May, S. W., & Phillips, R. S. (1980) *J. Am. Chem. Soc. 102*, 5981–5983.
May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981a) *J. Biol. Chem. 256*, 2258–2261.
May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981b) *J. Biol. Chem. 256*, 8470–8475.
May, S. W., Phillips, R. S., Herman, H. H., & Mueller, P. W. (1982) *Biochem. Biophys. Res. Commun. 104*, 38–44.
May, S. W., Mueller, P. W., Padgette, S. R., Herman, H. H., & Phillips, R. S. (1983) *Biochem. Biophys. Res. Commun. 110*, 161–168.
Njus, D., & Radda, G. K. (1979) *Biochem. J. 180*, 579–585.
Njus, D., Knoth, J., Cook, C., & Kelly, P. M. (1983) *J. Biol. Chem. 258*, 27–30.
Padgette, S. R., Herman, H. H., & May, S. W. (1984a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1880.
Padgette, S. R., Herman, H. H., Han, J. H., Pollock, S. H., & May, S. W. (1984b) *J. Med. Chem. 27*, 1354–1357.
Padgette, S. R., Wimalasena, K., Herman, H. H., Sirimanne, S. R., & May, S. W. (1985a) *Biochemistry 24*, 5826–5839.
Padgette, S. R., Roberts, S. F., Wimalasena, K., Herman, H. H., & May, S. W. (1985b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1054.
Perina, I., Bregant, N., & Balenovic, K. (1973) *Bull. Sci., Sect. A (Zagreb) 18*, 4.
Pollock, S. H., Padgette, S. R., Herman, H. H., Han, J. H., & May, S. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2260.
Pollock, S. H., Herman, H. H., Han, J. H., & May, S. W. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1101.
Pollock, S. H., Herman, H. H., & May, S. W. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 879.
Reich, H. J. (1978) *Oxidation in Organic Chemistry* (Trahanovsky, W., Ed.) Part C, pp 1–129, Academic, New York.
Rosenberg, R. L., & Lovenberg, W. (1980) *Essays Neurochem. Neuropharmacol. 4*, 163–209.
Sharpless, K. B., Young, M. W., & Lauer, R. F. (1973) *Tetrahedron Lett. 22*, 1979–1982.
Skotland, T., & Ljones, T. (1979) *Inorg. Perspect. Biol. Med. 2*, 151–180.
Skotland, T., & Ljones, T. (1980) *Biochim. Biophys. Acta 630*, 30–35.
Tirrell, J. G., & Westhead, E. W. (1979) *Neuroscience (Oxford) 4*, 181–186.
Van der Schoot, J. B., & Creveling, C. R. (1965) *Adv. Drug Res. 2*, 47–88.
Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 263–289, Wiley, New York.
Wehrmeister, H. L. (1963) *J. Org. Chem. 28*, 2589–2592.
Yamazaki, I. (1962) *J. Biol. Chem. 237*, 224–229.

# Chemical Modification of Bovine Transducin: Effect of Fluorescein 5′-Isothiocyanate Labeling on Activities of the Transducin $\alpha$ Subunit†

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ABSTRACT: Fluorescein 5′-isothiocyanate (FITC) was used to modify the lysine residues of bovine transducin (T), a GTP-binding protein involved in phototransduction of rod photoreceptor cells. The incorporation of FITC showed a stoichiometry of approximately 1 mol of FITC/mol of transducin. The labeling was specific for the $T_\alpha$ subunit. There was no significant incorporation on the $T_{\beta\gamma}$ subunit. The modification had no effect on the transducin–rhodopsin interaction or on the binding of guanosine 5′-($\beta,\gamma$-imidotriphosphate) [Gpp(NH)p] to transducin in the presence of photolyzed rhodopsin. The dissociation of the FITC–transducin–Gpp(NH)p complex from rhodopsin membrane remained unchanged. However, the intrinsic GTPase activity of $T_\alpha$ and its ability to activate the cGMP phosphodiesterase were diminished by FITC modification. The rate of FITC labeling of the transducin–Gpp(NH)p complex was about 3-fold slower than that of transducin. Limited tryptic digestion and peptide mapping were used to localize the FITC labeling site. The majority of the FITC label was on the 23-kilodalton fragment, and a minor amount was on the 9-kilodalton fragment of the $T_\alpha$ subunit. These results indicate that FITC labeling does not alter the activation of transducin by photolyzed rhodopsin but does affect the GTP hydrolytic activity as well as the GTP-induced conformational change of $T_\alpha$, which ultimately leads to the activation of cGMP phosphodiesterase.

Central to the phototransduction process in vertebrate retinal rod cells is a light-activated cGMP enzyme cascade which involves the rhodopsin molecule, a GTP-binding protein called transducin (T)[1] and the cGMP phosphodiesterase (PDE) [for a review, see Stryer (1986) and Chabre (1985)]. Subunits of transducin ($T_\alpha$, $M_r$ 39 000, and $T_{\beta\gamma}$, $M_r$ 36 000 and 8000)

