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Purification and Characteristics of Photoreceptor Light-Activated Guanosinetriphosphatase†

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ABSTRACT: We describe a reconstitution of light-activated vertebrate photoreceptor GTPase and a purification of the GTP-binding protein (G protein), which is a component of the GTPase and modulates the light-activated phosphodiesterase (PDE) enzyme system. Rod outer segments (ROS) of bull frogs were treated with ethylenediaminetetraacetic acid (EDTA), and the GTPase and PDE fractions were solubilized (EDTA supernatant). When the EDTA supernatant and EDTA-treated membrane fraction (EDTA-washed membranes) were recombined, light-dependent GTPase activity appeared. In the reconstituted system, the K_m for GTP as substrate was 0.5 μM; the optimum pH was 7.5-8.0. The isoelectric point of GTPase in EDTA supernatant was 4.8. G protein was purified 400-fold from ROS, and the molecular weight of G protein was determined to be 40 000 by polyacrylamide gel electrophoresis. The amount of G protein in ROS was calculated as at least 1 molecule per 400 rhodopsin molecules. By recombining (in the presence or absence of GTP) purified G protein, PDE, H fraction (an additional component of GTPase), and illuminated or unilluminated EDTA-washed membranes (as a source of rhodopsin), we showed that illuminated rhodopsin, G protein, PDE, and GTP are the minimum requirements for light-dependent PDE activity. We discuss the significance of these findings in the regulation of the light-activated GTPase and PDE activities, especially with regard to the mechanism of activation.

The capacity of GTP to serve as an activator for a variety of multienzyme complexes is receiving increasing attention. Examples of this phenomenon include the hormone-activated adenylate cyclase (Londos et al., 1974), light-activated phoshpodiesterase (PDE) (Wheeler et al., 1977), the involvement of GTP in the messenger RNA translation system (Nomura et al., 1974), and the polymerization of tubulin (Roberts & Hyams, 1979). In the hormone-activated adenylate cyclase and light-activated PDE systems, a model has emerged which depicts an oscillating control mechanism that depends upon the binding and hydrolysis of GTP. In this model, the regulated enzyme (adenylate cyclase or PDE) is active when the GTP is bound to an independent activator locus. The bound GTP is hydrolyzed by a GTPase,[1] which appears to be contiguous with the activator locus (Wheeler & Bitensky, 1977; Cassel & Selinger, 1976; Wheeler et al., 1977; Shinozawa et al., 1979). The light-activated photoreceptor PDE bears a striking resemblance to the epinephrine-activated adenylate cyclase found in turkey erythrocytes. Both systems are activated by nonhydrolyzable GTP analogues such as guanylyl imidodiphosphate [Gpp(NH)p] (Wheeler & Bitensky, 1977; Schramm & Rodbell, 1975), and they exhibit similar GTP affinities, which are in the micromolar range (Wheeler & Bitensky, 1977; Cassel & Selinger, 1976). The rate constants for the activation and inactivation steps for the epinephrine-activated adenylate cyclase of turkey red cells have been recently described (Cassel et al., 1977).

The light-activated GTPase has an action spectrum which is indistinguishable from that of the light-activated PDE (λ_{max} is close to 500 nm). Maximal activation of both the GTPase and PDE is observed following the bleaching of 1 molecule in 1000 rhodopsin molecules (Wheeler & Bitensky, 1977;

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[1] Abbreviations used: GTPase, guanosinetriphosphatase; PDE, phosphodiesterase; ROS, rod outer segments; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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Wheeler et al., 1977). Several reports have appeared on the relationship between light-activated GTPase and light-activated PDE. We noted that GTP regulates PDE in an activation step and also that the hydrolysis of GTP causes the decay of activated PDE (Wheeler & Bitensky, 1977). Godchaux & Zimmerman (1979) have obtained evidence that the GTP-binding protein is related to the GTPase. Moreover, both GTP-binding activity and GTPase enzymatic activity require the presence of a rhodopsin-containing membrane fraction. Baehr et al. (1979) have described a purification of PDE from bovine ROS and noted that, in contrast to the native membrane-bound enzyme, the purified PDE is not activated by light or GTP.

We have reported that the GTPase activity in ROS can be resolved into a GTP-binding protein (G protein) and an H or helper protein (Shinozawa et al., 1980; Shinozawa & Bitensky, 1980). The G protein is necessary for PDE activation and GTP binding in the presence of illuminated rhodopsin but does not, by itself, exhibit GTPase activity. The H protein is not needed for light- or GTP-dependent PDE activation. Its presence is indispensable for the expression of GTPase activity in combination with the G protein and bleached rhodopsin (as contained within reconstituted vesicles or EDTA washed, GTPase free, disk membranes).

We describe here the reconstitution of frog photoreceptor light-activated GTPase (dependence of its activity on the presence of illuminated rhodopsin, its kinetic properties, and its activity on pH), purification of G protein and it molecular weight, the amount of G protein in ROS, and its participation in light-activated PDE.

