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A Purified Agonist-Activated G-Protein Coupled Receptor: Truncated Octopus Acid Metarhodopsin

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ABSTRACT—G-protein coupled receptors (GPCRs) mediate responses to many types of extracellular signals. So far, bovine rhodopsin, the inactive form of a GPCR, is the only member of the family whose three dimensional structure has been determined. It would be desirable to determine the structure of the active form of a GPCR. In this paper, we report the large scale preparation of a stable, homogenous species, truncated octopus rhodopsin (t-rhodopsin) in which proteolysis has removed the proline-rich C-terminal; this species retains the spectral properties and the ability for light-induced G-protein activation of unproteolyzed octopus rhodopsin. Moreover, starting from this species we can prepare a pure, active form of pigment, octopus t-Acid Metarhodopsin which has an all-*trans*-retinal as its agonist. Photoisomerization of t-Acid Metarhodopsin leads back to the inactive form, t-rhodopsin with the inverse agonist 11-*cis*-retinal. Octopus t-Acid Metarhodopsin can activate an endogenous octopus G-protein in the dark and this activity is reduced by irradiation with orange light which photoregenerates t-Acid Metarhodopsin back to the initial species, t-rhodopsin.

Key words: G-protein coupled receptor, octopus rhodopsin, metarhodopsin, G-protein, agonist

INTRODUCTION

G-protein coupled receptors (GPCRs) form a large superfamily of seven transmembrane helix proteins that mediate responses to hormones, neurotransmitters, and in the case of rhodopsin, photons. It has been proposed that most GPCRs exist in two functionally distinct states in equilibrium, inactive (R) and active (R*) (Samama *et al*., 1993; Gether and Kobilka, 1998; Seifert and Wenzel-Seifert, 2002). Agonists maximally stabilize the R* state of GPCRs and shift the equilibrium far towards R*. In contrast, inverse agonists maximally stabilize the R state and inhibit G-protein activation. GPCRs fully occupied with agonists or inverse agonists are rare. Rhodopsin is one of the exceptions because it has an inverse agonist, 11-*cis*-retinal, which is covalently bound to the receptor within its binding pocket (Sakmar, 1998) and photoisomerization of the chromophore leads to the all-*trans* isomer which acts as an agonist.

The determination of the high-resolution structure of bovine rhodopsin opened an avenue to a deeper understanding of GPCR activation and transmembrane signaling (Palczewski *et al*., 2000). But bovine rhodopsin is the inactive form of the GPCR, and it would be desirable to determine the structure of its 'active form'. Although the inverse agonist 11-*cis*-retinal with bovine opsin forms inactive rhodopsin, the agonist all-*trans*-retinal does not lead to a stabilized active form of bovine opsin. Rather the all-*trans* species formed by light activation, metarhodopsin II, decay in minutes (Kliger and Lewis, 1995). In contrast to bovine rhodopsin, for octopus rhodopsin photoisomerization of the chromophore to the all-*trans* species leads to a stable, active form of the pigment, Acid Metarhodopsin. Like bovine rhodopsin, the inverse agonist 11-*cis*-retinal stabilizes octopus opsin in forming octopus rhodopsin (Tsuda *et al*., 1982; Tsuda, 1987; Koutalos *et al*., 1989). Because octopus Acid Metarhodopsin absorbs at considerably longer wavelengths than octopus rhodopsin, shorter wavelength light can convert most of the octopus rhodopsin to Acid Metarhodopsin while longer wavelength light can reverse this, driving all the pigment back to rhodopsin. Thus octopus rhodopsin is an appealing system to use to elucidate the molecular nature

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of the processes involved in receptor activation.

MATERIALS AND METHODS

Isolation of microvillar membranes and octopus rhodopsin

Microvillar membranes of octopus photoreceptors were prepared from eyes of *Octopus defleini* as described previously (Tsuda *et al*., 1992). Octopus rhodopsin was purified as described previously (Kikkawa *et al*., 1996).