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[1] Abbreviations: ROS, rod outer segment; FITC, fluorescein 5′-isothiocyanate; Gpp(NH)p, guanosine 5′-($\beta,\gamma$-imidotriphosphate); T, transducin; $T_\alpha$, $\alpha$ subunit of transducin; $T_{\beta\gamma}$, $\beta$ and $\gamma$ subunits of transducin; PDE, cyclic GMP phosphodiesterase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; MOPS, 4-morpholinepropanesulfonic acid; DEAE, diethylaminoethyl; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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and the PDE ($P_{\alpha\beta}$, $M_r$ 88 000 and 84 000, and $P_\gamma$, $M_r$ 14 000) have been purified and functionally reconstituted (Fung, 1983; Hurley & Stryer, 1982). The photoactivation of the cGMP cascade occurs in a two-stage amplification cycle. Each photolyzed rhodopsin catalyzes the exchange of GTP for bound GDP in hundreds of transducin molecules. The incorporation of GTP by $T_\alpha$ leads to the dissociation of the transducin subunits from the rhodopsin membrane. The $T_\alpha$–GTP complex activates the latent PDE by removing the inhibitory constraint of the $P_\gamma$ subunit of the phosphodiesterase complex. The activated PDE then hydrolyzes thousands of cGMP to 5′-GMP. The transient decrease of cGMP is believed to regulate the $Na^+$ permeability across the plasma membrane of the rod cells (Fesenko et al., 1985; Yau & Nakatani, 1985). The cascade is turned off after the hydrolysis of the bound GTP or the phosphorylation of the photolyzed rhodopsin by rhodopsin kinase (Liebman & Pugh, 1980; Sitaramayya & Liebman, 1983).

It is obvious that in the above coupling mechanism, the $T_\alpha$ subunit plays a pivotal role in mediating the light signal from photolyzed rhodopsin to the PDE. $T_\alpha$ should therefore contain binding sites for the $T_{\beta\gamma}$ subunit, rhodopsin, guanine nucleotide, and the PDE. We surmise that these binding sites are distinct and are located in different parts of the $T_\alpha$ molecule. Our aim is to identify these functional domains as well as the essential amino acid residues participating in the coupling action. In spite of the availability of the amino acid sequence of the transducin subunits via molecular cloning (Hurley et al., 1984a; Tanabe et al., 1985; Medynski et al., 1985; Yatsunami & Khorana, 1985; Yatsunami et al., 1985; Fong et al., 1986; Sugimoto et al., 1985), information concerning the topological organization of the functional domains and their interactions during the cascade cycle has not yet been deduced. Biochemical approaches using limited proteolysis (Fung & Nash, 1983) and chemical modification, combined with detailed reconstitution assays, have allowed us to dissect the $T_\alpha$ molecule and to identify various functional sites. We have demonstrated that modification of the sulfhydryl groups of $T_\alpha$ by *N*-ethylmaleimide leads to the inhibition of the initial events of enzyme cascade including the binding of transducin to rhodopsin and the exchange of GTP into $T_\alpha$. However, sulfhydryl modification does not block the second stage of the cascade, the activation of the phosphodiesterase (Ho & Fung, 1984). In this paper, we demonstrate that lysine residues of $T_\alpha$ can be modified by fluorescein 5′-isothiocyanate (FITC) and the modification inhibits only the second-stage reactions of the cascade cycle and has no effect on the activation of transducin by photolyzed rhodopsin.

## Experimental Procedures

*Materials.* Frozen bovine retinas were obtained from G. A. Hormel Co., Austin, MN, and Brown Packing Co., South Holland, IL. Hexylagarose was a product of Miles Laboratories. [$\gamma$-$^{32}$P]GTP (10 Ci/mmol) and [8-$^3$H]Gpp(NH)p (15 Ci/mmol) were from Amersham. Guanine nucleotides, GTP, GDP, cGMP, and Gpp(NH)p were from P-L Biochemicals. TPCK–trypsin was from Boehringer Mannheim, and soybean trypsin inhibitor was purchased from Cooper Biomedical. FITC was from Sigma. All other reagents were of the highest purity available.

*Preparation of Membranes.* Rod outer segment (ROS) membrane was isolated from bovine retina by sucrose floatation according to Hong and Hubbell (1973). "Stripped ROS membrane" was prepared by washing the purified ROS membrane 4 times with buffer containing 2 mM MOPS, 1 mM DTT, and 1 mM EDTA, pH 7.5. The stripped ROS membrane was devoid of any light-activated PDE activity. Reconstituted membrane containing purified rhodopsin and phosphatidylcholine at a molar ratio of 1:100 was prepared by the dialysis method of Hong and Hubbell (1973). Phosphatidylcholine and the detergent tridecyltrimethylammonium bromide (TrTAB) used in the reconstitution were prepared according to Singleton et al. (1965) and Hong and Hubbell (1973), respectively. All three types of membranes were stored at −70 °C in 10 mM MOPS, 200 mM NaCl, 2 mM DTT, and 2 mM $MgCl_2$, pH 7.5 (buffer A). 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).

*Protein Isolation.* Retinal PDE was extracted from photolyzed ROS membrane in low ionic strength buffer (10 mM Tris, 1 mM DTT, and 1 mM $MgCl_2$, pH 7.5). Subsequently, transducin was released from ROS membrane with the same buffer in the presence of 0.1 mM GTP. Transducin was purified by hexylagarose column chromatography (1.0 cm × 8.0 cm) according to Fung (1983). The column was equilibrated with buffer A. The bound transducin was eluted from the column with buffer A containing 0.3 M NaCl. The yield from 500 retinas was routinely 20 mg. The transducin–Gpp(NH)p complex was prepared by incubating purified transducin with 500 $\mu$M Gpp(NH)p in the presence of 10 $\mu$M photolyzed rhodopsin in reconstituted membrane and then purified by hexylagarose chromatography. The PDE was purified by a modified procedure of Hurley and Stryer (1982) using DEAE-Sephadex, $\omega$-aminooctylagarose, and gel filtration chromatographies (Fung, 1983). Purified proteins were concentrated by positive pressure, using Amicon filters (YM 10), to approximately 5 mg/mL and 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 39, 36, and 8 kDa and the PDE contained three polypeptides of 88, 84, and 14 kDa. Purified PDE was a latent enzyme which could be activated by trypsin treatment or in the presence of transducin–Gpp(NH)p complexes. The purified transducin–Gpp(NH)p complex contained all three subunits with 0.95 mol of Gpp(NH)p/mol of transducin. In this sample, the $T_\alpha$–Gpp(NH)p is dissociated from $T_{\beta\gamma}$ (Fung, 1983).

