Improved purification of transducin subunits from bovine retinal rod outer segments

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Transducin serves as a mediator between the receptor protein, rhodopsin, and the effector protein, cGMP phosphodiesterase, in the visual process. Transducin is a protein composed of three polypeptides: T_α, T_β, and T_γ, and acts as two functional units, the α-subunit and the βγ-complex. In the present study, I describe an efficient and fast method of purifying T_α and $T_{\beta\gamma}$ using chromatography on a blue agarose column connected ***in tandem*** *with an ω-amino octylagarose column. The recombination of T_α and $T_{\beta\gamma}$ reconstitutes the functional heterotrimeric holoprotein, as demonstrated by the recovery of three native properties of transducin: 1) its capacity to exchange guanine nucleotide, 2) its GTP hydrolytic activity, and 3) the ADP-ribosylation of T_α catalysed by pertussis toxin.*

Key terms*: G-proteins, GTP binding proteins, protein purification, signal transduction, transducin, visual process.*

INTRODUCTION

Guanine-nucleotide-binding proteins (G-proteins)[1] represent a group of regulatory proteins that serve as intermediaries between receptor and effector proteins in a variety of transmembrane signaling processes in eukaryotic cells (Simon *et al*, 1991; Hepler and Gilman, 1992; Birnbaumer, 1992; Neer, 1995).

Transducin (T) is the visual G-protein, which couples the light receptor protein, rhodopsin, with the cGMP phosphodiesterase (Chabre and Deterre, 1989; Hargrave and McDowell, 1992). The holoenzyme consists of three subunits (T_α, T_β, and T_γ). The molecular masses of T_α, T_β, and T_γ are approximately 40, 36, and 8 KDa, respectively. Each photolyzed rhodopsin activates hundreds of transducin molecules by a GDP/GTP exchange reaction (Fung *et al*, 1981). In the presence of GTP, holotransducin dissociates to form T_α:GTP and the complex $T_{\beta\gamma}$. The α-subunit complexed with GTP activates the cGMP phosphodiesterase, which hydrolyzes thousands of cGMP molecules to 5'-GMP. The diminution in the levels of cGMP closes cGMP-gated channels on the plasmatic membrane, with a concomitant decrease in the cationic conductance (Yau and Nakatani, 1985; Fesenko *et al*, 1985). As a consequence, there is a hyperpolarization of retinal rod cells, which produces a neuronal signal (Liebman *et al*, 1987).

Several laboratories have reported the purification of transducin subunits, but the protocols employed present some dis-

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Abbreviations: EDTA, ethylenediaminetetra-acetate; **G-proteins**, guanine-nucleotide-binding regulatory proteins; **T**, transducin; **GMP-PNP**, βγ-imido-guanosine 5'-triphosphate; **GTPγS**, guanosine 5'-[γ-thio] triphosphate; **HEPES**, N-[2-hydroxyethyl] piperazine-N'-2-ethanesulfonic acid; **HPLC**, high performance liquid chromatography; **NAD⁺**, oxidized nicotinamide-adenine dinucleotide; **ROS**, rod outer segments; **SDS**, sodium dodecyl sulphate.

advantages. Fung *et al* (1981) separated the α-subunit complexed with ßγ-imido-guanosine 5'-triphosphate (GMP-PNP), from the ßγ-unit, using high performance liquid chromatography (HPLC) on a TSK gel filtration column. Since GMP-PNP is a non-hydrolyzable analog of GTP, the GTPase activity of T_α:GMP-PNP, purified using this procedure, cannot be measured. Moreover, T_α:GMP-PNP cannot reassociate with $T_{\beta\gamma}$ to reconstitute the holoenzyme, impairing the function of the isolated T_α in the transducin visual cycle. Shichi *et al* (1984) and Yamazaki *et al* (1987) used affinity chromatography on blue sepharose CL6B for the purification of T_α and $T_{\beta\gamma}$. However, $T_{\beta\gamma}$ isolated using this method is contaminated with cGMP phosphodiesterase. Fung (1983) reported a different method of purification of transducin subunits using hydrophobic chromatography on ω-amino octylagarose. This procedure needs to be repeated several times to obtain transducin subunits purified to homogeneity.