Purification of truncated rhodopsin

Octopus microvillar membranes (containing 5mg/ml rhodopsin) were incubated with 1mg/ml *Staphylococcus aureus* V-8 protease (Wako) with a ratio of rhodopsin: enzyme of 280:1 (mol/mol) in 50mM Tris-HCl buffer, pH7.4 at 15°C for 1 hr. Under these conditions, about half of the rhodopsin was not digested, but with longer incubation times cleavage of the 5-6 intracellular loop of rhodopsin started (Tsuda, 1988, 1979). After the digestion, the membranes were suspended with a buffer containing 0.5% CHAPS (Wako), 50mM Tris-HCl pH7.4, and 1mM DTT and peripheral proteins were removed by centrifugation at 200,000g for 30min. Washed membranes were solubilized with 1.2%(w/v) Nonyl-glucoside (NG, Anatrace) in 50mM Tris-HCl pH7.4, and 1mM DTT and diluted 3-fold with 50mM Tris-HCl pH7.4, and centrifuged at 200,000g for 30min to remove the insoluble fraction. The mixture was fractionated with 61% ammonium sulfate (Sigma). The supernatant was dialyzed against a solution containing 0.01% Dodecyl-maltoside (DDM, Anatrace), 10mM Tris-HCl pH7.4 and 1mM DTT and applied to a monolithic DEAE column (1.5cm × 4.5cm, BIA Separations) which had been equilibrated with 10mM Tris-HCl pH7.4, 1mM DTT, and 0.05% DDM. The column was thoroughly washed with the equilibrate buffer until absorbance at 280 nm returned to the baseline; then the proteins were eluted with NaCl. The fractions were assayed by absorption spectroscopy and SDS-PAGE. The t-rhodopsin eluted at about 40mM NaCl as a single peak.

Purification of truncated octopus Acid Metarhodopsin

The t-rhodopsin in 10mM Tris-HCl pH7.4, 1mM DTT, and 0.05% DDM was irradiated with blue light (maximum transmittance at 440 nm; band path filter: V44 Toshiba) from 100 W slide projector for 1min. The amount of t-rhodopsin and t-Acid Metarhodopsin in the photosteady state mixture under these conditions was estimated to be 30% and 70%, respectively (Tsuda, 1979; Kitagawa and Tsuda, 1980). To isolate t-Acid Metarhodopsin from the photosteady state mixture, the irradiated t-rhodopsin solution was loaded onto a DEAE column which had been equilibrated with 10mM Tris-HCl pH7.4, 0.05% DDM, 1mM DTT. Elution was performed with a linear concentration gradient of 0-200mM NaCl.

Electrophoresis and immunoblot analysis

SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (Laemmli, 1970). Protein blotting to a PVDF membrane was performed following Towbin *et al*.'s method (Towbin *et al*., 1979) using a transfer buffer containing 0.1%(w/v) SDS and 15%(v/v) methanol. Monoclonal antibodies which recognized the proline-rich C-terminus (C15), and the core domains (C1) of octopus rhodopsin (gifts from Dr. Tadashi Ishibashi of Hitachi) were used. For immunological detection, horseradish peroxidase-conjugated anti-IgG antibodies and the ECL chemiluminescence detection system (Amersham) were used according to manufacturer's directions.

GTPγS binding assay

The preparation of the octopus G-protein, G_q, and the GTPγS binding assay was performed as described previously (Kikkawa *et al*., 1996). The reaction mixture containing the octopus retinal G-protein G_q (about 45 nM), 0.5 μM [^{35}S]GTPγS, and the t-rhodopsin or t-Acid Metarhodopsin (400nM) in 10mM Tris-HCl pH7.4, 1mM DTT, and 0.05% DDM were incubated at 20°C. The t-rhodopsin was irradiated with blue flash light (V-44 glass filter, Toshiba) and t-Acid Metarhodopsin was irradiated with orange light (O–58 glass filter, Toshiba) from 100W slide projector for 5min. At fixed times, a 20 μl aliquot was withdrawn and diluted with 250 μl of ice-cold buffer containing 20mM Tris-HCl pH7.4, 25mM $MgCl_2$, and 100mM NaCl. Each diluted sample was filtered through a nitrocellulose filter, and the filter counted for ^{35}S with a liquid scintillation counter.