*FITC Modification of Transducin.* To ensure that the FITC labeling was directed to the $\epsilon$-amino group of the lysine residues, the modification was carried out at pH 8.0–8.5. Prior to labeling, transducin was diluted in buffer A (pH 8.0) to a concentration of 3 mg/mL. A 20-fold molar excess of FITC in pH 8.0 buffer A was added to the transducin. The reaction was allowed to proceed at room temperature in the dark for up to 2 h. The reaction was stopped by quenching the unreacted FITC with a hundredfold excess of diethylamine or glycine. The FITC-labeled transducin was separated from the small molecular weight products by gel filtration chromatography on a Bio-Rad P-6 column equilibrated with buffer A, pH 7.5. In the control experiments, transducin was treated in an identical manner with the FITC replaced by buffer, or the FITC was quenched by diethylamine prior to mixing with transducin.

*Assays.* Protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976) using $\gamma$-globulin from Bio-Rad Laboratories as a standard. SDS–polyacrylamide gel (13%) electrophoresis was performed according to Laemmli (1970). Lysozyme ($M_r$ 14 000), soybean trypsin inhibitor ($M_r$ 21 000), carbonic anhydrase ($M_r$ 31 000), ovalbumin ($M_r$ 45 000), bovine serum albumin ($M_r$ 68 000),

and phosphorylase *b* ($M_r$ 92000) were used as molecular weight standards.

The [$^3$H]Gpp(NH)p-binding activity of transducin was assayed by filtration on nitrocellulose filters (Millipore HA 0.45-$\mu$m pore) (Fung & Stryer, 1980). The reaction mixture containing 0.2 mg/mL transducin and 6 $\mu$M rhodopsin, in reconstituted membrane in 30 $\mu$L of buffer A, was photolyzed at 0 °C for 10 min. Reactions were initiated by the addition of 30 $\mu$L of 30 $\mu$M [$^3$H]Gpp(NH)p (15 $\mu$M final concentration). After 10-min incubation at 25 °C, under ambient light, the reaction was quenched by dilution into 3 mL of ice-cold buffer and immediately followed by filtration through the nitrocellulose filter and two 3-mL washes of ice-cold buffer. The amount of radioactive nucleotide that was bound to transducin and retained by the nitrocellulose filter was quantitated by counting the filter in 10 mL of Liquiscint (National Diagnostics). Under these conditions, the incorporation of [$^3$H]Gpp(NH)p was linear up to 20 min. Therefore, the assayed activity at the 10-min time point represents the initial rate of [$^3$H]Gpp(NH)p uptake. The maximal incorporation reached a stoichiometry of 0.95 mol of [$^3$H]Gpp(NH)p/mol of transducin. In the absence of photolyzed rhodopsin, there is no incorporation of [$^3$H]Gpp(NH)p to transducin.

The GTPase assays were performed by measuring the release of [$^{32}$P]$P_i$ from [$\gamma$-$^{32}$P]GTP (Fung & Stryer, 1980). Reaction conditions were the same as described in the binding assay except 30 $\mu$M [$\gamma$-$^{32}$P]GTP was added to initiate the reaction. The reaction was stopped after 10 min by the addition of 0.2 M perchloric acid and followed by molybdate precipitation. The inorganic phosphate molybdate 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 25 min. Therefore, the assayed activity at the 10-min time point represents the initial rate of GTP hydrolysis. The GTPase activity of the control transducin sample was 1.8 nmol min$^{-1}$ (mg of protein)$^{-1}$ and was used as 100% activity. In the absence of photolyzed rhodopsin, the purified transducin does not hydrolyze GTP.

The release of FITC-labeled transducin from photolyzed ROS membrane in the presence Gpp(NH)p was performed by first reconstituting FITC-labeled transducin (0.1 mg/mL) with photolyzed stripped ROS membrane (50 $\mu$M photolyzed rhodopsin). More than 90% of the soluble FITC–transducin was found to bind to the stripped ROS membrane. The FITC–transducin-reconstituted membranes were then washed twice with buffer A. Gpp(NH)p or GDP at 100 $\mu$M concentration was added to the membrane suspension. After 5-min incubation at 25 °C, the solubilized FITC–transducin was separated from the stripped ROS membrane by centrifugation in a Beckman Airfuge (5 min at 20 psi at room temperature). The amount of solubilized FITC–transducin was quantitated by measuring the relative fluorescence in the supernatant using a Perkin-Elmer MFP-44P fluorospectrometer and by densitometric analyses of the transducin subunits separated by SDS–polyacrylamide gel electrophoresis using a Biomed Instrument laser scanning densitometer.

The PDE activity was monitored by the decrease of medium pH due to the hydrolysis of cGMP (Yee & Liebman, 1978). The reaction mixture contained 25 $\mu$M photolyzed rhodopsin in reconstituted membrane, 5 mM cGMP, 0.2 mg/mL transducin, and 0.18 mg/mL PDE in buffer A. Reactions were initiated by the addition of 25 $\mu$M Gpp(NH)p. 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 cGMP hydrolysis. The activity for unmodified transducin was 15.3 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$ and was used as 100% activity.