In this work, I show a fast and efficient method to isolate the α-subunit and the ßγ-complex of transducin. This procedure combines the methods described by Shichi *et al* (1984), and by Fung (1983), and consists of a chromatography through blue agarose followed in series by an ω-amino octylagarose column. The procedure which was partially described (Bubis and Khorana, 1990), is shown in detail, and compared with the procedure described by Fung (1983), which uses exclusively iterative chromatography through ω-amino octylagarose. The transducin subunits purified using the technique described here, were able to reconstitute the functions of the heterotrimeric holoenzyme.

METHODS

Materials.

Bovine eyes were obtained from the nearest slaughterhouse (Matadero Caracas, C.A.). Retinae were extracted in the dark, under red light, and were maintained frozen at -70°C. Chemical reagents were obtained from the following suppliers: ß, γ-imido-[^{3}H] guanosine 5'-triphosphate ([^{3}H] GMP-PNP, 12.8 Ci/mmol), and [γ-^{32}P] GTP (30 Ci/mmol), Amersham; nicotinamide [^{32}P] adenine dinucleotide ([^{32}P] NAD$^+$, 800 Ci/mmol), and [^{35}S] guanosine 5'-[γ-thio]triphosphate ([^{35}S] GTPγS, 1280 Ci/mmol), Dupont-NEN Research Products; [8,5-^{3}H] GTP (15 Ci/mmol), American Radiolabeled Chemicals Inc.; ω-amino octylagarose, Sigma or ICN Biomedicals; diethylaminoethylcellulose DE 52, Whatman; blue agarose, Bethesda Research Laboratories; pertussis toxin, List Biological Laboratories, INC.; OptiPhase Hisafe II (scintillation liquid), LKB. All other reagents were of analytical grade.

Rod outer segments and washed membranes.

Rod outer segments (ROS) were isolated from frozen bovine retinae by flotation and subsequent centrifugation on discontinuous sucrose gradients, as described previously (Bubis and Khorana, 1990; Bubis *et al*, 1993). ROS membranes were washed with 2 mM EDTA (Baehr *et al*, 1979), or 5 M urea (Shichi and Somers, 1978), to remove ROS peripheral proteins. Washed ROS were used as the source of rhodopsin in GTPase and guanine nucleotide binding assays.

Transducin isolation.

Transducin was isolated from ROS membranes prepared under room light, at 4°C, following the affinity binding procedure carried out by Kühn (1980). GTP, or in some cases [^{3}H] GTP, (100 µM) was used to elute transducin from the washed illuminated ROS membranes, and transducin was further purified to homogeneity by ion-exchange chromatography on diethylaminoethylcellulose DE 52, as described elsewhere (Bubis and Khorana, 1990; Bubis *et al*, 1993). Transducin was stored at -20°C in storage buffer [20 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 100 mM NaCl, 10 mM ß-mercaptoethanol, 50% glycerol].

Separation of T_α and $T_{\beta\gamma}$ using ω-amino octylagarose

Transducin extracted from ROS with [^{3}H] GTP, or [35S] GTPγS, was chromatographed

on an ω-amino octylagarose column (Sigma, 25 ml), as described by Fung (1983). Briefly, the column was washed with 200 mM NaCl in Buffer A [5 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 5 mM ß-mercapto-ethanol], and transducin subunits were eluted with a linear gradient from 200 mM NaCl (125 ml) to 600 mM NaCl (125 ml), in the same buffer. Fractions corresponding to the overlapping region containing T_α and $T_{\beta\gamma}$, were pooled and rechromatographed on a second ω-amino octylagarose column (ICN Biomedicals, 25 ml), under the same conditions, with the exception of the linear gradient used, which was from 200 mM NaCl (125 ml) to 500 mM NaCl (125 ml). Protein fractions were stored at -20°C in storage buffer.