Spectroscopic measurements

Absorption spectra were recorded with a Shimadzu MPS 2000 spectrophotometer equipped with a cross-illumination attachment. The samples were irradiated with light of appropriate wavelength, orange light (O–58 glass filter, Toshiba) for t-rhodopsin and blue light (V-44 glass filter, Toshiba) for t-Acid Metarhodopsin. The temperature of the sample was maintained at 15°C by a temperature controller (Lauda RMS6).

Chromophore analysis

The configurations of chromophores extracted from the purified t-rhodopsin and t-Acid Metarhodopsin were analyzed by an Waters HPLC system (Okano, 2002). Briefly, 500 μl of 0.17mg/ml samples were mixed with 200 μl of 2M hydroxylamine and 1ml of methanol to convert the chromophores into retinal oximes. They were extracted with 3ml of dichloromethane and 6ml of *n*-hexane and an aliquot of the extract was then subjected to YMC-Pack SIL A-012-3 column (150 × 6.0mm, YMC), and eluted with *n*-hexane containing 10% diethyl ether at a flow rate of 2.0 ml/min while being monitored for absorbance at 360nm.

RESULTS AND DISCUSSION

Purification of the truncated octopus rhodopsin

Octopus rhodopsin contains multiple copies of a repeated proline-rich sequence on its C-terminus; this motif is unique to cephalopod rhodopsins and its function is unknown (Ovchinnikov *et al*., 1988; Hara-Nishimura *et al*., 1993). Several isoforms of octopus rhodopsin are observed in SDS-PAGE, probably due to different lengths of the proline-rich C-terminus. Truncated octopus rhodopsin (t-rhodopsin) is formed by proteolytically removing the proline rich C-terminal tail of octopus rhodopsin with V8 protease. Fig. 1 shows SDS-PAGE and immunoblots of the purified preparations of native (unproteolyzed) octopus rhodopsin and truncated octopus rhodopsin (t-rhodopsin). Coomassie blue staining of purified rhodopsin shows several bands located at 51-53kDa on SDS-PAGE (lane 2 in Fig. 1A). After digestion by V-8 protease and subsequent purification (see elution profile from DEAE column in Fig. 2A), t-rhodopsin appears as a single band at 44kDa on the gel (lane 3 of Fig. 1A). Figs. 1B and 1C show immunoblots of rhodopsin and t-rhodopsin with monoclonal antibodies against the core domain and the C-terminus of octopus rhodopsin, respectively. The monoclonal antibody against the core domain bound to both rhodopsin and t-rhodopsin (Fig. 1B). On the other hand, the monoclonal antibody against the proline-rich C-terminus reacted with rhodopsin, but not with t-rhodopsin (Fig. 1C). These results suggest that the V-8 protease removed the C-terminus of octopus rhodopsin. The molecu-

