Purification and characterization of transducin from capybara Hydrochoerus hydrochaeris

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Received 4 June 2007; received in revised form 31 July 2007; accepted 1 August 2007
Available online 14 August 2007

Abstract

Polypeptides of ~39, 36 and ≤14.4 kDa remained tightly bound to illuminated Hydrochoerus hydrochaeris retinal rod outer segment (ROS) membranes following extensive isotonic and hypotonic washes, and were specifically released in the presence of GTP. These results identified them as the α-, β- and γ-subunits of transducin (T). Once purified to homogeneity by anion-exchange chromatography, capybara T showed light-dependent β,γ-imido-guanosine 5'-triphosphate (GMPPNP) binding and GTPase activities in the presence of bovine rhodopsin, was recognized by anti-bovine T polyclonal antibodies, and was ADP-ribosylated by pertussis toxin. Capybara T bound GMPPNP with an apparent Kd of 18 nM, βγ-sulfate; SDS; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS; Kd, dissociation constant; R, rhodopsin; R*γ, photoactivated rhodopsin; metarhodopsin II; ROS, rod outer segments; SDS, sodium dodecyl sulfate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS; T, transducin; T*α, activated transducin.

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Keywords: Capybaras; G-protein-coupled signaling; Hydrochoerus hydrochaeris; Rhodopsin; Rodent; Transducin; Visual process

1. Introduction

Transducin (T) mediates vision in retinal rods by transmitting light signals detected by the receptor molecule rhodopsin (R) to a latent type VI cGMP phosphodiesterase (PDE6). Here, signal transduction begins with the absorption of a photon by the 11-cis-retinal chromophore of R. Rapid photoisomerization to all-trans retinal triggers a series of structural changes that lead to the formation of photoexcited rhodopsin (R*), which corresponds to the metarhodopsin II photointermediate. R* binds to the heterotrimeric GDP-bound form of T and catalyzes the exchange of GTP for GDP, resulting in the dissociation of the holoenzyme into the α-subunit complexed with GTP and the βγ dimer. In turn, the α-subunit:GTP complex or activated T (T*α) binds and stimulates the potent PDE6. The resulting decrease in the cytosolic concentration of the second messenger cGMP causes the closure of cation-specific cGMP-gated channels located in the plasma membrane, leading to the hyperpolarization of the rod cell.

Abbreviations: PDE6, retinal rod type VI cGMP phosphodiesterase; GMPPNP, β,γ-imido-guanosine 5'-triphosphate; GTPγS, guanosine 5'-O-(3-thiotriphosphate); Kd, dissociation constant; R, rhodopsin; R*, photoactivated rhodopsin or metarhodopsin II; ROS, rod outer segments; SDS, sodium dodecyl sulfate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS; T, transducin; T*α, activated transducin.

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doi:10.1016/j.cbpb.2007.08.001
5 G-protein β-subunit and membrane anchor R9AP (RGS9- GβS-R9AP) (He et al., 1998; Makino et al., 1999; Hu and Wensel, 2002). The resulting multiprotein complex, T*α-PDE6γ- RGS9-GβS-R9AP, rapidly hydrolyzes GTP to GDP, and consequently both, activated PDE6 and T*α, are simultaneously inactivated. Then, the α-subunit bound to GDP recombines with the βγ dimer and is ready for another round of activation. Additional molecules are also involved in modulating the duration of the signal and the achievement of the appropriate response (Pugh et al., 1999). The phosphorylation of R* by rhodopsin kinase, and its interaction with arrestin-1, contributes to signal desensitization. Phosducin and recoverin also participate in the regulation of the signal at different levels. 