*Isolation of T_α and $T_{\beta\gamma}$ by chromatography **in tandem** through blue agarose followed by ω-amino octyl agarose*

GTP-extracted transducin in Buffer A, was supplemented with 100 µM EDTA and 10% glycerol (Buffer B) and chromatographed on a blue agarose column (30 ml) followed by an ω-amino octylagarose column (30 ml). After washing both columns *in tandem* with 600 ml of 100 mM NaCl in Buffer B, the columns were disconnected. The α-subunit, which was bound to the blue agarose column, was eluted with a linear gradient from 100 mM NaCl (125 ml) to 2 M NaCl (125 ml), following the procedure of Shichi *et al* (1984). The ßγ-complex was eluted from the ω-amino octylagarose with a linear gradient from 200 mM NaCl (125 ml) to 500 mM NaCl (125 ml). T_α and $T_{\beta\gamma}$ were stored at -20°C in storage buffer. Similar chromatographies were carried out using GMP-PNP- or GTP$_\gamma$S-extracted transducin. In these cases, both transducin functional units were bound exclusively to the ω-amino octylagarose.

Binding of [^{3}H] GMP-PNP to transducin and to its subunits

Guanine nucleotide binding was measured by Millipore filtration and equilibrium dialysis using [^{3}H] GMP-PNP, as described previously for cyclic nucleotide binding to cAMP-dependent protein kinase (Bubis and Taylor, 1985; 1987). The reaction mixture contained 0.2 µM transducin or $T_\alpha + T_{\beta\gamma}$, 0.1 µM rhodopsin (as urea-washed ROS membranes), and various concentrations of [^{3}H] GMP-PNP (0-1.2 µM). The binding reaction was carried out in Buffer C [10 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM magnesium acetate, 5 mM ß-mercapto-ethanol]. After an overnight incubation, at 4°C, under light, an aliquot of the reaction mixture (100 µl) was transferred to the nitrocellulose filter reservoir (Millipore HA, 0.45 µm) containing 5 ml of Buffer C. The filters were washed under vacuum with 25 ml of additional Buffer C, dried, placed in scintillation vials, and counted in 8 ml of Optiphase Hisafe II. Binding mixtures omitting transducin, or including only one transducin functional unit (T_α or $T_{\beta\gamma}$), were used in control experiments.

[^{3}H] GMP-PNP binding was also measured by equilibrium dialysis. Equilibrium dialyses were carried out in a 250-µl eight-cell chamber apparatus obtained from Hoefer Scientific. The binding reaction mixture (200 µl) containing protein and [^{3}H] GMP-PNP was placed in one side of the dialysis membrane, and 200 µl of Buffer C was placed on the opposite side. After an overnight incubation at 4°C, under illumination, a 20-µl aliquot was transferred into scintillation vials and counted in 4 ml of Optiphase Hisafe II.

Competition for [^{3}H] GTP binding to transducin by Cibacron blue

Competition experiments were carried out in Buffer C, or in Buffer C containing 2 M NaCl. Transducin (0.1 µM) was incubated with rhodopsin (0.1 µM), and [^{3}H] GTP (160 nM), in the presence or absence of Cibacron blue (0.12 mM). The reaction was initiated by the addition of urea-washed ROS membranes, as the source of rhodopsin, under light. The binding of [^{3}H] GTP was assayed after incubating for 2 h at 4°C by using the filter disk assay described above.

GTPase assay

GTP hydrolysis assays were performed as described by Franke *et al* (1992), with slight

modifications. Briefly, 0.2 μM transducin or T_α + $T_{\beta\gamma}$ were incubated with rhodopsin (0.1 μM, as EDTA-washed ROS membranes) and 20 μM [^{32}P] GTP in Buffer C. All the components except [^{32}P] GTP were mixed in the dark. After continuous illumination for 1 min, at room temperature, the reaction was started by the addition of [^{32}P] GTP. Aliquots (20 μl) were removed at 1, 2, 4, 6, 8, and 10 min, and added to 200 μl of molybdic acid solution (10.1 mM molybdic acid, 1.3 M H_2SO_4). A reducing solution (100 μl) was added, and the solution was mixed. The reducing solution contained 230 mM $Na_2S_2O_5$, 15.9 mM Na_2SO_3, and 4.2 mM 1-amino-2-naphthol-4-sulfonic acid. The mixture was then extracted by vortexing with 700 μl of isoamyl alcohol. After phase separation by centrifugation, 600 μl of the organic layer was analyzed for [^{32}P] Pi by scintillation counting in 8 ml of Optiphase Hisafe II. Assay mixtures omitting transducin, or including only one transducin functional unit (T_α or $T_{\beta\gamma}$), were used in control experiments.