Experimental Procedures

Materials

[γ-^{32}P]GTP, [γ-^{32}P]ATP, [^{3}H]cAMP, and Formula-963 were purchased from New England Nuclear Corp. DEAE Sephadex A-50, Blue Sepharose CL-6B, AH-Sepharose 6B, Sepharose 6B, Sephacryl S-200, crystallized ovalbumin, and standard protein for molecular weight determination by NaDodSO$_4$–polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals. Ultrogel ACA34, Ultrogel ACA44, and Ampholine were purchased from LKB Instruments Co. Reagents for gel electrophoresis (acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylenediamine, ammonium persulfate, sodium dodecyl sulfate (NaDodSO$_4$), and Coomassie brilliant blue R250) were obtained from Bio-Rad Laboratories. Infrared image converters (Model 9902E) were purchased from Varo, Inc., Garland, TX.

Methods

Preparation of EDTA-Washed Membranes, LiCl/Urea-Washed Membranes, and the EDTA Supernatant. Preparation of EDTA-washed membranes and EDTA supernatant from frog (*Rana catesbeiana*) ROS was as previously described (Shinozawa et al., 1980; Shinozawa & Bitensky, 1980).

The GTPase activity of illuminated EDTA-washed membranes, either alone or with the addition of H fraction, was minimal, but this activity increased when G protein was added. This indicated that significant amounts of H fraction are retained within the EDTA-washed membranes. For removal of the remaining G and H fractions, EDTA-washed membranes were further washed with a solution of 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM DTT which alternatively contained either of the following reagents: 2.7 M CsCl, 3.7 M RbCl, 2 M LiCl, 2 M LiCl plus 4 M urea, or 4 M LiCl. The washed membranes were further washed by 100 mM Tris-HCl (pH 7.5), 5 mM MgSO$_4$, and 1 mM DTT to remove the above cheotropic reagents.

The GTPase activities of these washed membranes were measured without further addition to detect residual G and H proteins. These membranes were also assayed for GTPase activity with the addition of a purified G protein to detect residual H protein. Finally, the purged membranes were assayed with the addition of purified G protein and H fraction to test the ability of the washed membranes to activate GTPase. It was found that treatment with 2 M LiCl and 4 M urea completely removed the G protein and virtually all of the H protein remaining in EDTA-washed membranes (these membranes are referred to as LiCl/urea-washed membranes).

Preparation of Partially Purified PDE Free from G or H Proteins. Preparation of partially purified PDE without contamination by G or H proteins was as previously described (Shinozawa et al., 1980; Shinozawa & Bitensky, 1980).

Preparation of Purified Bovine Rhodopsin Reconstituted in Phosphatidylcholine Vesicles. Purified reconstituted bovine rhodopsin in phosphatidylcholine vesicles was a gift from Dr. Wayne Hubbell and was prepared by the method of Hong & Hubbell (1972).

Assay of GTPase. GTPase activity was measured by the method of Abrams et al. (1974). The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 13 mM MgSO$_4$, 1 mM DTT, 0.5 mM EDTA, 75–220 mM KCl, 25% (v/v) glycerol, 25–33 μg of protein contained in EDTA-washed membranes, and 0.5 μM [γ-^{32}P]GTP (1.22×10^5 cpm/assay) in a final volume of 100 μL. The reaction was started by the addition of [γ-^{32}P]GTP. After 3 min at 37 °C, the reaction was stopped with a slurry of 500 μL of 10% Cl_3CCOOH containing 5 mM NaH_2PO_4 and 6 g/100 mL charcoal. This mixture was centrifuged, and 200 μL of the supernatant was mixed with Formula-963 and counted in a Beckman cpm 200. In experiments which used EDTA-washed membranes, the measured background of GTPase activity from these membranes was subtracted. GTPase assay data obtained by this method corresponded perfectly with those obtained by the method of Neufeld & Levy (1969). Under these conditions, GTPase activity was linear for more than 3 min.

PDE Assay. PDE activity was measured as described by Miki et al. (1975), with cAMP as the substrate, since its lower hydrolysis rate permits convenient measurement of PDE activity at enzyme concentrations which retain light regulation. Assays were done at 30 °C for 5 min in a volume of 40 μL. The assay mixture contained 90 mM Tris-HCl (pH 7.5), 11 mM MgSO$_4$, 1 mM DTT, 0.5 mM EDTA, 40 mM KCl, 30% glycerol, 2.5 mM [^{3}H]cAMP (25 000 cpm/assay), EDTA-washed membrane, G protein, H protein, and PDE. Assays were done in duplicate and agreed within 5%.