*Hydrochoerus hydrochaeris*, commonly known as capybaras or “chigüires”, are caviomorph rodents widespread in the Neotropics, ranging from Central America to the Buenos Aires Province in Argentina (Ojasti, 1973). The capybara is the world’s largest living rodent with 50–70 Kg of weight, 1–1.3 m of length and about 50 cm of height. This wild animal is abundant on cattle ranches in the Venezuelan seasonally flooded savannas, where it is commercially exploited under a program against other animals sharing the same territory and are easily domesticated. Offal is almost certainly capybaras most significant sense, whereas vision and audition are probably sensitive only at low-distances. Yet, capybaras vision must be very important in open habitats. Here, we report for the first time the purification of T from capybara retinal rod outer segments. Similar to other vertebrates, capybara T is a protein composed of three polypeptides, the α-, β- and γ-subunits, with analogous properties to other transducins described in the literature.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following sources: β, γ-imido-[1H] guanosine 5’-triphosphate ([8-3H] GMPPPNP, 12.8 Ci/mmol), Amersham; [35S] guanosine 5’-γ-thio] triphosphate ([35S] GTPγS, 1280 Ci/mmol), and nicotinamide [32P] adenosine dinucleotide ([32P] NAD+, 800 Ci/mmol), Dupont-NEN Research Products; anti-mouse IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium, Promega; β,γ-imido-guanosine 5’-triphosphate (GMPPNP), and guanosine 5’-O-(3-thiotriphosphate) (GTPγS), Boehringer Mannheim; diethylaminoethylcellulose DE 52, Whatman; α-amino octylagarose column, ICN Biomedicals; pertussis toxin, List Biological Laboratories, INC.; OptiPhase Hisafe II (scintillation liquid), LKB. All other chemical compounds were analytical grade. Capybara eyes were obtained from animals collected in March 1992 (dry season), during the annual harvest carried out at Hato El Cedral (Apure State, Venezuela; 7° 25’ N, 69° 20’ W) under authorization of the Wildlife Service of the Venezuelan Ministry of Environment (Salas and Herrera, 2004). Bovine eyes were obtained from a local abattoir (Matadero Caracas, C.A.). Capybara and bovine retinas were extracted in the dark, under red light, and were maintained frozen at −70 °C.

2.2. Preparation of bovine rod outer segments and washed membranes

Rod outer segments (ROS) were isolated from frozen bovine retinas by flotation and subsequent centrifugation on discontinuous sucrose gradients (Bubis, 1998). Urea-washed ROS membranes were prepared accordingly with Shichi and Somers (1978). ROS membranes and urea-washed ROS membranes were stored in the dark, at −70 °C. Rhodopsin concentration was calculated from its UV/visible absorption spectra, using the molar extinction coefficient of the protein (Wald and Brown, 1953).

2.3. Purification of retinal T from capybaras

ROS were prepared under room light, at 4 °C, starting from 200 capybara retinas, following the procedure described by Bubis (1998) for bovine ROS. Then, T was isolated according to the affinity binding methodology carried out by Kühn (1980). Briefly, the purified illuminated ROS membranes were extracted with isotonic buffer [100 mM potassium phosphate (pH 6.8), 1 mM magnesium acetate, 5 mM β-mercaptoethanol, 0.1 mM PMSF] by manually resuspending the membranes in a glass homogenizer followed by three passes through a N° 21 needle. The washed ROS membranes were recovered by centrifugation at 50,000 g, for 30 min. This procedure was repeated three consecutive times to obtain the isotonically depleted-ROS membranes, which were then subjected to four successive extractions with a hypotonic buffer [5 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 5 mM β-mercaptoethanol, 0.1 mM PMSF]. Following centrifugation at 50,000 ×g, for 30 min, the sediments containing the hypotonically stripped-ROS were further subjected to three consecutive washes with 100 μM GTP in hypotonic buffer in order to release T from the washed and photoexcited membranes (Bubis, 1995). Finally, T was further purified to homogeneity by anion-exchange chromatography on diethylaminoethylcellulose DE 52 (Bubis, 1995). Capybara T was stored at −20 °C in storage buffer [20 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 100 mM NaCl, 10 mM β-mercaptoethanol, 50% glycerol].