ADP-ribosylation of transducin catalyzed by pertussis toxin

ADP-ribosylation of transducin or its subunits was carried out at 35°C, for 90 min, using 4.7 μg of pertussis toxin/ml in 160 μl of Buffer D [40 mM potassium phosphate (pH 7.0), 25 mM glycine, 0.4 mM ATP, 0.4 mM GTP, 15 mM thymidine, 10 mM dithiothreitol] containing 0.97 μM [^{32}P] NAD^+. Pertussis toxin was preactivated at 35°C, for 30 min, in 12.5 mM HEPES (pH 8.0) and 25 mM dithiothreitol. An aliquot (80 μl) of the incubation mixture was filtered through nitrocellulose papers (Millipore HA, 0.45 μm). The filters were dried, placed in scintillation vials, and counted in 8 ml of Optiphase HiSafe II. The rest of the labeling reaction (80 μl) was separated by electrophoresis in polyacrylamide gels with SDS. The gel was stained with Coomassie blue R-250, dried, and exposed to x-ray film (Kodak X-OMAT) for autoradiography.

Electrophoresis on polyacrylamide gels with SDS

Electrophoresis on polyacrylamide slab gels (10%, 1.5 mm thick) were carried out in the presence of SDS according to Laemmli (1970).

RESULTS

Purification of T_α and $T_{\beta\gamma}$ by ω-amino octylagarose chromatography

Fractions enriched in T_α and $T_{\beta\gamma}$ were obtained using the chromatographic procedure on ω-amino octylagarose reported by Fung (1983) (Data not shown). The use of [^{3}H] GTP during transducin isolation, identified the first peak, which corresponded to T_α, as the protein subunit containing the radioactive nucleotide. As described previously (Fung, 1983), it was necessary to pool these fractions and rechromatographed them separately on a second ω-amino octylagarose column. By repeating the procedure, fractions containing pure T_α and $T_{\beta\gamma}$ were obtained. However, the separation of both transducin units was not complete yet. Furthermore, the resolving power of the resin depended on the lot and/or supplier company. The resin from ICN Biomedicals separated the α-subunit and the βγ-complex better than the resin obtained from Sigma. This procedure yielded 1-2 mg of each transducin functional unit starting from 200 retinae, after repeating twice the separation on ω-amino octylagarose.

In agreement with Fung (1983), the use of $GTP_\gamma S$, a non-hydrolyzable analog of GTP, during the isolation of transducin, produced a better separation of T_α and $T_{\beta\gamma}$ by chromatography on ω-amino octylagarose, compared with the case in which GTP was used (Data not shown). The use of [^{35}S] $GTP_\gamma S$ also identified T_α as the subunit with the bound nucleotide. In this case, it was not necessary to repeat the chromatographic procedure.

Effect of Cibacron blue on transducin [^{3}H] GTP binding activity

As illustrated in Table I, transducin incubated with Cibacron blue showed a 47% inhibition on its [^{3}H] GTP binding capacity, under isotonic conditions. However, this inhibition was completely abolished in the presence of 2

TABLE I

Inhibition of transducin GTP binding activity by Cibacron blue [a]

Compound added	GTP binding (cpm)	Percent GTP binding [b]
None (Control)	20,402	100
Cibacron blue	10,865	53
NaCl	21,876	107
Cibacron blue and NaCl	21,145	104

a Transducin 0.1 μM incubated with rhodopsin 0.1 μM and [^{3}H]GTP 160 nM, in presence of Cibacron blue 0.12 mM and/or NaCl 2 mM, for 2 h at 4°C. Neither Cibacron blue nor NaCl contained within control sample. [^{3}H]GTP binding measured employing filter disk assay described under Methods. Values shown correspond to average radioactivity in cpm, from duplicates, retained in filters under different conditions.

b Value of GTP binding determined for control sample taken as 100 %.

