μM Mg(II)Gpp(NH)p or 20 μM Cr(III)Gpp(NH)p.

tivation may be limited. As a result, a delay of the onset of the cGMP hydrolysis may occur. Such a delay would be greater when the concentration of photolyzed rhodopsin is much less than that of transducin, such as under a low photobleaching level, but would be reduced under high bleached condition where photolyzed rhodopsin is in approximately 10-fold excess over transducin. The onset of the PDE activation by Cr(III)Gpp(NH)p under high and low bleached conditions is shown in Figure 5. Indeed, a delay of the PDE activation was observed under the low bleached condition and is consistent with the above suggestion.

Mechanism of T_α–GTP Activation of PDE. The observation that Cr(III)Gpp(NH)p is capable of activating latent PDE but lacks the ability to dissociate the transducin subunits from the ROS disk membrane implies that the formation of a soluble complex of T_α–GTP–P_γ is not an obligatory step in the activation of PDE. The PDE activation remains a membrane-associated event, and the dissociation of T_α–Gpp(NH)p from the ROS disk membrane is related only to signal amplification and movement from one disk to another. We have designed experiments to further examine this possibility.

The amounts of T_α and PDE that remained bound to ROS membrane or were released from the photolyzed ROS membranes in the presence of GTPγS were quantitated by Western immunoblotting using specific antisera against purified T_α and PDE. The GTPγS-activated PDE activity and the trypsin-activated PDE activity in membrane-bound and soluble fractions were assayed. The results allow us to estimate the fraction of PDE that was activated by T_α–GTPγS and the remaining latent PDE that was activated by trypsin treatment. Figure 6 shows that 90% of the T_α was released into solution as T_α–GTPγS with approximately 10% remaining tightly bound to the ROS membrane. Additional washes of the activated ROS membrane in buffer containing GTPγS did not cause further release of the tightly bound T_α. The PDE

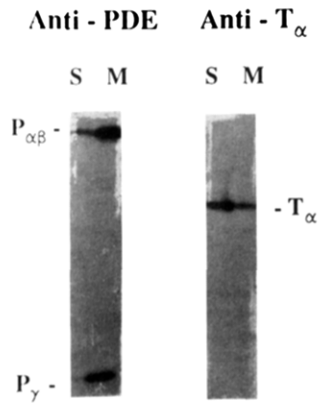

FIGURE 6: Distribution of T_α and PDE in the ROS membrane-bound and soluble fractions during GTPγS activation of the cGMP cascade by Western immunoblotting. Photolyzed ROS membranes containing 5 μM rhodopsin were incubated with 1 mM GTPγS in buffer containing 10 mM Tris, 150 mM NaCl, and 1 mM $MgCl_2$, pH 7.5, for 5 min. The soluble and ROS membrane fractions were separated by means of centrifugation with a Beckman Airfuge (20 psi, 5 min). After the soluble fraction was removed, the ROS membrane pellet was resuspended to the original volume with the above buffer. The proteins in the soluble fraction (S) and the ROS membrane fraction (M) were separated by SDS–polyacrylamide gel electrophoresis (13% gel) and electroblotted onto nitrocellulose paper for Western immunoblotting analysis using antisera against purified T_α and PDE. The distribution of the T_α and PDE in these two fractions was quantitated from the autoradiogram via densitometric scanning.

complexes including the P_γ peptides were found associated mainly with the ROS membrane with less than 2% washed into the soluble fraction. Hence, the soluble T_α–GTPγS complexes were not bound to the P_γ peptides. The PDE activities associated with the soluble and membrane fractions were assayed. Approximately 85% of the PDE associated with the ROS membrane was activated by the membrane-bound T_α–GTPγS. Trypsin treatment activated the remaining 10% latent PDE on the membrane-bound fraction. No T_α–GTPγS-stimulated PDE activity was found in the soluble fraction, and only a few percent of the PDE activity was found after trypsin treatment. This result clearly demonstrates that the 10% membrane-bound T_α–GTPγS is sufficient in activating the majority of the latent PDE on the ROS membrane. The P_γ peptides did not follow the majority of the T_α–GTPγS complexes that was released into solution.