2.4. Separation of the α-subunit and the βγ dimer of capybara T using α-amino octylagarose

Purified capybara T was incubated with illuminated urea-washed bovine ROS and further extracted with [35S] GTPγS. GTPγS-disalbed T was then chromatographed on an α-amino octylagarose column (25 ml), as described by Fung (1983). The column was washed with 200 mM NaCl in hypotonic buffer, and T subunits were eluted with a linear gradient from 200 mM NaCl (125 ml) to 500 mM NaCl (125 ml), in the same buffer. The use
of $[^{35}S]$ GTPγS facilitated the identification of the α-subunit as the polypeptide containing the bound nucleotide.

2.5. Guanine nucleotide binding assays

The guanine nucleotide binding activity of capybara T was measured by Millipore filtration using various concentrations of the nonhydrolyzable analog of GTP, $[^{3}H]$ GMPPNP (0–0.3 μM). The binding reaction was carried out as previously described (Bubis et al., 1993; Ortiz and Bubis, 2001) in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM magnesium acetate, and 5 mM β-mercaptoethanol and contained 60 nM of capybara T and an excess of bovine R (0.2 μM, as urea-washed bovine ROS). Blanks were prepared in the absence of T. Blank values ranged between 3 and 15% of the total $[^{3}H]$ GMPPNP binding and were subtracted in all assays performed.

2.6. GTPase assay

Capybara T GTP hydrolysis was determined by using a coupled enzymatic spectrophotometric assay similar to the one employed by Roskoski (1983) to measure cAMP-dependent protein kinase activity. With this technique, the formation of GDP in the GTPase reaction of T and the exchange of GTP for GDP catalyzed by bovine R* (Eq. (1)) is coupled to the pyruvate kinase (PK) reaction to produce pyruvate (Eq. (2)), which is, in turn, coupled to the lactate dehydrogenase (LDH) reaction with the concomitant oxidation of NADH to NAD+ (Eq. (3)). The decrease in absorbance at 340 nm was used to determine the reaction rate using a molar extinction coefficient of 6220 M$^{-1}$ cm$^{-1}$. The optimum concentrations of LDH and PK were determined according to Cleland (1979) and validated by varying the concentration of T. The sequence of reactions was:

\[
\text{GTP} \rightarrow \text{GDP} \quad (1)
\]

\[
\text{GDP + phosphoenolpyruvate} \rightarrow \text{GTP + pyruvate} \quad (2)
\]

\[
\text{pyruvate + NADH} \rightarrow \text{lactate + NAD} \quad (3)
\]

In a final volume of 1 ml, the reaction mixtures contained 2.5 μM of capybara T, 0.6 μM of bovine R (as urea-washed bovine ROS), 20 units of PK, 15 units of LDH, 100 mM MOPS (pH 7.0), 100 mM KCl, 2 mM phosphoenolpyruvate, 10 mM MgCl$_2$, 0.5 mM GTP and 200 μM NADH. Usually the reaction was initiated by adding GTP and the absorbance was monitored at 340 nm, for 3 h, at room temperature, in a Beckman DU-70 spectrophotometer. An initial fall in absorbance, which was instantaneous and independent of the presence of T, was attributed to a minimal amount of GDP contaminating the GTP stock. As controls, GTPase activity was assayed in the absence of R, or in the presence of R* and 1 mM GMPPNP instead of GTP. In this last control, the reaction was initiated by adding GMPPNP and phosphoenolpyruvate together in order to minimize the decrease of absorbance at 340 nm due to the release of the T endogenous GDP. This assay has been standardized using bovine T (data not shown).