M NaCl. This result suggested that a blue agarose column, which contains Cibacron blue covalently bound, could be used as an affinity matrix for the purification of T_α.

*Purification of T_α and $T_{\beta\gamma}$ by chromatography **in tandem** through blue agarose followed by ω-amino octylagarose*

Transducin, obtained by GTP elution from illuminated ROS membranes, was chromatographed on a blue agarose column connected *in tandem* with an ω-amino octylagarose column. Both columns were washed *in tandem* with 100 mM NaCl to elute any residual amount of βγ-complex bound non-specifically to the blue agarose, and the columns were disconnected. As shown in Figure 1, T_α was eluted from the blue agarose with a linear gradient from 100 mM to 2 M NaCl. On the other hand, $T_{\beta\gamma}$ was eluted from the ω-amino octylagarose column with a linear gradient from 200 to 500 mM NaCl (Fig 2). This procedure yielded 8-10 mg of each subunit starting from 200 retinae. As illustrated in both figures (lane T), purified transducin always presents small amounts of other contaminating proteins, including the cGMP phosphodiesterase (bands of apparent molecular masses ≈ 95 kDa). Following this procedure, all the contaminating proteins were absent in the fractions containing the isolated transducin units (Figs 1 and 2).

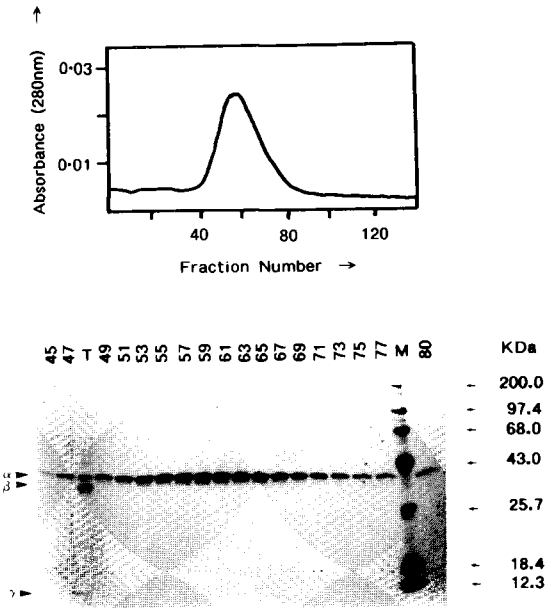

Fig 1. *Purification of T_α from blue agarose.* GTP-extracted transducin, obtained from ROS, was passed through a column of blue agarose followed by an ω-amino octylagarose column. Both columns were washed *in tandem*, and the columns were then disconnected (See Methods). Shown are the elution profile from the blue agarose column (upper panel), and the separation by SDS-polyacrylamide gel electrophoresis of the peak fraction (lower panel). T = 10 μg of DE 52-purified transducin. M = molecular weight protein markers.

When transducin was separated from illuminated ROS membranes using GMP-PNP or GTPγS, instead of GTP, T_α complexed with the non-hydrolyzable GTP