Native Gel Electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was done by a modification of the method of Davis (1964). We employed an acrylamide content of 3.0% in the stacking gel and 6.0% in the resolving gel. The acrylamide was supplemented with 25% glycerol. Gels were prerun overnight (2 mA per column) prior to sample application. Preruns and analytical runs were carried out at 2 °C. Protein samples were dialyzed against a basal buffer (BB) [10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 1 mM EDTA] supplemented with 50% glycerol and applied to the column with bromophenol blue. Electrophoresis was carried out at 1 mA for 3.5 h. Completed gels were sectioned longitudinally and one-half stained with Coomassie brilliant blue R250. The other half was serially sliced, and each slice was assayed for GTPase activity in the presence of

supplemental H fraction and EDTA-washed membranes. Prior to assay, the entire GTPase reaction mixture containing the gel slice, H fraction supplement, and EDTA-washed membranes was gently shaken for 5 h on ice to extract the G protein from the gel. After the addition of [γ-^{32}P]GTP, assays were carried out at 37 °C for 6 min.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with disc type apparatus. We used 3% acrylamide for the stacking gel and 8.3% acrylamide for the resolving gel and preran the gels (2 mA per column) for 3 h at room temperature. Protein samples were boiled for 8 min in a solution of 0.4% $NaDodSO_4$, 40 mM DTT, and 4.0 mM EDTA and then applied to the gels with bromophenol blue. After electrophoresis (1.5 mA per column) for 5 h at room temperature, the gels were stained with Coomassie brilliant blue R250. The molecular weight of the purified G protein was determined by running with known molecular weight standards which contained α-lactalbumin, M_w 14 400; trypsin inhibitor, M_w 20 100; carbonic anhydrase, M_w 30 000; ovalbumin, M_w 43 000; albumin, M_w 67 000; phosphorylase *b*, M_w 94 000.

Experiments Carried Out in the Absence of Visible Light. Frogs (*Rana catesbeiana*) were dark adapted overnight at room temperature and sacrificed by decapitation immediately before use in the absence of visible light. All subsequent manipulation, including dissection of retina, isolation of ROS, and enzyme assays, were carried out under infrared light image converters.

Isoelectric Focusing. Isoelectric focusing was carried out with ISCO Model 212 apparatus as described by Kawakita et al. (1978). Electrophoresis was done in a glycerol gradient (20–65%) with a pH gradient from 3.8 to 7.5. Thirty-one fractions were harvested, and the pH was immediately determined to avoid the effect of contamination by CO_2. Each fraction was neutralized by Tris base, and the GTPase activity was measured in the presence of EDTA-washed membranes.

Protein Determination. Proteins were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Results

Reconstitution of Light-Dependent GTPase Activity. EDTA-washed membranes were prepared from fully dark-adapted frogs, and their preparation was completed in the absence of light. These unilluminated EDTA-washed membranes were combined with partially purified GTPase (this was used in order to avoid the effect of contaminating rhodopsin in the EDTA supernatant), and their GTPase activities were examined. In an accompanying experiment, the membranes were completely illuminated with fluorescent light on ice, and we compared the GTPase activity in illuminated and unilluminated EDTA-washed membranes. GTPase activities were measured in total darkness. It was found that GTPase activity requires the presence of illuminated rhodopsin (Figure 1). A similar result was obtained with the use of bovine rhodopsin purified by hydroxylapatite chromatography and reconstituted into phosphatidylcholine vesicles.

When [γ-^{32}P]ATP at 0.5 μM was used as substrate, no hydrolysis of ATP by the EDTA supernatant was found. This was not altered by the addition of EDTA-washed membranes. The K_m for GTP, obtained by Lineweaver–Burk analysis in a reconstituted GTPase system from EDTA supernatant and EDTA washed membranes, was 0.5 μM.

Dependence of GTPase Activity on pH. We examined the pH dependence of the GTPase activity formed by combining

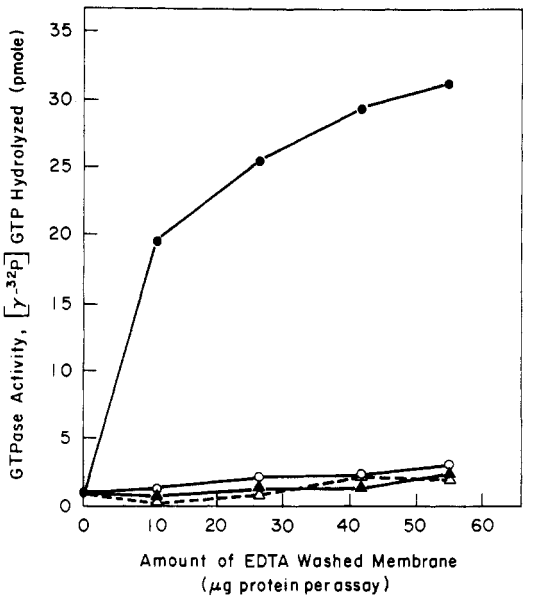

FIGURE 1: Light dependence of ROS GTPase activity. Unilluminated EDTA-washed membranes were prepared from fully dark-adapted *R. catesbeiana*. Other aliquots of unilluminated EDTA-washed membranes were illuminated with fluorescent light before use (illuminated EDTA-washed membranes). The GTPase fraction from Ultrogel ACA34 column chromatography was used. The activity of GTPase was assayed in the dark at 37 °C for 3 min. (▲) Unilluminated EDTA-washed membranes alone; (○) unilluminated EDTA-washed membranes + 10.8 μg of GTPase fraction; (△) illuminated EDTA-washed membranes alone; (●) illuminated EDTA-washed membranes + 10.8 μg of GTPase fraction.