2.7. Thin-layer chromatography

Free guanine nucleotides, present in the fractions from the DE 52 column, were analyzed on 1-mm thick thin-layer plates of polyethyleneimine (PEI)-cellulose (Polgram cel 300 PEI, Macherey-Nagel C.O.). PEI–cellulose plates were previously run in 2 N formic acid containing 0.5 M LiCl, which was used as the mobile phase, and dried at 60 °C for 12 h. Fractions (~3 ml) were lyophilized to dryness and resuspended in 100 μl of water. Aliquots of 1 μl of each fraction were applied to PEI-cellulose thin-layer plates until a total of 5 μl was loaded. Samples of GDP (20 mM, 5 μl) and GTP (20 mM, 5 μl) were also applied as standards. The plates were dried by air ventilation following each application. Once all samples were applied, the separation was carried out using the same solvent mentioned above. Plates were revealed using 0.002% fluorescein in methanol and photographed under UV light (254 nm) employing a red filter (Kodak gelatin filter frame).

2.8. Other procedures

Protein concentration was measured according to Bradford (1976), using bovine serum albumin as protein standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 1.5-mm thick slab gels containing 10% polyacrylamide (Laemmli, 1970). Coomassie blue R-250 was used for protein staining. For Western blot analyses, the proteins were electrotransferred from the gels to nitrocellulose filters (Towbin et al., 1979). For immunodetection, the filters were incubated with polyclonal antibodies.
against bovine T (dilution 1:1000), prepared in mice ascitic fluids (Bubis et al., 1993). These antibodies recognize the α-subunit very specifically, and also cross-react with rhodopsin. The antigenically reacting polypeptides were then treated with alkaline phosphatase-conjugated secondary antibodies against mouse IgG, at a dilution of 1:5000. Finally, the bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates. ADP-ribosylation of capybara T catalyzed by pertussis toxin was carried out as described in Bubis (1995).

3. Results

As shown in Fig. 1, three polypeptides with molecular masses of about 39, 36 and ≤14.4 kDa remained tightly bound to the illuminated capybara ROS membranes following three isotonic (S1–S3) and four hypotonic washes (S4–S7). Interestingly, the ∼39, 36 and ≤14.4 kDa polypeptides were specifically released following three consecutive washes with 100 μM GTP in hypotonic buffer (S8–S10). In view of that, the higher molecular weight polypeptide bands must correspond to the α- and β-subunits of capybara T, respectively. Since the formation of a tight complex between the β- and γ-subunits of T is already known, the polypeptide running with an apparent molecular mass of ≤14.4 kDa must contain the γ-subunit of capybara T. The major polypeptide band with an apparent molecular mass of approximately 35 kDa, which is observed in the original ROS sample and remained in the pellets following all washes, must be attributed to capybara R (Fig. 1).

All GTP washes were then pooled and applied to a DE 52 column (Fig. 2), which removed the excess of non-bound nucleotides and purified capybara T to homogeneity. Proteins and other components eluting from the column were monitored with an in-line spectrophotometer that measured the absorbance at 280 nm. As illustrated in Fig. 2a, there were two major absorbing peaks that appeared following a low ionic strength elution with hypotonic buffer supplemented with 100 mM NaCl. As demonstrated by thin-layer chromatography on PEI-cellulose, the first peak corresponded to GDP and the second peak to GTP (Fig. 2b). In addition, a unique absorbing peak appeared after a high ionic strength elution of the column with hypotonic buffer supplemented with 500 mM NaCl (Fig. 2a). The protein content of this peak was determined at 595 nm according to Bradford (1976) (Fig. 2a, inset, top), and the R- and light-dependent [3H] GMPPNP binding activity of the eluting fractions was also measured (Fig. 2a, inset, bottom). Analysis by SDS-PAGE revealed that the same fractions comprising the GMPPNP binding activity also contained the ~39, 36 and ≤14.4 kDa polypeptides that were previously identified as the α-, β- and γ-subunits of capybara T, and proved the purity of