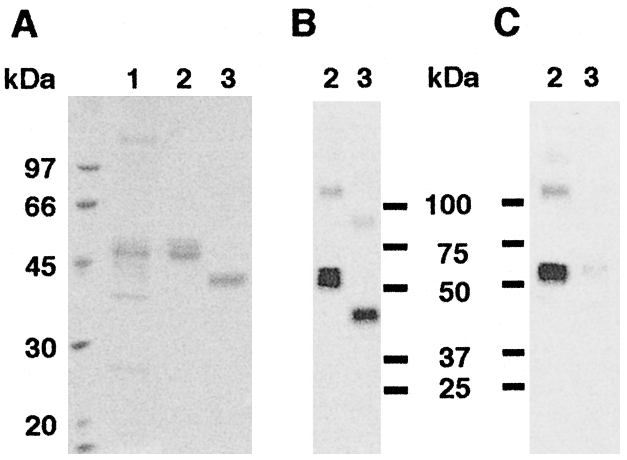

Fig. 1. SDS-PAGE and western blots of octopus rhodopsins. A: SDS-PAGEs stained with Coomassie Blue. Lane 1; octopus photoreceptor microvillar membranes, Lane 2; purified rhodopsin, Lane 3; purified t-rhodopsin. B: Western blots with a monoclonal antibody against the core domain of octopus rhodopsin, lanes 2 and 3 as above. C: Western blots with a monoclonal antibody against the proline-rich C-terminus of octopus rhodopsin, lanes 2 and 3 as above.

lar weights of unproteolyzed rhodopsin and t-rhodopsin were analyzed by MALDI-TOF MS (mass spectra were run by Shimadzu Biotech.). Though three distinct molecular masses for rhodopsin (50814, 51542, 52587) were estimated in the mass spectra, t-rhodopsin showed a single molecular mass of 43773. An analysis by a protein sequencer showed that N-terminus of t-rhodopsin was not digested by the V8 protease. These results suggested that the different molecular weights of rhodopsin seen on the gel were due to inhomogeneity in the proline-rich C-terminus. The t-rhodopsin prepared as above was a single species as analyzed by mass spectroscopy and by SDS-PAGE. As with many GPCRs, octopus rhodopsin has the post-translational modifications of fatty acid acylation (Nakagawa *et al*., 1997), and glycosylation (Zhang *et al*., 1997; Nakagawa *et al*., 2001). Taking into consideration these modifications, the likely cleavage site of t-rhodopsin was at Glu^{372}. Thus the proline-rich C-terminus, unique to cephalopod rhodopsins, is readily cleaved to yield a “core” receptor which is similar in size (40 - 45 kDa) and topology to other rhodopsins (Ovchinnikov *et al*., 1982; Zuker *et al*., 1985). Though the function of the proline-rich C-terminus is not clear, removal of the C-