Discussion

The purpose of using exchange-inert Cr(III) guanine nucleotides to study the activation of the retinal cGMP cascade was twofold. First, we wanted to characterize the nucleotide-binding site of transducin, especially in terms of the coordination of metal ion with guanine nucleotides. Second, we wanted to examine the effect of Cr(III) nucleotides on the coupling function of transducin in order to gain a deeper understanding of the signal transduction process. The Cr(III) β,γ-bidentate GTP or Gpp(NH)p is a relatively poor substrate for transducin with a 30–50-fold decrease in affinity as compared to its Mg(II) counterpart. Although Cr(III)Gpp(NH)p can activate PDE up to 75% of the maximal velocity obtained with Mg(II)Gpp(NH)p at saturating concentrations and Cr(III)GTP is hydrolyzed by transducin at a rate similar to that of Mg(II)GTP, Cr(III) guanine nucleotides do not cause the dissociation of transducin subunits from the ROS membrane. Moreover, no stereoselectivity among the diastereomers of the β,γ-bidentate complexes is observed.

The decrease in affinity and lack of stereoselectivity of the Cr(III) β,γ-bidentate isomers suggest that the β,γ-bidentate ligands may not represent the configuration of metal ion nu-

cleotide complex at the transducin binding site. Under physiological conditions, the Mg^{2+} and GTP may bind to transducin independently. Furthermore, it is possible that the bound Mg^{2+} may exist as a monodentate complex with the γ phosphate of GTP rather than the tightly associated β,γ-bidentate complex used in this study. There are several lines of indirect evidence that support the above suggestions and provide an explanation of the observed results on Cr(III) β,γ-bidentate complexes.

(1) Transducin belongs to the family of GTP-binding proteins that are involved in various biological coupling processes and include the G proteins in hormonal regulation and olfaction, the *ras* oncogene p21 protein, and the elongation factor Tu (EF-Tu) (Halliday, 1984; Stryer & Bourne, 1986; Gilman, 1987). The GTP-binding sites among these proteins show a high degree of structural similarity. The tertiary structure of EF-Tu has been solved to 2.7-Å resolution via X-ray crystallography (Jurnak, 1985; la Cour et al., 1985). This information provides a basis for structural modeling of the nucleotide-binding sites of other GTP-binding proteins (McCormick et al., 1985; Masters et al., 1986). It is likely that the mode of binding of metal ion and GTP in this class of GTP-binding proteins is the same. A structural model of the transducin GTP-binding domain has been proposed (Hingorani & Ho, 1987). The proposed GTP-binding site is quite exposed to the surface of the protein and has sufficient room to accommodate the different configurations of the Cr(III)GTP stereoisomers. The Mg^{2+}-binding site is associated specifically with an aspartic acid residue (Asp-196) and interacts with the β phosphate of GDP in a monodentate fashion. The monodentate nature of the Mg^{2+} and guanine nucleotide interaction has also been revealed in electron paramagnetic resonance studies of the Mn^{2+}-substituted EF-Tu interacting with ^{17}O-labeled nucleotides and water molecules (Eccleston et al., 1981). It is likely that transducin shares the same characteristics.

(2) The binding of GTP and Mg^{2+} to transducin and G_0 has been shown to occur independently (Yamazaki et al., 1987; Higashijima et al., 1987; Wessling-Resnick & Johnson, 1987; Codina et al., 1984). Although the binding of Mg^{2+} to the GTP-binding site greatly enhances the hydrolysis of the bound GTP, the binding of the nucleotide substrate does not seem to require a preformed Mg^{2+}–nucleotide complex in solution, as it is believed to occur in many other nucleotide-utilizing enzymes such as kinases.