![Fig. 2. Purification of capybara T by DE 52 column chromatography. (a). Supernatants S8–S10 from Fig. 1 were pooled and applied to a DE 52 anion-exchanger. The elution profile was monitored at 280 nm. Two peaks emerged after an initial wash with buffer containing 100 mM NaCl. Then, a unique absorbing peak appeared following elution with 500 mM NaCl (arrow). Fractions were analyzed for protein content (Inset, top), and for GMPPNP binding activity in the absence (Δ) or presence (▲) of light-activated R (Inset, bottom). (b) Analysis by thin-layer chromatography on PEI-cellulose of the fractions eluting with 100 mM NaCl. Arrows show the migration of GDP and GTP. (c) Analysis by SDS–PAGE of the fractions eluting with 500 mM NaCl. Tα, Tβ and Tγ = α-, β- and γ-subunits of T.](https://example.com/fig2.png)
the protein (Fig. 2c). In addition, anti-bovine T polyclonal antibodies that preferentially detect the $\alpha$-subunit of bovine T (Bubis et al., 2001) also favored the recognition of the $\alpha$-subunit in the fractions containing this protein peak (Fig. 3a). T $\beta$-subunit was only slightly recognized by these antibodies (Fig. 3a). Moreover, the purified protein was ADP-ribosylated by pertussis toxin on its $\alpha$-subunit (Fig. 3b). All these results demonstrated that this peak represented the purified T from capybaras.

A fraction of the purified capybara T was incubated with urea-washed bovine ROS and further extracted with $[^{35}S]$ GTP$\gamma$S. GTP$\gamma$S-released capybara T was then chromatographed on an $\omega$-amino octylagarose column (Fung, 1983). As shown in Fig. 4, the $\alpha$-subunit of T bound to $[^{35}S]$ GTP$\gamma$S was clearly separated from the $\beta\gamma$ complex of T. Thus, capybara T also acts as two functional units, the $\alpha$-subunit and the $\beta\gamma$ dimer.

To characterize the purified capybara T, the protein was assayed to determine its guanine nucleotide exchange reaction (Fig. 5) and intrinsic GTPase activity (Fig. 6). As expected, capybaras T did not bind GMPPNP in the absence of $R^\ast$ (data not shown).
not shown). However, as illustrated in Fig. 5a, the purified protein bound GMPPNP in the presence of light-activated bovine R. The apparent Kd for GMPPNP was found to be $1.8 \times 10^{-8}$ M, and the Scatchard plot of the data revealed positive cooperativity for R* binding (Fig. 5b). A Hill coefficient of 1.4 was determined by the corresponding Hill plot (data not shown). Additionally, a coupled enzymatic spectrophotometric assay was utilized to determine the GTP hydrolytic capacity of the purified capybara T in the absence of GAP proteins. As shown in Fig. 6, the oxidation of NADH measured by the loss of absorbance at 340 nm was proportional to the GTPase activity of the purified protein (Fig. 6c). Capybara T did not exhibit GTP hydrolytic activity in the absence of GMPPNP instead of GTP (Fig. 6b). Moreover, no activity was observed when the reaction was performed in the presence of GMPPNP and the Scatchard plot of the data revealed positive cooperativity for the binding of GMPPNP in the presence of light-activated bovine R. An allosteric behavior in the R-catalyzed activation of T has been recently reported for the protein isolated from bovine retinas (Wessling-Resnick and Johnson, 1987a, 1987b; Willardson et al., 1993), which was predicted to result from oligomeric association of T (Wessling-Resnick and Johnson, 1987b). The trapping of oligomers by the formation of disulfide bonds (Wessling-Resnick and Johnson, 1989) or cross-linking bridges induced with bifunctional maleimides (Millán and Bubis, 2002) has provided physical evidence for specific intermolecular interactions between the α-subunits of T.