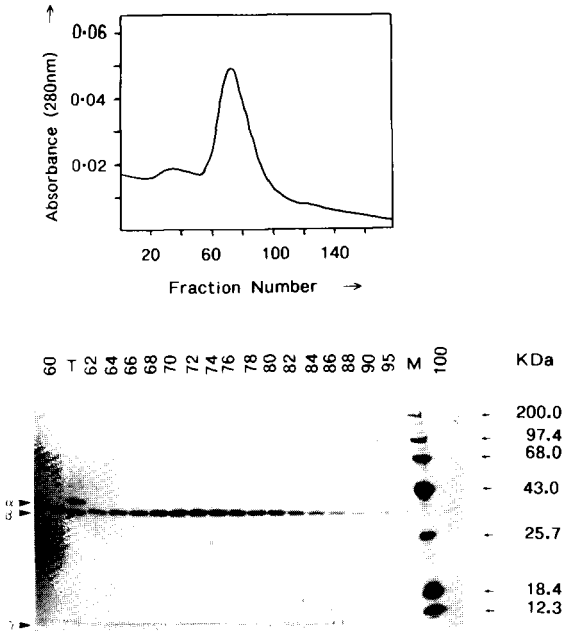

Fig 2. *Purification of $T_{\beta\gamma}$ from ω-amino octylagarose.* Transducin obtained with GTP from illuminated ROS, was chromatographed on a blue agarose column followed by an ω-amino octylagarose column. Shown are the elution profile from the ω-amino octylagarose column (upper panel), and the analysis by SDS-polyacrylamide gel electrophoresis of the peak fraction (lower panel). T = 10 ∝g of DE 52-purified transducin. M = molecular weight protein markers.

analogs did not bind to the blue agarose column. Both transducin functional units were bound to the ω-amino octylagarose column, and they were eluted as described above, according to the procedure of Fung (1983). The blue agarose column did not contain any bound protein (Data not shown).

Reconstitution of transducin function by recombining T_α and $T_{\beta\gamma}$

To prove the functionality of transducin subunits, the proteins eluted from the blue agarose (T_α) and the ω-amino octylagarose ($T_{\beta\gamma}$) connected in series, were assayed to determine their guanine nucleotide exchange reaction, GTPase activity, and ADP-ribosylation by pertussis toxin. As shown in Figures 3, 4 and 5, these activities were null or extremely low when the two functional units of transducin were separated. Addition of βγ-complex to the α-subunit, restored transducin GTPase activity, GMP-PNP binding, and ADP-ribosylation of T_α by pertussis toxin. These results confirm that both functional units of transducin are necessary for the reconstitution of the protein activities.

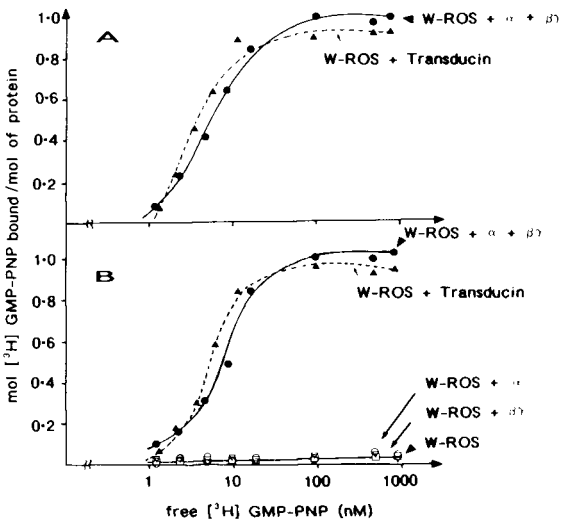

Fig 3. *Reconstitution of [³H] GMP-PNP binding activity of transducin with purified T_α and $T_{\beta\gamma}$* Transducin or its functional units (0.2 μM) were incubated with urea-washed ROS containing 0.1 μM rhodopsin, and various concentrations of [³H] GMP-PNP (0-1.2 μM) as described under Methods. Panel A: equilibrium dialysis. Panel B: Millipore filtration assay. W-ROS = washed ROS.