(3) Phosphorothioate analogues of GTP have been used to study the GTP-binding site of transducin (Yamanaka et al., 1985). The binding affinities of these chiral phosphorothioate GTP compounds to transducin extend over a large range, with GTPγS = (S_P)-GTPαS > (R_P)-GTPαS > (S_P)-GTPβS $\gg$ (R_P)-GTPβS in the presence of Mg^{2+}. When Cd^{2+} is used instead of Mg^{2+}, neither an increase in the affinity of the β-substituted analogues nor a reversal of the effectiveness of the (S_P)-GTPβS $\gg$ (R_P)-GTPβS in binding to transducin is observed. 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 the divalent metal ion (Tsai et al., 1985). A corollary of this interpretation is that Mg^{2+} and GTP do not form a β,γ-bidentate complex at the binding site of transducin.

The T_α subunit should consist of three functional domains, one for the rhodopsin/$T_{\beta\gamma}$ interaction, another for guanine nucleotide binding, and a third for the activation of PDE, and the signal coupling mechanism must rely on the communication between these three distinct domains. The binding of GTP to T_α results in two major events. First, it dissociates T_α from the photolyzed rhodopsin and the $T_{\beta\gamma}$ subunit. Second, the PDE activation site of the T_α is exposed for interaction with the latent PDE. The observation that the incorporation of Cr(III)GTP to transducin only leads to the activation of PDE but not to the dissociation of the transducin subunits from the ROS membrane suggests that Cr(III)GTP and Cr(III)Gpp(NH)p are capable of differentiating between these two signal coupling events. We have proposed a molecular mechanism for the coupling action of the T_α that could provide an explanation for the action of Cr(III)GTP (Hingorani & Ho, 1987). We suggest that the communication between the three functional domains of T_α is mechanically linked through two flexible hinge regions of the protein structure. Binding of ligands to one domain could trigger a conformational change that is conveyed to another domain via the movable hinge region. Cr(III)GTP, when it is bound to T_α, is sterically large enough to activate one of the flexible hinge regions that leads to PDE activation. However, the exchange-inert Cr(III) β,γ-bidentate GTP complexes eliminate the interaction of the β and γ phosphates with the bound Mg^{2+}. It is plausible that such an interaction, especially between the Mg^{2+} and the γ phosphate of GTP, is essential for the dissociation of the transducin subunits from photolyzed rhodopsin. As a result, Cr(III) activates PDE but does not dissociate transducin from ROS membrane. We propose that the Mg^{2+}-binding site of T_α may play a more important role in regulating the subunit interactions than in the activation of PDE. This is supported by the fact that increasing the Mg^{2+} concentration alone in vitro causes dissociation of the transducin subunits and allows the separation of the T_α and $T_{\beta\gamma}$ subunits (Deterre et al., 1984).

It is generally accepted that the activation of the latent PDE is due to the removal of the inhibitory constraint of the P_γ peptide imposed on the catalytic $P_{\alpha\beta}$ subunits of PDE by the T_α–GTP complex (Hurley & Stryer, 1982). However, the exact mechanism is still elusive. The T_α–GTP may physically remove the P_γ peptide from the PDE complex (Yamazaki et al., 1985). In frog ROS membrane, it has been reported that the T_α–GTP–P_γ is released from ROS membrane in a soluble form. Such a complex can be isolated from low ionic strength extract of ROS membrane treated with GTPγS (Deterre et al., 1986). On the other hand, the T_α–GTP complex may bind to the $P_{\alpha\beta}$ subunits and trigger a conformational change to remove the P_γ inhibition and/or prevent the P_γ peptide from reassociating with the activated PDE (Sitaramayya et al., 1986). It has been suggested that a small fraction of GTP-activated transducin remains tightly bound to ROS membrane and can efficiently activate PDE (Wensel & Stryer, 1985). The ratio of transducin to PDE on ROS membrane is approximately 10 to 1, so the membrane-bound T_α alone is sufficient to stoichiometrically activate all the PDE. The observation that Cr(III)GTP activates PDE without dissociating transducin subunits from ROS membrane suggests that the formation of a soluble complex of T_α–GTP–P_γ is not necessary. In other words, if the putative T_α–GTPγS–P_γ does exist under physiological condition, this complex should remain tightly associated with the ROS membrane.