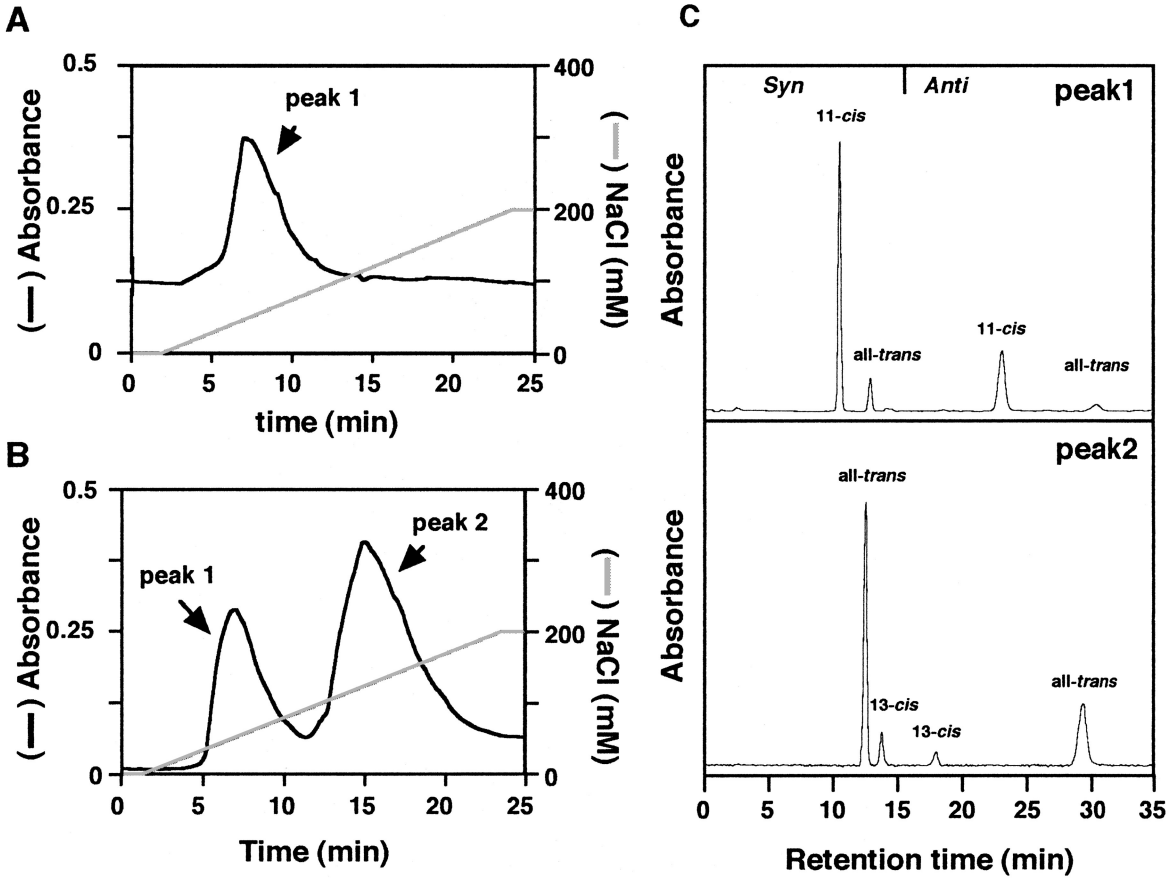

Fig. 2. Elution profiles of octopus t-rhodopsin and t-Acid Metarhodopsin from a DEAE column and also HPLC analysis of the peak fractions. A: The ammonium sulfate fraction of t-rhodopsin was applied to a DEAE column. Absorbance at 280 nm of the eluted proteins was monitored. The peak containing t-rhodopsin is indicated by an arrow 1. B: Illuminated t-rhodopsin was applied to a DEAE column. The peaks containing t-rhodopsin and t-Acid Metarhodopsin are indicated by arrow 1 and arrow 2, respectively. C: The configurations of the chromophores in the peak1 and peak2 fractions were analyzed by HPLC after extraction of the chromophores as retinal oximes.

terminus from squid rhodopsin did not affect its absorption spectra (Naito *et al.*, 1981) and a similar excision of octopus rhodopsin did not affect its phosphorylation by octopus rhodopsin kinase (Ohguro *et al.*, 1998). The phosphorylation sites are located upstream of proline rich C-terminus.

t-Rhodopsin retained the spectral properties and the ability to light-activate a G-protein of unproteolyzed rhodopsin

We investigated the spectral properties of octopus rhodopsin with and without the C-terminus. Curve 1 in Fig. 3A shows that the absorption spectrum of the t-rhodopsin is the same as that of rhodopsin. The absorption maximum of t-rhodopsin shifted from 476 nm to 508nm upon illumination with blue light (λ~440nm) to form a photosteady state mixture containing predominately Acid Metarhodopsin (curve 2). When this photoproduct was illuminated with orange light (λ>580nm), the absorption spectrum returned to the original one with its maxima at 476 nm (curve 3), corresponding to that of rhodopsin. Thus, the spectral properties of the t-rhodopsin were not affected upon removal of the C-terminus.