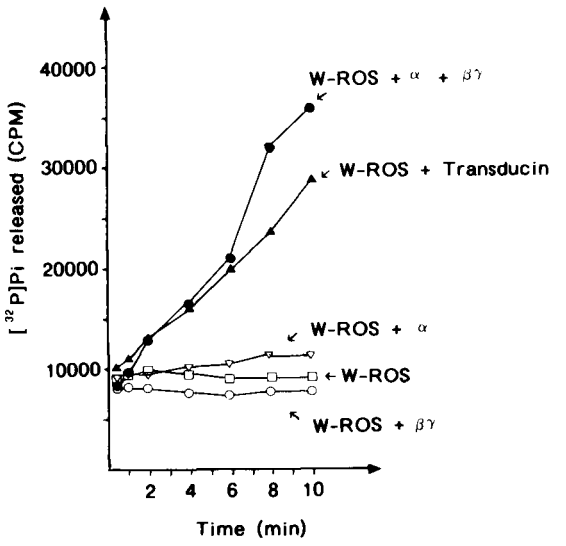

Fig 4. *Reconstitution of GTPase activity of transducin with purified T_α and $T_{\beta\gamma}$* Transducin or T_α + $T_{\beta\gamma}$ (0.2 μM) were incubated with rhodopsin (0.1 μM, as EDTA-washed ROS) and 20 μM [³²P] GTP as described under Methods. Aliquots were removed at various times (1-10 min), to determine the release of inorganic phosphate by scintillation counting. W-ROS = washed ROS.

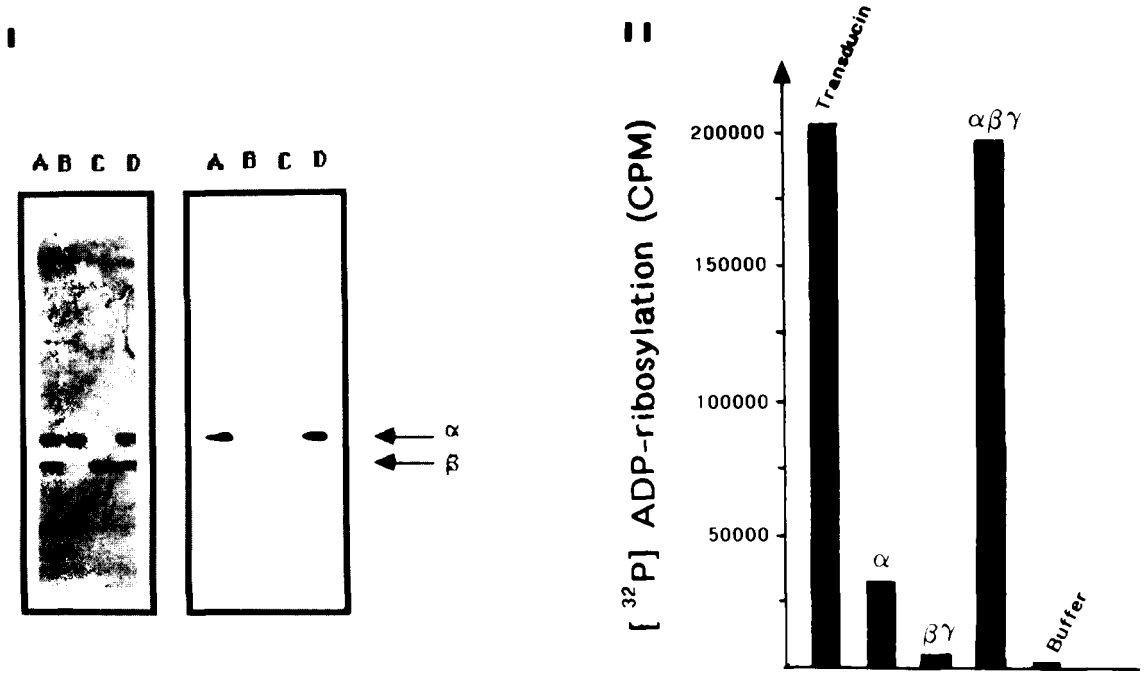

Fig 5. *ADP-ribosylation by pertussis toxin of purified T_α and $T_{\beta\gamma}$ and the reconstituted enzyme.* Panel I: SDS-polyacrylamide gel electrophoresis of the samples treated with [^{32}P] NAD$^+$ and pertussis toxin. Left, staining with Coomassie blue R-250; right, autoradiography of the same gel. A, transducin; B, T_α; C, $T_{\beta\gamma}$; D, T_α + $T_{\beta\gamma}$. Panel II: Measurement of radioactivity incorporated into the samples by the filter disk assay described under Methods.