Cr(III) nucleotides have been extensively used to probe the mechanism of many ATP-requiring enzymes. Enzymes such as kinases in which Cr(III)ATP is used as a substrate for phosphoryl-transfer reactions generally use only one of the screw sense isomers. On the other hand, myosin ATPase, which does not transfer a phosphate group to another substrate,

has been shown to lack the stereoselectivity for Cr(III)ATP isomers (Connolly & Eckstein, 1981). The use of Cr(III)GTP to study GTP-requiring enzymes has not been as thorough. Judging from the stereoselectivity, one could assume that the conformation of the transducin GTP-binding site is more closely related to the ATP-binding site of myosin ATPase than the sites found in kinases. Cr(III)GTP has been used to probe the GTP-induced polymerization of tubulin (MacNeal & Purich, 1978). Microtubules assembled with Cr(III)GTP have nearly total resistance to calcium-induced depolymerization. Since the nucleotide-binding site of tubulin is related to that of elongtion factor Tu and transducin, the observed resistance to the calcium-induced depolymerization in Cr(III)GTP microtubules may be analogous to the lack of subunit dissociation in the case of transducin. It is of interest to expand the Cr(III)GTP study to include elongation factor Tu, *ras* p21, G_o, G_i, and G_s. Such a comparative study could provide new insight into the general principles that govern the coupling functions of G proteins.

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Viscoelasticity of F-Actin and F-Actin/Gelsolin Complexes†

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ABSTRACT: Actin is the major protein of eukaryote peripheral cytoplasm where its mechanical effects could determine cell shape and motility. The mechanical properties of purified F-actin, whether it is a viscoelastic fluid or an elastic solid, have been a subject of controversy. Mainstream polymer theory predicts that filaments as long as those found in purified F-actin are so interpenetrated as to appear immobile in measurements over a reasonable time with available instrumentation and that the fluidity of F-actin could only be manifest if the filaments were shortened. We show that the static and dynamic elastic moduli below a critical degree of shear strain are much higher than previously reported, consistent with extreme interpenetration, but that higher strain or treatment with very low concentrations of the F-actin severing protein gelsolin greatly diminish the moduli and cause F-actin to exhibit rheologic behavior expected for independent semidilute rods, and defined by the dimensions of the filaments, including shear rate independent viscosity below a critical shear rate. The findings show that shortening of actin filaments sufficiently to permit reasonable measurements brings out their viscoelastic fluid properties. Since gelsolin shortens F-actin, it is likely that the effect of high strain is also to fragment a population of long actin filaments. We confirmed recent findings that the viscosity of F-actin is inversely proportional to the shear rate, consistent with an indeterminate fluid, but found that gelsolin abolishes this unusual shear rate dependence, indicating that it results from filament disruption during the viscosity measurements. The viscosity of gelsolin/F-actin complexes at very low shear rates is proportional to approximately the fifth power of the filament length. Therefore, proteins that control actin filament length can powerfully regulate the rheologic behavior of cytoplasmic actin.

Actin is the most abundant protein in the peripheral cytoplasm of nucleated cells where other proteins specifically regulate its reversible assembly into linear polymers and the attachment of these filaments to each other or to the plasma membrane (Stossel et al., 1985; Pollard & Cooper, 1986). These attributes of actin have led to the belief that it is responsible for various cell movements and for the ability of cells to resist external forces and maintain their shapes. Since this proposed function of actin is mechanical, it is important to understand actin's architectural properties, and as a starting point, investigators have tried to study the mechanics of purified actin solutions in vitro. This approach, rather than defining the basic mechanical properties of actin itself, by which to understand more complex protein mixtures, has led to controversy and quite disparate views (Stossel et al., 1987; Elson, 1988).

Purified monomeric actin polymerizes in vitro to form a 10 nm diameter filament (F-actin) population with a heterodisperse length distribution, the longest strands of which may be as long as 30 μm (Lanni & Ware, 1984; Zaner & Hartwig, 1988) and which are relatively stiff along their contour (Takebayashi et al., 1977; Fujime, 1970, Egelman, 1985). A tradition of colloid science and polymer chemistry predicts that dilute monomeric actin should be a Newtonian fluid of viscosity very near that of its solvent and that F-actin exhibits

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