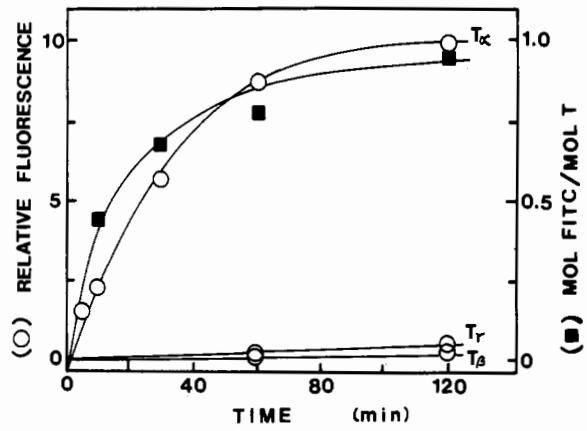



FIGURE 1: Stoichiometry and selectivity of FITC labeling of transducin. The labeling was initiated by the addition of 10 $\mu$L of a saturated solution of FITC in buffer A (approximately 24 mM) to a 315-$\mu$L protein sample (3 mg/mL) at pH 8.0. At various time points, 60-$\mu$L aliquots of the reaction mixture were removed and quenched with 26 $\mu$L of glycine solution (0.188 M) and immediately applied to a desalting column. The absorbance at 493.2 nm was measured for the labeled protein and was used to determine the moles of FITC present by using a molar extinction coefficient of $8.52 \times 10^4$ M$^{-1}$ cm$^{-1}$ (Andersson et al., 1971). The stoichiometry of labeling was plotted as moles of FITC per mole of transducin (■). In order to determine the subunit specificity of labeling, the transducin–FITC was separated on a 13% SDS–polyacrylamide gel. The fluorescence incorporated into each subunit was measured by densitometer scanning as described under Experimental Procedures and expressed as relative fluorescence (○).

Limited tryptic proteolysis of FITC-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. The tryptic fragments were analyzed by SDS–polyacrylamide gel electrophoresis. Tryptic fragments that contained the FITC label were visualized under short-wavelength UV irradiation. The fluorescence pattern was photographed, using a green filter, with Polaroid Type 55 film, and the distribution of FITC label on each fragment was analyzed by densitometric scanning of the negative.

## RESULTS

*Selective Modification of the $T_\alpha$ Subunit of Transducin by FITC.* Purified bovine transducin was allowed to react with FITC in buffer A at pH 8.0 containing 1 mM DTT as described under Experimental Procedures. Figure 1 shows the time course of the FITC modification. The incorporation of FITC to transducin reached a plateau corresponding to 1 mol of FITC/mol of transducin. Further addition of fresh FITC after 120 min of reaction or a higher FITC to transducin ratio (100:1) during modification did not increase the stoichiometry of labeling. To localize the modification site, transducin subunits were separated by SDS–polyacrylamide gel electrophoresis. The relative fluorescence of each protein band, corresponding to the transducin subunits, was quantitated by means of a scanning densitometer. It was found that more than 95% of the FITC was on the $T_\alpha$ subunit and approximately 5% FITC labeling occurred on the $T_\gamma$ subunit. There was no significant labeling on the $T_\beta$ subunit.

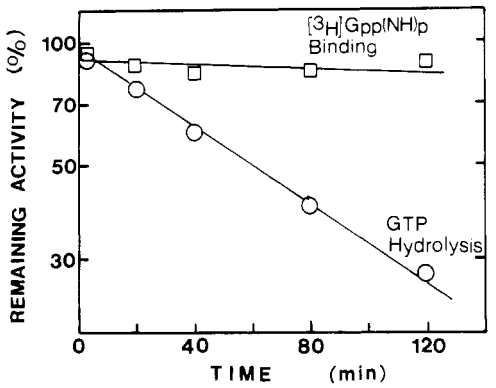



FIGURE 2: Effect of FITC modification on the GTPase- and [$^3$H]-Gpp(NH)p-binding activities of transducin. Conditions for FITC modification of transducin are the same as those described in the legend of Figure 1. The free FITC was removed from the modified transducin by means of a P-6 desalting column. Both the [$^3$H]Gpp(NH)p-binding and GTPase activities were expressed as the percent of the control sample without FITC modification and plotted as a function of time of the FITC labeling reaction. The [$^3$H]Gpp(NH)p binding (□) was carried out in 60 $\mu$L of buffer containing 0.1 mg/mL labeled transducin, 15 $\mu$M [$^3$H]Gpp(NH)p, and 3 $\mu$M photolyzed rhodopsin in reconstituted membranes. After 10 min of incubation at 27 °C, the protein was absorbed onto nitrocellulose filters. The filters were washed with cold buffer, and the radioactivity associated with the filter was measured with a scintillation counter. The GTPase activity (○) was determined under the same condition, except that 15 $\mu$M [$^{32}$P]GTP was used. Under these assay conditions, the reported activities represent the initial rates of Gpp(NH)p uptake and GTP hydrolysis. A semilog plot of transducin activities vs. FITC reaction time is used to indicate that the inhibition of the GTPase activity by FITC modification follows pseudo-first-order kinetics up to 30% remaining activity. The activity of the control sample (100%) was 0.66 nmol min$^{-1}$ (mg of protein)$^{-1}$ for [$^3$H]Gpp(NH)p binding and 1.8 nmol min$^{-1}$ (mg of protein)$^{-1}$ for GTP hydrolysis.

*Effect of FITC Labeling on Transducin Activity.* The participation of transducin in the two-stage cascade cycle of the phototransduction mechanism can be divided into four distinct steps which can be assayed independently. The first step of the cascade involves the interaction between rhodopsin and transducin which can be assayed by transducin binding to rhodopsin membranes and by [$^3$H]Gpp(NH)p binding to transducin catalyzed by photolyzed rhodopsin. The second step involves conformational changes of the $T_\alpha$–GTP complex which decrease its affinity for the ROS membrane. This can be easily monitored by SDS–polyacrylamide gel electrophoresis of the soluble proteins in the presence of the nonhydrolyzable Gpp(NH)p. The third step is the activation of the latent PDE by the transducin–GTP complex which can be assayed by the phosphodiesterase activity using a micro-pH electrode (Yee & Leibman, 1978). The final step of the cycle involves the hydrolysis of the bound GTP which deactivates the transducin and returns the cascade to its latent stage. This step can be monitored by measuring the transducin GTPase activity catalyzed by the photolyzed rhodopsin. Using these assays, we have been able to pinpoint the effect of FITC modification on the cascade cycle.