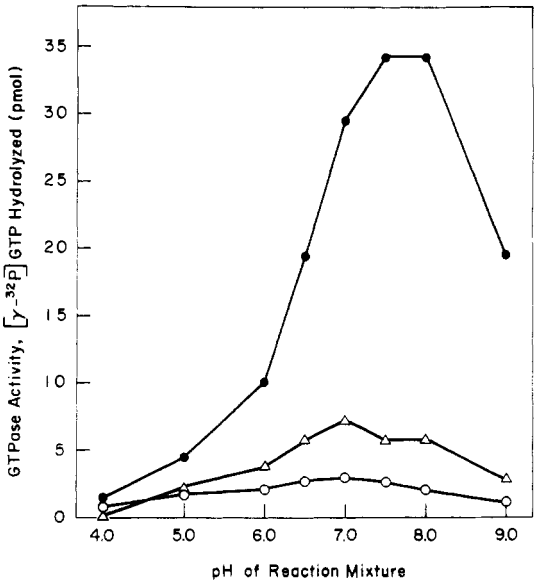

FIGURE 2: pH dependency of GTPase activity in a reconstituted system. A 33-μg sample of EDTA-washed membranes alone, 3.72 μg of EDTA supernatant alone, and both in combination were incubated at 37 °C for 3 min at the indicated pH in 100 mM Tris–acetate buffer containing 10 mM $MgSO_4$ and 1 mM DTT. (△) EDTA-washed membranes alone; (○) EDTA supernatant alone; (●) EDTA-washed membranes + EDTA supernatant.

the EDTA supernatant with EDTA-washed membranes. A pH range between 4 and 9 was examined with the use of a 100 mM Tris–acetate buffer. The optimal pH for the activity of the reconstituted GTPase was observed between 7.5 and 8.0 (Figure 2).

Determination of the Isoelectric Point of the ROS GTPase. Figure 3 shows the data from the isoelectric focusing of rod outer segment GTPase contained in EDTA supernatant. We established a pH gradient from 3.8 to 7.5 with ampholines in

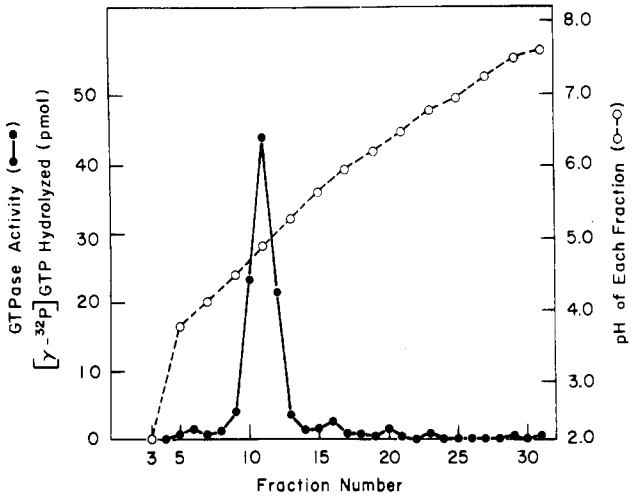

FIGURE 3: Isoelectric focusing of GTPase in EDTA supernatant. A 748-μg sample of EDTA supernatant was applied to isoelectric focusing. (●) GTPase activity; (○) pH of each fraction.

the presence of glycerol. The isoelectric point for GTPase activity measured by this method was 4.8. The assay was done in the presence of EDTA-washed membranes at pH 7.5.

Purification of Photoreceptor Rod Outer Segment GTPase. The following steps resulted in a 400-fold purification of G protein from ROS suspensions. In the first step, we released a number of peripheral proteins from the disk membrane by incubation with EDTA. The EDTA supernatant, thus obtained, contained 90% of the total GTPase activity found in outer segment suspension. Further purification steps were carried out with the use of this EDTA supernatant which was stored in lyophilized form.

The lyophilized EDTA supernatant (containing 30 mg of protein from 100 frogs) was dissolved into 20 mL of BB supplemented with 0.1 M KCl and 25% glycerol and applied to a DEAE-Sephadex column (3 × 10 cm) previously equilibrated with the same buffer. The column was washed with 200 mL of BB supplemented with 6 mM $MgSO_4$, 0.1 M KCl, and 50% (v/v) glycerol and then rewashed with 200 mL of the same buffer except that the KCl concentration was 0.15 M. After the wash with the latter buffer, linear gradient elution from 0.15 (200 mL) to 0.35 M KCl (200 mL) was done. GTPase activity was measured with the supplementation of EDTA-washed membranes. The G protein appeared at a concentration range of 0.23 M KCl, as already reported by us (Shinozawa & Bitensky, 1980). We emphasize that when the G fraction is separated from the H fraction, there is a striking loss of GTPase activity which can only be recovered by recombination of the two fractions. The recovery of GTPase activity from the DEAE-Sephadex column chromatography step (assayed with added H fraction) was 85%. All the fractions in the column chromatography were collected in plastic test tubes, and the pipetting procedures were done with plastic pipets because of the viscosity of glycerol. Since the G fraction is unstable, even at 4 °C, we stored G protein containing samples with 50% glycerol at −20 °C.