DISCUSSION

Here, an efficient procedure to purify T_α and $T_{\beta\gamma}$, in milligram amounts is reported. Using chromatography *in tandem* through blue agarose followed by ω-amino octylagarose, a high improvement in the yield of both transducin functional units was obtained, as compared to the procedure that uses exclusively hydrophobic chromatography. This method yielded 8-10 mg of each, T_α and $T_{\beta\gamma}$, starting from 200 retinae, instead of only 1-2 mg of each, obtained when iterative chromatography through ω-amino octylagarose was employed.

The purification procedure for T_α and $T_{\beta\gamma}$, described in this article, is simple, fast, and renders both transducin functional units, T_α and $T_{\beta\gamma}$, purified to complete homogeneity. No contamination with other ROS proteins (for example, the cGMP phosphodiesterase) was observed in the fractions containing the purified subunits. Furthermore, this method does not present the disadvantages of the other methods described in the literature (Fung *et al*, 1981; Fung, 1983; Shichi *et al*, 1984; Yamakazi *et al*, 1987; see Introduction).

When transducin was isolated from illuminated ROS membranes using $GTP_\gamma S$, it was observed that T_α:$GTP_\gamma S$ did not bind to the blue agarose column. The α-subunit complexed with $GTP_\gamma S$, remained in the non-adherent fractions of the column, and was trapped by the ω-amino octylagarose column placed *in tandem*. Blue agarose only worked as an affinity adsorbent matrix for the α-subunit of transducin when T_α was complexed with an exchangeable nucleotide, GTP or GDP. Cibacron blue, which is the dye covalently ligated to the resin, must replace the guanine nucleotide bound to T_α. This result implies that T_α eluted from the blue agarose column with NaCl must be free of bound nucleotide.

Recently, the three dimensional structure of a 325-amino acid fragment of T_α bound to $GTP_\gamma S$ (Noel *et al*, 1993) and to GDP (Lambright *et al*, 1994) has been solved. These two T_α crystal structures furnish contrasting freeze-frame pictures of two key intermediates in the transducin cycle (Bour-

ne, 1993; 1994). The main differences between both structures (only 14% of the amino acids, corresponding to the switch regions I, II, and III) result from changes in a small surface area located on one face of T_α (Lambright *et al*, 1994). However, the structure of the empty state of T_α (without nucleotide), the conformation stabilized by activated rhodopsin and $T_{\beta\gamma}$, that serves as a transition state in the GTP/GDP exchange reaction is still not resolved. T_α purified with the blue agarose column, appears to be free of nucleotide, as discussed above, and could be used by interested crystallographers to get this structure.

Purified T_α and $T_{\beta\gamma}$, using the improved procedure described here, retained light-dependent GMP-PNP binding and GTPase activities, when recombined with membranes containing rhodopsin. Addition of $T_{\beta\gamma}$ to T_α also restored transducin's capacity to be ADP-ribosylated by pertussis toxin on its α-subunit. The purity of the α-subunit and of the βγ-complex was verified since no light-dependent guanine nucleotide binding and GTP hydrolysis activities could be measured, when the individual transducin units were incubated with rhodopsin. Furthermore, the α-subunit can not act as a substrate for pertussis toxin, unless the heterotrimeric transducin is reconstituted by recombining T_α and $T_{\beta\gamma}$.

The availability of purified T_α and $T_{\beta\gamma}$ will allow detailed analyses of the function and biochemical properties of each individual transducin unit in phototransduction. Furthermore, the purified T_α and $T_{\beta\gamma}$ will also be used to continue our studies (Bubis and Khorana, 1990; Bubis *et al*, 1994, 1995) on the structural organization of transducin.

ACKNOWLEDGEMENTS

This research was supported by grants from CONICIT (N° S1-2171) and Decanato de Investigación y Desarrollo, Universidad Simón Bolívar (N° S1-CB-241). I thank Rafael Rangel-Aldao and his laboratory for supplying reagents and equipment throughout the course of this work, and Ramón Sánchez for helping with the illustrations.

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