Figure 2 shows the effect of FITC modification on the [$^3$H]Gpp(NH)p-binding and GTPase activities of transducin catalyzed by photolyzed rhodopsin. As can be seen, the incorporation of [$^3$H]Gpp(NH)p into transducin was not affected by FITC modification; however, the GTPase activity was inhibited. The inhibition of the GTP hydrolytic activity followed pseudo-first-order kinetics up to approximately 75% inhibition. The remaining 25% activity is relatively insensitive to FITC labeling. There is no intrinsic GTPase activity in the purified transducin sample or in the reconstituted rhodopsin membrane. This remaining activity is clearly not due to any

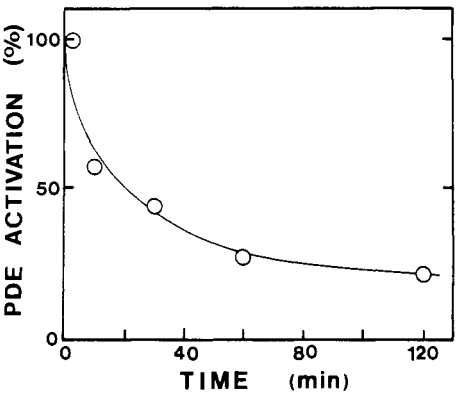



FIGURE 3: Effect of FITC modification of transducin on the activation of cGMP phosphodiesterase. FITC modification was carried out under identical conditions as described in Figure 1. The phosphodiesterase activity was determined by the rate of proton release due to cGMP hydrolysis using a pH electrode, as described under Experimental Procedures. The activity is expressed as the percent of phosphodiesterase activation by the control transducin sample without FITC modification and plotted as a function of FITC reaction time. The activity of the control transducin sample (100%) was 15.3 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$.

contaminant GTPase in the protein sample. Since the FITC incorporation leveled off at 120 min as shown in Figure 1, we conclude that the maximum inhibition of the GTPase activity by FITC has been reached. It is important to point out the difference between the [$^3$H]Gpp(NH)p-binding and the GTPase activity assays. The former represents a single turnover of the transducin molecule in which the transducin–Gpp(NH)p complex is trapped in the activated form. The latter represents the multiple turnover of the transducin by the photolyzed rhodopsin in the presence of GTP. Therefore, the observed result could be due to the inhibition of the intrinsic GTPase activity of $T_\alpha$ rather than to a decrease in the ability of transducin to participate in the guanine nucleotide exchange reaction.

Since Gpp(NH)p can be incorporated into transducin, it is likely that FITC modification occurs on a domain which is not involved in the interaction with rhodopsin and the $T_{\beta\gamma}$ subunit, or in the binding of guanine nucleotide. We have examined the effect of FITC modification on the later steps of the cascade cycle, namely, the dissociation of transducin from the ROS membrane in the presence of Gpp(NH)p and the activation of the latent PDE by the transducin–Gpp(NH)p complex. FITC modification did not alter the dissociation of transducin from the ROS membrane in the presence of Gpp(NH)p. Gpp(NH)p was capable of dissociating more than 90% of the bound FITC–transducin whereas GDP or buffer alone removed less than 15% of the bound FITC–transducin. This result suggests that the conformational change on the $T_\alpha$ subunit triggered by the bound Gpp(NH)p that leads to the dissociation of transducin from rhodopsin was not affected by FITC modification.

Figure 3 shows the effect of FITC modification on the ability of transducin to activate the PDE. The experiment was carried out in a reconstituted system containing purified PDE, photolyzed rhodopsin, FITC–transducin, and cGMP. The activation of the PDE activity decreased with increasing FITC incorporation on transducin. Similar to the reduction in the GTPase activity, the ability of transducin to activate PDE was reduced to approximately 25% of the control sample after 120 min of FITC modification. It is likely that these two inhibited functions of transducin are spatially related. The FITC modification may alter the conformation of a region, near the binding site of the $\gamma$-phosphate of GTP, which is essential for

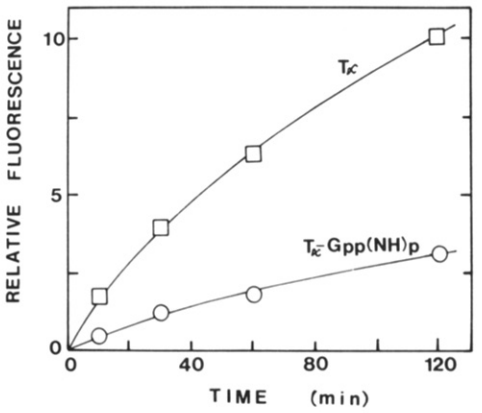
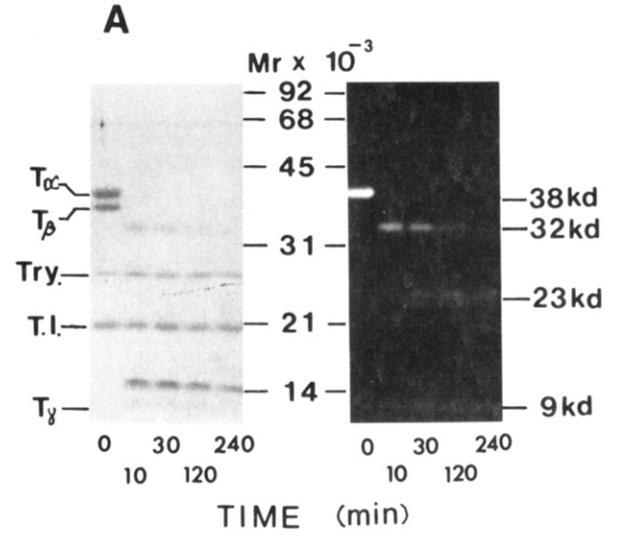