DEAE-Sephadex column chromatography is a critical step in the purification since it separates the G and H fractions. This step was repeated twice in order to remove H fraction completely. G protein fractions of two batches of DEAE-Sephadex column chromatography, described above, were joined and dialyzed against BB supplemented with 6 mM $MgSO_4$, 0.1 M KCl, and 50% glycerol and then applied to DEAE-Sephadex column (2 × 10 cm). The column was washed with 200 mL of the same buffer, and the linear gradient elution from 0.15 (120 mL) to 0.35 M KCl (120 mL) was done.

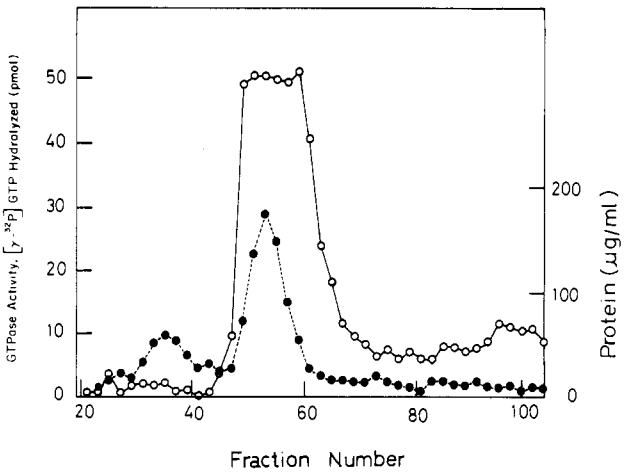

FIGURE 4: Elution profile of G protein in Ultrogel ACA44 gel filtration chromatography. G fraction obtained from the second DEAE-Sephadex column chromatography was applied to Ultrogel ACA44 column and eluted. GTPase activity (○) in 25 μL of each fraction was assayed at 37 °C for 3 min with 0.7 μg of H fraction and 33 μg of EDTA-washed membranes. (●) Protein concentration. (Note: The GTPase activity in Figures 4 and 5 shows the maximal value at 50 pmol of [γ-32P]GTP hydrolyzed per assay. This is because of the fact that the total amount of [γ-32P]GTP as substrate in assay mixture is 50 pmol.)

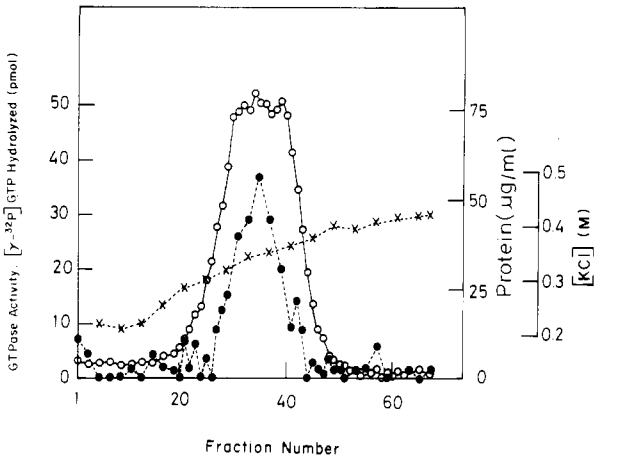

FIGURE 5: Elution profile of G protein in AH-Sepharose 6B column chromatography. G fraction obtained from Ultrogel ACA44 chromatography was applied to an AH-Sepharose 6B column. Proteins were eluted by KCl gradient elution (220–450 mM). GTPase activity (○) in 30 μL of each fraction was determined by incubation at 37 °C for 3 min with 0.5 μg of H fraction and 33 μg of EDTA-washed membranes. (●) Concentration of protein; (×) concentration of KCl.

The G protein fraction of the second DEAE-Sephadex column chromatography was concentrated by Amicon PM30 membrane filter and dialyzed against a BB supplemented with 1.5 mM EDTA, 0.15 M KCl, and 25% glycerol; 2.39 mg of dialyzed protein was applied to an Ultrogel ACA44 column (0.9 × 60 cm) and eluted by the same buffer (Figure 4). As shown in the elution profile of protein in Figure 4, contaminating high molecular weight proteins were removed by this step.

The fractions containing maximal activity in the previous step were pooled (0.68 mg of protein) and applied to an AH-Sepharose 6B column (1 × 15 cm) which was already washed with a BB supplemented with 0.2 M KCl and 50% glycerol. The column was washed with 40 mL of the same buffer, and a gradient elution from 0.22 (51 mL) to 0.45 M KCl (51 mL) was done. GTPase activity emerged at a KCl concentration range of 0.35 M (Figure 5). While the G protein could be

Table I: Purification of G Protein

step fraction	total protein (mg)	total act.[a] (units)	sp act. (units/mg of protein)	yield (%)
(1) flotation of rod outer segments	351	562	1.60	100
(2) treatment with EDTA	60.1	483	8.03	85
(3) DEAE-Sephadex chromatography	5.12	435 (122)[b]	85.0	77
(4) second DEAE-Sephadex chromatography	2.39	304 (77.4)[b]	127	54
(5) Ultrogel ACA44 chromatography	0.68	122 (43.3)[b]	179	21
(6) AH-Sepharose 6B chromatography	0.23	91.5 (12.5)[b]	398	16
(7) Blue Sepharose CL-6B chromatography	0.057	45.8 (6.23)[b,c]	805	8