Next, we examined whether removal of the C-terminus affected light-induced G-protein activation. After a period of dark preincubation, GTPγS-binding to the octopus G-protein G_q was measured after a Xenon flash transformed part of the pigment (rhodopsin or t-rhodopsin) to Acid Metarhodopsin (Fig. 4A). Controls were also run without any pigment present. GTPγS-binding to the G_q in the absence of a

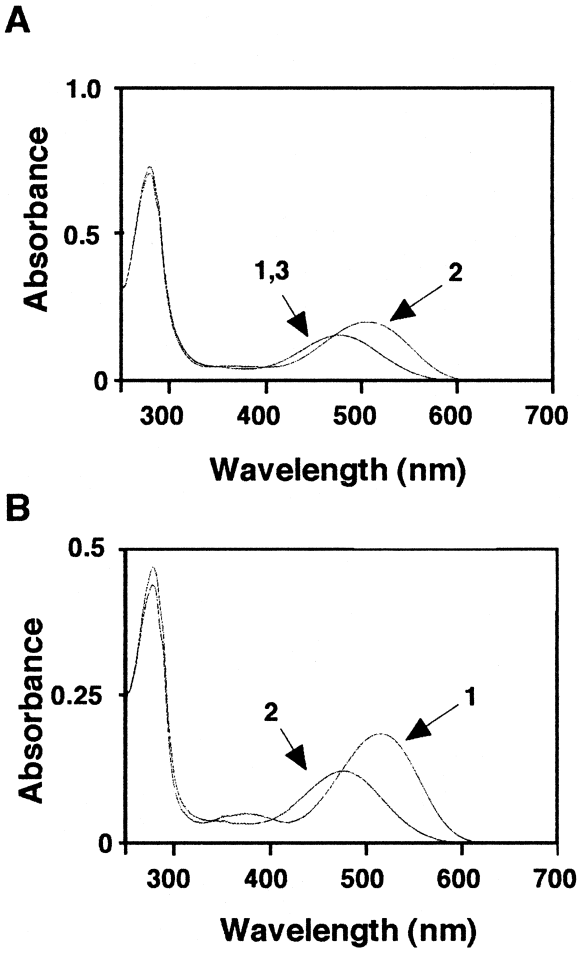

Fig. 3. Absorption spectrum of peak1 and peak2. A: Absorption spectra of the peak 1 fraction corresponding to t-rhodopsin (curve 1). The absorption maximum of t-rhodopsin (476 nm) shifted to about 508 nm upon illumination with blue light (λ~440 nm) which showing the formation of Acid Metarhodopsin as well as some residual rhodopsin (curve 2). Illumination of this photoproduct with orange light converted the pigment back to the starting spectrum (curve 3). B: Absorption spectrum of the peak 2 fraction (curve 1). The absorption maximum was located at 514nm, in agreement with the calculated absorption maximum for Acid Metarhodopsin. When the peak 2 fraction was irradiated with orange light, the absorption spectrum converted to the original spectrum of t-rhodopsin (curve 2).