FIGURE 4: Effect of Gpp(NH)p binding to transducin on the rate of FITC modification. Transducin–Gpp(NH)p was prepared as described under Experimental Procedures. The FITC modification was initiated by the addition of 4 $\mu$L of FITC suspension (24 mM) to 400 $\mu$L of protein sample (1 mg/mL). At various time points, 100 $\mu$L of the reaction mixture was quenched with 20 $\mu$L of diethylamine (0.96 M). The subunits were separated by SDS–polyacrylamide gel electrophoresis, and the fluorescence of the $T_\alpha$ subunit was measured as described previously. The degree of FITC modification is expressed as the relative fluorescence of the $T_\alpha$ subunits and plotted as a function of FITC reaction time. (□) represents the FITC labeling on transducin and (○) for transducin–Gpp(NH)p.

GTP hydrolysis and PDE activation.

If the FITC modification site is associated with the GTP-induced conformational change of the $T_\alpha$ subunit, one might expect the binding of Gpp(NH)p to alter the rate of FITC incorporation to transducin. We have carried out experiments to test this possibility. The reaction was carried out with a 3-fold decrease of the FITC and transducin concentrations, so that the initial rate of FITC incorporation could be accurately monitored. It was found that FITC labeling to the transducin–Gpp(NH)p complex is approximately 3–5 times slower than to transducin as shown in Figure 4. This observation is consistent with the above hypothesis.

*Localization of the FITC Labeling Site by Tryptic Peptide Mapping.* Limited tryptic digestion has been used by Fung and co-workers (Fung & Nash, 1983) to dissect the transducin molecule in order to probe the structural and functional relationship of the protein. A set of the tryptic fragments can be identified by SDS–polyacrylamide gel electrophoresis. The linear peptide map of the $T_\alpha$ subunit has been constructed (Hurley et al., 1984b) from these fragments. The alignment of the $T_\alpha$ fragments from the amino terminal to the carboxy terminal was established as 1-, 23-, 9-, and 6-kDa fragments. Under the same conditions, the $T_\beta$ peptide was cleaved into 26- and 16-kDa fragments, and $T_\gamma$ was not cleaved. We used this tryptic peptide mapping method to localize the FITC labeling site. The results are summarized in Figure 5. Panel A shows the tryptic fragmentation pattern of FITC–transducin as revealed by Coomassie blue staining (left) and FITC fluorescence (right). First, it is important to note that the rate of the final cleavage of a transient 32-kDa fragment yielding the 23- and 9-kDa fragments was greatly reduced. The reaction was not completed after 240 min of tryptic digestion. Under identical conditions, the tryptic digestion of unmodified transducin reached its final stage in 40 min (data not shown). It has been demonstrated that this cleavage site is protected by Gpp(NH)p binding. The observation that FITC labeling reduces the accessibility of this site to tryptic digestion suggests that the FITC site may be located within a region in which the Gpp(NH)p-induced conformational change occurs. It was surprising to find that both the 23- and 9-kDa fragments contained the FITC label. This observation would imply that



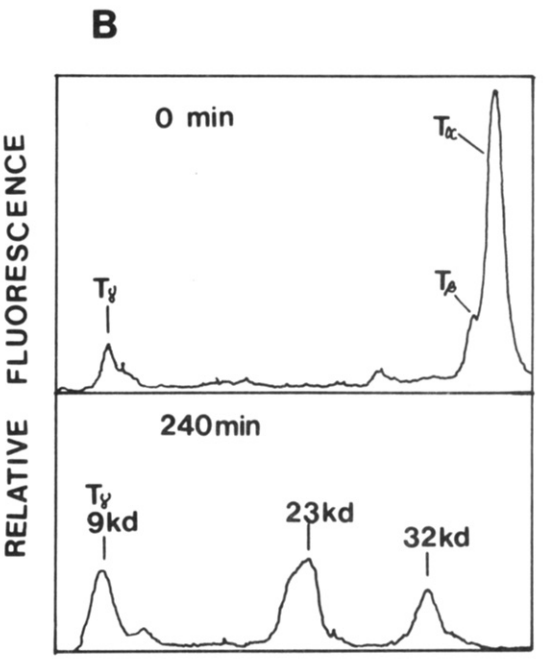




FIGURE 5: Limited tryptic proteolysis of FITC–transducin. FITC modification was carried out as described in the legend of Figure 1 for 120 min. The desalted FITC–transducin was digested with TPCK–trypsin (Try.) at 0 °C with a substrate to protease ratio of 25:1. At various time points, a 10-fold excess of soybean trypsin inhibitor (T.I.) was added to quench the proteolysis. The tryptic fragments were then separated by SDS–polyacrylamide gel electrophoresis. The Coomassie blue staining and UV-activated fluorescence of the polyacrylamide gel as a function of the tryptic digestion time are shown in panel A. Densitometer scans of the fluorescent bands of the undigested sample (0 min, top panel) and the digested sample (240 min, bottom panel) are shown in panel B.

there are at least two separate lysine residues on the $T_\alpha$ molecule that can be labeled by FITC. However, data shown in Figure 1 clearly demonstrate that the incorporation of FITC to transducin reached a maximum stoichiometry of one FITC per transducin. There are two possible explanations for this observation. (1) There are two distinct FITC-binding sites, one located on the 23-kDa fragment and the other on the 9-kDa fragment. Modification of one of these sites could trigger a conformational change in the $T_\alpha$ molecule which could block the interaction of FITC with the second site. (2) There is only one FITC-binding site which contains lysine residues from both the 23- and 9-kDa fragments. Binding of FITC to this site can modify a single lysine residue on either

fragment and sterically exclude modification of the other. To distinguish these two possibilities, further information on the exact modified residues as well as on the tertiary structure of the $T_\alpha$ molecule is needed.