[a] GTPase activity was measured at 37 °C for 3 min in the presence of EDTA-washed membranes. The assay was done by several graded dilutions of G protein, and the activity was calculated on the basis of the fact that the hydrolysis of [γ-^{32}P]GTP was linear to the incubation time and was less than 10%. After step 3, GTPase activity was also measured with the excessive amount of supplemented H fraction. One unit equals 1 nmol of [γ-^{32}P]GTP hydrolyzed per 3 min. Values are the averages of the two or more determinations that agreed within 5%. EDTA-washed membranes of the same batch were used in all of these assays. [b] Total activity calculated from the assay without H fraction supplementation. [c] GTPase activity measured using LiCl/urea-washed membranes without supplementation of H fraction was zero and that with H fraction supplementation was 40.0.

eluted from this column by 0.25 M KCl in the presence of 6 mM $MgSO_4$, the resolution of the G protein peak was more precise with a $MgSO_4$-free buffer. The separation of proteins by AH-Sepharose 6B is based on its hydrophobicity and ionic character. Therefore, the change in these characters of G protein depends on the presence or absence of Mg^{2+}.

The pooled peak fraction of GTPase activity from the preceding column step were dialyzed against a BB supplemented with 50% glycerol; 0.23 mg of the dialyzed protein was then supplemented with 3 mM $MgSO_4$ and applied to a Blue Sepharose CL-6B column (1 × 10 cm). The column was washed with 50 mL of the above buffer supplemented with 3 mM $MgSO_4$. Finally, the G protein was eluted with the above (EDTA-containing) buffer in the absence of $MgSO_4$. H protein does not bind to Blue Sepharose CL-6B even in the presence of $MgSO_4$.

A summary of the purification scheme and the degree of purification achieved with retinas from 200 frogs are shown in Table I. During the purification, the levels of GTPase activity, measured in the absence of supplementation by H fraction, decreased progressively. With the use of purified G protein and illuminated LiCl/urea-washed membranes, it was shown that the presence of G and H fractions and illuminated rhodopsin are all indispensable for the reconstitution of GTPase activity (Table I, footnote *c*) as already reported by us (Shinozawa & Bitensky, 1980). The GTPase activity was saturated when the excessive amount of H protein was added (Shinozawa & Bitensky, 1980). In this reconstituted GTPase system, there was no added PDE or detectable PDE activity. These experiments also show that PDE is not necessary for the expression of GTPase activity.

Figure 6 illustrates the Coomassie brilliant blue staining pattern (lower gel picture) and GTPase activity profile of 7.5 μg of the purified G protein in the nondenaturing polyacrylamide gel electrophoresis system. The R_f of GTPase activity was 0.48 and corresponded exactly to that of the Coomassie brilliant blue stained protein band. Electrophoresis was also done with 3.8 μg of the purified G protein, and a similar Coomassie brilliant blue staining band was obtained. The R_f of H protein in this nondenaturing polyacrylamide gel electrophoresis system was 0.28 (unpublished result), and the G protein was completely separated from H protein in this gel system. The $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified G protein is also shown in Figure 6 (upper gel picture). The Coomassie brilliant blue staining pattern in this system revealed a band of M_r 40 000. The molecular weight of G protein was also confirmed by using a GTP photoaffinity analogue, P^3-(4-azidoanilido)-5′-GTP (AAGTP) (S. Uchida et al., unpublished data). R_fs of G protein and H protein in

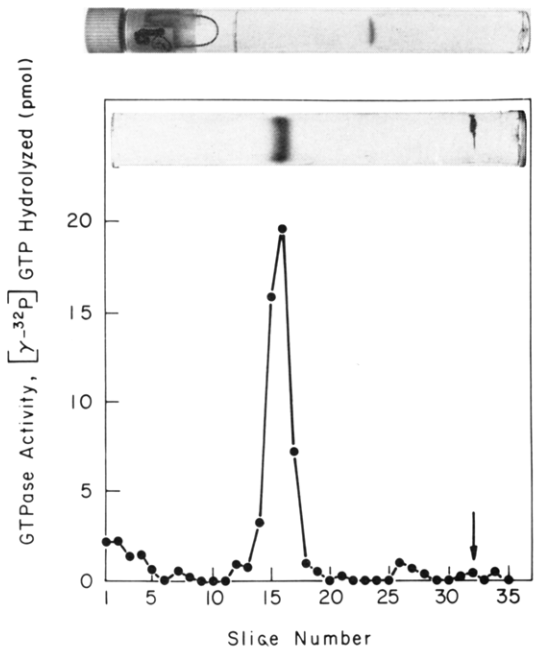

FIGURE 6: Native gel electrophoresis of G protein. A 7.5-μg sample of purified G protein was applied to native gel. After electrophoresis, the gel was sliced longitudinally. One piece of it was stained with Coomassie brilliant blue (lower gel picture) and another was further sliced horizontally. Each piece was added to a GTPase assay mixture containing 3.1 μg of H fraction and 33 μg of EDTA-washed membranes. GTPase assay was done at 37 °C for 6 min. The arrow shows the position of the dye front. (●) GTPase activity. Upper gel picture shows the Coomassie brilliant blue staining pattern of 10 μg of purified G protein in $NaDodSO_4$–polyacrylamide gel electrophoresis.