To determine the GTPase activity of capybara T, we have standardized a coupled enzymatic spectrophotometric assay comparable to a technique routinely used for the measurement of protein kinase activities (Roskoski, 1983). This assay is valuable in enzyme kinetic studies because it is continuous. Additionally, it is colorimetric and not radioisotopic, and consequently, does not require the use of radiolabeled GTP that is expensive, has a very short lifetime, and needs a special handling due to its toxicity. This assay requires the use of relatively high concentrations of the enzyme, since the GTP-hydrolytic activity of heterotrimeric G-proteins is too slow when performed in the absence of GAP proteins. However, given that the GTPase activity of bovine T* α is highly stimulated when T* α is bound to PDE6γ and to the complex RGS9-γ5-R9AP (He et al., 1998; Makino et al., 1999; Hu and Wensel, 2002), better results will probably be attained with this assay following the addition of these GAP proteins to the purified capybara T.

ADP-ribosylation catalyzed by different toxins from bacterial origin has been used as markers for the various heterotrimeric G-protein subtypes. In particular, the α-subunit members of the Gi/Go subgroup, which include the α-subunit of T, serve as substrates for pertussis toxin, the exotoxin of Bordetella pertussis. Pertussis toxin transfers the ADP-ribosyl moiety of NAD$^+$ to the α-subunit at a cysteine situated just four residues from its C-terminal. ADP-ribosylation by pertussis toxin has the effect of interrupting the communication between the receptor and the G-protein, freezing it in its GDP-bound state. Similar to other transducins, capybara T was also ADP-ribosylated by pertussis toxin on its α-subunit indicating the presence of the characteristic cysteine near its C-terminal end.

When the primary structures of T α-, β- and γ-subunits were compared across vertebrate species using the TBLASTN 2.2.17 program (http://www.ncbi.nlm.nih.gov/Entrez/), they show conservation approaching complete identity. For the α-subunit, 98–100% amino acid identity was seen among terrestrial mammals (bovine, equine, mouse, rat, dog, monkey, opossum, and man). There was some sequence divergence when the bovine T α-subunit, which was used as the query, was compared to T α-subunits from vertebrates located at a lower evolutionary level: birds (96%), frogs (92–94%), the semi-aquatic mammal duck-billed platypus (87%), salamanders and lizards (80–92%), and fish (77–93%). A much higher percent amino acid identity was observed among all members of the vertebrate T β-subunit subfamily (96–100%). The T β-subunits from cow, monkey, mouse, rat, man, dog, frog, horse, and opossum share a 99–100%
homology. Other vertebrates (hamster, birds, platypus, salaman-
der, lizard, and fish) have a slightly lower homology (96–98%) when compared to the bovine T β-subunit sequence. In contrast, there was a greater sequence divergence between members of the vertebrate T γ-subunits (75–98%) than for the members of the T α- and β-subunits. None of the reported T γ-subunit sequences had a 100% amino acid identity with the corresponding bovine homologue. A 93–98% amino acid identity was seen when the sequences for the T γ-subunit of equine, mouse, rat, dog, monkey, and man were compared to the bovine T γ-subunit. Other vertebrates (platypus, frog, bird, and fish) only shared 75–87% amino acid identity. However, this is also considered a very high level of conservation. This similarity of primary structure over extended periods of evolutionary history demonstrates the importance of the particular sequences of the T subunits for their specific role in the visual process, and suggests that the few amino acid sequence differences observed among the various vertebrate subunits probably are of functional significance. Since this caviomorph rodent, the capybara, has a conventional T similar to those of other mammal species, its subunits probably share a high level of amino acid sequence homology with the sequences reported for other organisms. It would be extremely valuable to determine the primary structures of the three subunits of capybara T, in order to compare them with those of other species that are available in the literature.

Acknowledgements

This work was supported by a grant from FONACIT, N° S1-2000000514, Caracas, Venezuela. We would like to thank Dr. Emilio A. Herrera and members of his laboratory for their assistance during the field trip to collect capybara eyes, and the owners of Hato El Cedral for permission to work in their property.

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