We have estimated the distribution of the FITC label associated with each tryptic fragment by measuring the relative fluorescence with densitometric scanning. The result is shown in panel B. The top scan represents the undigested FITC-labeled transducin, and the bottom scan shows the distribution of the FITC label in the tryptic fragments after 240 min of tryptic digestion. As can be seen in the undigested sample, the FITC label is mainly associated with the $T_\alpha$ subunit, with approximately 5% attached to the $T_\gamma$ peptide. After 240 min of tryptic digestion, most of the $T_\alpha$ peptide was cleaved into its final product of 23- and 9-kDa fragments with a small amount of the undigested 32-kDa fragment. All of the fluorescence associated with $T_\alpha$ can be accounted for in these three tryptic fragments, suggesting that the terminal 1- and 6-kDa fragments were not modified by FITC. The distribution of the FITC label on the final tryptic 9- and 23-kDa peptides can be estimated by subtracting the contribution of fluorescence from the $T_\gamma$ peptide on the unresolved $T_\gamma$ and 9-kDa fragment band on the SDS–polyacrylamide gel. It was found that the 23-kDa fragment contained 72% of the FITC label and the 9-kDa fragment carried 28%.

It is of interest to correlate the site of modification with the inhibition of transducin function. Reconstitution experiments (Fung, 1983) have demonstrated that $T_{\beta\gamma}$ is the modulator for the interaction between $T_\alpha$ and photolyzed rhodopsin in the initial steps of the cascade cycle. Since this activity was not affected by FITC modification at all, the minor incorporation of FITC on the $T_{\beta\gamma}$ subunit is clearly not responsible for the inhibition. In view of the fact that the maximum inhibition of the GTP hydrolysis and phosphodiesterase activation activities was only 75%, which correlates with the amount of FITC incorporation into the 23-kDa fragment, we believe that it is the modification of the 23-kDa peptide which diminishes the transducin activities. This conclusion is consistent with two other observations. (1) Similar to the FITC modification, cholera toxin catalyzed ADP-ribosylation of the $T_\alpha$ subunit occurs on the 23-kDa tryptic fragment which inhibits GTP hydrolytic activity but not GTP binding (Abood et al., 1982; Navon & Fung, 1984). (2) It is well documented that transducin belongs to a family of GTP-binding proteins including the *ras* p21 protein, elongation factor Tu, and the GTP-binding proteins (Ni and Ns) of the adenylate cyclase system (Gilman, 1984). The GTP hydrolytic site of the *ras* p21 proteins has been shown to be located near Gly-12 (Temeles et al., 1985). The homologous region on the $T_\alpha$ subunit is found on the 23-kDa fragment. These observations, together with our results, suggest that the 23-kDa fragment of transducin may contain the functional domain that regulates the phosphodiesterase activation and the GTP hydrolytic activity.

## Discussion

The general picture emerging from biochemical studies suggests that the $T_\alpha$ subunit consists of at least three functionally distinct domains which may communicate through conformational changes upon binding of ligands such as GTP/GDP or through interactions with the receptor (rhodopsin), the modulator ($T_{\beta\gamma}$), and the effector (PDE) proteins. A distinct region composed of the amino- and carboxy-terminal peptides can be identified as the rhodopsin- and $T_{\beta\gamma}$-binding domains. The major component of the guanine nucleotide binding domain is composed of the 9-kDa fragment which can be labeled by 8-[$\alpha$-$^{32}$P]azido-GTP (Ho and Fung, unpublished data). The domain that regulates PDE activation is likely to be located on the 23-kDa fragment. The key to elucidating the molecular mechanism of the coupling action of $T_\alpha$ is to understand how these three functional domains communicate. Modification of a single sulfhydryl group on the 9-kDa fragment blocked the interaction with rhodopsin and $T_{\beta\gamma}$ and the guanine nucleotide exchange reaction but had no effect on the phosphodiesterase activation (Ho & Fung, 1984; Y.-K. Ho and B. K.-K. Fung, unpublished data). In contrast, we demonstrate in this paper that FITC modification of the lysine residue, mainly located on the 23-kDa fragment, inhibits phosphodiesterase activation and the GTP hydrolytic activity but has no effect on the rhodopsin and $T_{\beta\gamma}$ interaction and the guanine exchange reaction.

Another approach to identify the functional domain of transducin is to compare the primary sequence of transducin to those of the homologous GTP-binding proteins such as *ras* p21 protein, elongation factor Tu, Ns, and Ni (Wierenga & Hol, 1983; Gay & Walker, 1983; Halliday, 1984; Leberman & Egner, 1984; Moller & Amons, 1985). The homologous regions among these proteins represent the nucleotide-binding site, and the variable regions may represent domains that are specialized for specific receptor and effector interaction. To the best of our knowledge, the prediction of functional domains from sequence homology is in complete agreement to those obtained from direct biochemical studies. Recently, the three-dimensional structure of elongation factor Tu has been elucidated at 2.7-Å resolution from X-ray crystallographic studies (Jurnak, 1985; la Cour et al., 1985). It may be possible to construct for transducin, on the basis of the available biochemical evidence and by comparison with the crystal structure of homologous proteins, a model comparable to that predicted for the *ras* p21 protein (McCormick et al., 1985). In light of this, the molecular mechanism of transducin and related GTP-binding proteins could be elucidated in the near future.