this $NaDodSO_4$–polyacrylamide gel electrophoresis were 0.52 and 0.56, respectively. G protein was separated from H protein by this $NaDodSO_4$–polyacrylamide gel electrophoresis. The purity of this G protein was estimated as almost homogeneous by these gel systems.

Kühn (1980) has reported that washing of illuminated bovine ROS with a GTP-containing buffer causes release of GTPase activity (assayed in the presence of membrane fraction) into the supernatant fraction. We have analyzed proteins released by GTP from frog ROS and detected two major species with molecular weight of 40 000 and 37 000 as measured by $NaDodSO_4$–polyacrylamide gel electrophoresis. A minor species which migrates with the dye front (bromophenol blue) was also detected. The GTP-released proteins were applied to a DEAE-Sephadex A-50 column and eluted with a continuous KCl gradient as described above. All fractions were analyzed both for GTP binding and H activity, in the

Table II: Reconstitution of PDE Activity

	PDE activity[a]			
	with GTP		without GTP	
supplemented components	light	dark	light	dark
none	2.6	0.4	0.4	0.2
G protein[b]	15.0	5.6	0.4	0.6
G protein[b] and H protein[c]	18.4	4.8	0.2	1.0
H protein[c]	5.0	3.2	0.2	1.0

[a] PDE activity was measured at 30 °C within a period of 5 min in the dark with 11 μg of illuminated or unilluminated EDTA-washed membranes, 0.6 μg of PDE partially purified by sucrose density gradient centrifugation, and the supplemented components in the presence or absence of 5 μM GTP. PDE assays were done in duplicate, and the values shown are the average of nanomoles of cAMP hydrolyzed per assay which agreed within 5%. The PDE activity was also measured in combinations of G plus PDE, H plus PDE, and G plus H plus PDE in the absence of EDTA-washed membranes where no activity was observed. [b] A total of 9.1 ng of purified G protein was added. [c] A 3.5-μg sample of H fraction of DEAE-Sephadex chromatography was added.

presence of LiCl/urea-washed membranes. In addition, the peak fractions for each activity were also analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis for molecular weight determination. The fraction with maximal GTP binding activity (which eluted at a range of 0.23 M KCl) contained a 40 000-dalton protein while the fraction with maximal H activity (which eluted at a range of 0.15 M KCl) contained a 37 000-dalton protein. The H fraction also contained a small amount of protein which migrates with the dye front.

Reconstitution of Light-Activated PDE. We attempted to identify those components of the photoreceptor system which are required for the demonstration of light-dependent PDE activity. In our experiments, we used illuminated or unilluminated EDTA-washed membranes as a source of rhodopsin. Additional disk membrane components used in this experiment included PDE partially purified by sucrose density gradient centrifugation which contained no G or H protein, H fraction not contaminated by G protein and PDE, and purified G protein. The results of these experiments are shown in Table II and indicate that (a) the presence of illuminated rhodopsin, G protein, and GTP are absolutely indispensable for the demonstration of light-activated PDE and (b) reconstitution of light-activated PDE *does not* depend on the presence of H protein. We have reported a similar experiment using purified bovine rhodopsin reconstituted into phosphatidylcholine vesicles (Shinozawa & Bitensky, 1980). The advantage of the latter material is that it is entirely free of H contamination and can be used to demonstrate that reconstitution of light-activated PDE is independent of H protein. In such an experiment, where illuminated reconstituted bovine rhodopsin was recombined (in the presence of GTP) with extensively purified G protein and partially purified PDE, we demonstrated a striking activation of PDE which was independent of H fraction in accord with previous findings.

Discussion

We have described the characteristics and the purification of light-activated GTPase and the reconstitution of light-activated PDE. ROS were treated with EDTA, and the peripheral protein fraction (EDTA supernatant) was separated from the precipitate (EDTA-washed membranes). When the EDTA supernatant was recombined with EDTA-washed membranes, light-dependent GTPase activity appeared. In the reconstitution system of light-dependent GTPase, the K_m for GTP as substrate was 0.5 μM, the optimum pH was 7.5–8.0, and the p*I* of the GTPase fraction in EDTA supernatant was 4.8.

Analysis of light-dependent GTPase in EDTA supernatant showed that illuminated rhodopsin, GTP-binding protein (G protein), and another protein component (H protein or helper protein) were minimum requirements for light-dependent GTPase activity. G protein was purified 400-fold from frog ROS as GTPase activity in the presence of H protein and illuminated EDTA-washed membranes. The molecular weight of purified G protein in NaDodSO$_4$–polyacrylamide gel electrophoresis was determined to be 40 000; this value was confirmed by the technique of photoaffinity labeling with [^{3}H]AAGTP.