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**Registry No.** FITC, 27072-45-3; GTP, 86-01-1; GTPase, 9059-32-9; PDE, 9068-52-4; L-lysine, 56-87-1.

## References

Abood, M. E., Hurley, J. B., Pappone, M.-C., Bourne, H. R. & Stryer, L. (1982) *J. Biol. Chem. 257*, 10540–10543.

Andersson, L.-O., Rehnstrom, A., & Eaker, D. L. (1971) *Eur. J. Biochem. 20*, 371–380.

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

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

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

Fong, H.-K., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F., & Simon, M. I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2162–2166.

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

Fung, B. K.-K., & Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2500–2504.

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

Gay, N. J., & Walker, J. E. (1983) *Nature (London) 301*, 262–264.

Gilman, A. G. (1984) *Cell (Cambridge, Mass.) 36*, 577–579.

Halliday, K. R. (1984) *J. Cyclic Nucleotide Protein Phosphorylation Res. 9*, 435–448.
Ho, Y.-K., & Fung, B. K.-K. (1984) *J. Biol. Chem. 259*, 6694–6699.
Hong, K., & Hubbell, W. L. (1973) *Biochemistry 12*, 4517–4527.
Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099.
Hurley, J. B., Fong, H.-K., Teplow, D. B., Dreyer, W. J., & Simon, M. I. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6948–6952.
Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., & Gilman, A. G. (1984b) *Science (Washington, D.C.) 226*, 860–862.
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.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lancet, D. (1986) *Annu. Rev. Neurosci. 9*, 329–355.
Leberman, R., & Egner, U. (1984) *EMBO J. 3*, 339–341.
Liebman, P. A., & Pugh, E. N., Jr. (1980) *Nature (London) 287*, 734–736.
McCormick, F., Clark, B. F., la Cour, T. F., Kjeldgaard, M., Norskov-Lauritsen, L., & Nyborg, J. (1985) *Science (Washington, D.C.) 230*, 78–82.
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.
Moller, W., & Amon, R. (1985) *FEBS Lett. 186*, 1–7.
Navon, S., & Fung, B. K.-K. (1984) *J. Biol. Chem. 259*, 6686–6693.
Reichert, J., & Hofmann, K. P. (1984) *FEBS Lett. 168*, 121–124.
Roof, D. J., Applebury, M. L., & Sternweis, P. C. (1985) *J. Biol. Chem. 260*, 16242–16249.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–58.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 1205–1209.
Sternweis, P. C., & Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806–13813.
Stryer, L. (1986) *Annu. Rev. Neurosci. 9*, 87–119.
Sugimoto, K., Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Minamino, N., Kangawa, K., Matsuo, H., Hirose, T., Inayama, S., & Numa, S. (1985) *FEBS Lett. 191*, 235–240.
Tanabe, T., Nukada, T., Nishikawa, Y., Sugimoto, K., Suzuki, H., Takahashi, H., Noda, M., Haga, T., Ichiyama, A., Kangawa, K., Minamino, N., Matsuo, H., & Numa, S. (1985) *Nature (London) 315*, 242–245.
Temeles, G. L., Gibbs, J. B., D'Alonzo, J. S., Sigal, I. S., & Scolnick, E. M. (1985) *Nature (London) 313*, 700–703.
Wierenga, R. K., & Hol, W. G. L. (1983) *Nature (London) 302*, 842–844.
Yatsunami, K., & Khorana, H. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4316–4320.
Yatsunami, K., Pandya, B. V., Oprian, D. D., & Khorana, H. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1936–1940.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 313*, 579–582.
Yee, R., & Liebman, P. A. (1978) *J. Biol. Chem. 253*, 8902–8909.

# Isolation and Characterization of a Nucleolar 2′-*O*-Methyltransferase from Ehrlich Ascites Tumor Cells†

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ABSTRACT: A 2′-*O*-methyltransferase that transfers the methyl group from *S*-adenosylmethionine to the 2′-hydroxyl group of ribose moieties of RNA has been purified from Ehrlich ascites tumor cell nucleoli. The partially purified enzyme is devoid of other RNA methylase activities and is free of ribonucleases. The enzyme has optimal activity in tris(hydroxymethyl)aminomethane buffer, pH 8.0, in the presence of 0.4 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, and 50 mM KCl, and has an apparent $K_m$ for *S*-adenosylmethionine of 0.44 μM. Gel filtration studies of this enzyme gave a Stokes radius of 43 Å. Sedimentation velocity measurements in glycerol gradients yield an $s_{20,w}$ of 8.0 S. From these values, a native molecular weight of 145 000 was calculated. The enzyme catalyzes the methylation of synthetic homoribopolymers as well as 18S and 28S rRNA; however, poly(C) is the preferred synthetic substrate, and preference for unmethylated sequences of rRNA was observed. For each RNA substrate examined, only methylation of the 2′-hydroxyl group of the ribose moieties was detected.

Nucleoli not only act as an organizational structure for rRNA genes but also contain the machinery for rRNA transcription, methylation, and processing (Perry, 1976; Attardi & Amaldi, 1970; Hadjilov & Nickolaev, 1976). Methylation of preribosomal RNA is confined to the conserved 18S and 28S sequences destined to become ribosomes (Weinberg & Penman, 1970; Maden & Salim, 1974). Although both base methylation and 2′-O-methylation occur, the extent of 2′-O-methylation is greater (Lane & Tamaoki, 1969; Brown &

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