Kühn (1980) reported that when illuminated bovine ROS were washed with the buffer-containing GTP, GTPase activity (with the supplementation of membrane fraction) appeared in the supernatant fraction. The M_w of the protein component in this supernatant was 37 000, 35 000, and 6000 in NaDodSO$_4$–polyacrylamide gel electrophoresis. We analyzed GTP-washed supernatant of frog ROS, obtained by the method of Kühn, which yielded a G protein with a molecular weight of 40 000 and a H protein fraction with an apparent M_w of 37 000. The H fraction also contained a small quantity of low molecular weight material which migrated with the dye front. Since the G fraction (M_w = 40 000) and the 37 000-dalton fraction gave GTPase activity when supplemented with LiCl/urea-washed membranes, we assigned the H fraction to the protein band with M_r 37 000. Fung et al. (1981) also reported the separation of GTPase of bovine ROS by the method of Kühn (1980) and showed that the GTPase consists of three polypeptides, T_α (M_w = 39 000), T_β (M_w = 36 000), and T_γ ($M_w \approx$ 10 000). Comparing the characters of GTPase of frog obtained by us with that of bovine, G protein corresponds to T_α and H protein to T_β.

The ratio of G protein to rhodopsin in ROS was determined from the M_w of G protein and rhodopsin, the specific activities of GTPase in ROS and purified G protein with the supplementation of H fraction and EDTA-washed membranes, and the amount of rhodopsin (which forms 90% of the ROS protein) in ROS. This ratio was at least 1 molecule of the G protein per 400 molecules of rhodopsins. Calculation was done on the assumption that the efficiency of coupling of GTPase in the reconstituted system is equal to that of native ROS.

PDE free of G and H proteins was obtained by the sucrose density gradient centrifugation of EDTA supernatant. In the reconstitution experiment, it was found that illuminated EDTA-washed membranes, G protein, PDE, and GTP were minimum requirements for light-dependent PDE activity. The requirement of GTP and GTP-binding protein, and not of GTP hydrolysis (by the function of H protein), for the activation of PDE corresponds to the activation of PDE by a nonhydrolyzable GTP analogue, Gpp(NH)p, reported by us (Wheeler & Bitensky, 1977) and also by a Gpp(NH)p treated bovine T_α protein, reported by Fung et al. (1981).

The physiological significance of the hydrolysis of GTP includes two possibilities. When a small amount of GTP is supplied to the light-activated PDE system, PDE activity decreases as hydrolysis progresses and GTP is exhausted. However, PDE is activated again by the further supplementation of GTP (Wheeler & Bitensky, 1977). Therefore, PDE activity is regulated by the hydrolysis of GTP by the function of H protein. The other possibility is that the hydrolysis of GTP is coupled to other systems, and the energy of the hydrolysis of GTP is used as proposed by Robinson & Hagins (1979).

Light-activated GTPase and PDE are interesting as a model of protein complex systems on membrane. The number of rhodopsin molecules in ROS is much larger than that of G protein or PDE. In the dark, rhodopsin is not bleached, and when some rhodopsin molecules capture light, G and H proteins and PDE must find those rhodopsin molecules. Light-induced structural change in the rhodopsin molecules must be detected by G and H proteins. Some questions remain unanswered, however. The first question is whether or not G and H proteins and PDE are already complexed before rhodopsin captures the light. Analysis with a cross-linking reagent, as elucidated in the case of ribosomal structure (Nomura et al., 1974), will answer this question. The next question is how the association and dissociation of these components on the disk membranes is regulated.

A number of different systems use GTP as a cofactor, including hormone-activated adenylate cyclase, light-activated PDE, messenger RNA translation, and tubulin assembly. In these systems, hydrolysis of GTP is not necessary for activation (Londos et al., 1974; Wheeler & Bitensky, 1977; Nomura et al., 1974). In each case, this has been demonstrated by the substitution of GTP analogues which are resistant to hydrolysis.

In both the light-dependent PDE system and the messenger RNA translation system, the binding of GTP or GDP is observed with the GTP-binding protein component alone. However, the hydrolysis of GTP requires the presence of other components (Nomura et al., 1974; Godchaux & Zimmerman, 1979; Shinozawa et al., 1980; Shinozawa & Bitensky, 1980). It is clear that GTP binding and hydrolysis is a widely utilized molecular device for flip-flop regulation of oscillating biochemical systems. Yet, several interesting questions remain: Are other components (such as the H protein of ROS) present in the hormone-activated adenylate cyclase system or the tubulin assembly system? Are these components (GTP-binding protein or H protein) interchangable? Furthermore, the evoluationary interrelations of these GTP-dependent systems are an interesting topic for future study.

We emphasize that in spite of the growing number of components which we have detected as participants in the light activation of disk membrane proteins, it is by no means a foregone conclusion that all the components have been now identified. Clearly more work is needed to characterize all of the components which interact to support light-dependent PDE in vertebrate rod.

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