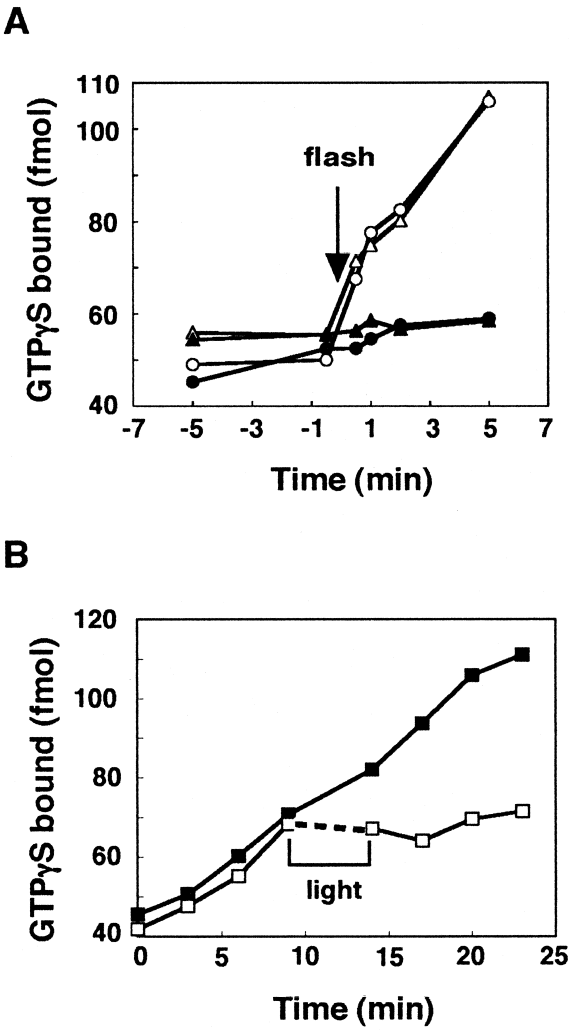

Fig. 4. Time course of GTPγS-binding to the octopus retinal G-protein G_q in the presence of rhodopsin and t-rhodopsin. A. GTPγS bound to the G_q upon illumination of rhodopsin (open circles) or t-rhodopsin (open triangles). Basal GTPγS-binding without illumination was not observed either in the presence of rhodopsin (closed circles) or t-rhodopsin (closed triangles). B. GTPγS bound to the G_q in the presence of t-Acid Metarhodopsin in the dark (closed squares). GTPγS binding to the G_q was inhibited when t-Acid Metarhodopsin was illuminated with orange light (for 5 min from 9 to 14 min) to form rhodopsin (open squares).

rhodopsin was not observed in the dark or light. In contrast, GTPγS-binding to the G_q occurred immediately after irradiation of rhodopsin or t-rhodopsin. These results show that the proline rich C-terminus of octopus rhodopsin is not required for G-protein activation. Thus, the purified t-rhodopsin is a homogenous species which retains rhodopsin's spectral properties and its ability to activate a G-protein.

Purification of t-Acid Metarhodopsin

The next task was to prepare and purify Acid Metarhodopsin. The maximal content of Acid Metarhodopsin in photosteady state mixture depends on the wavelength of the actinic light (Hamdorf *et al*., 1973). In case of *Octopus defleini,* the maximal content of Acid Metarhodopsin formed, about 70%, occurred when rhodopsin was illuminated with blue light (440 nm)(Tsuda, 1979). In order to isolate a stable, active form of octopus rhodopsin, we attempted to separate the t-Acid Metarhodopsin from t-rhodopsin in this photosteady state mixture.

A sample of t-rhodopsin or irradiated t-rhodopsin was loaded onto a DEAE column which had been equilibrated with 10mM Tris-HCl pH7.4, 1mM DTT, and 0.05% of the detergent dodecyl maltoside (DDM). The t-rhodopsin bound to the DEAE-cellulose and could be eluted with a linear concentration gradient of 0 - 200mM NaCl. As noted earlier, Fig. 2A shows the elution profile of t-rhodopsin: it eluted at about 40mM NaCl. Fig. 2B shows the elution profile of the photosteady state mixture of t-rhodopsin after irradiation at 440 nm. In addition to peak 1 (t-rhodopsin) eluting at about 40mM NaCl, another species (peak 2) eluted at about 100mM NaCl. The isomeric conformations of the retinal chromophores of the various octopus rhodopsin samples eluting from the column were analyzed by HPLC. These showed that the isomeric form of the retinal of peak 1 was more than 92.5% of 11-*cis* retinal and peak 2 was solely all-*trans* retinal (Fig. 2C). Curve 1 in Fig. 3B shows the absorption spectrum of peak 2. The absorption maximum was located at 514 nm, in agreement with the absorption maximum calculated for Acid Metarhodopsin (Tsuda *et al*., 1982) and that of Acid Metarhodopsin reconstituted with all-*trans* retinal and octopus opsin (Koutalos *et al*., 1989). The present results suggest that peak 2 from the DEAE column was pure t-Acid Metarhodopsin. When this purified t-Acid Metarhodopsin was illuminated with orange light (λ>580nm), the absorption spectrum converted to the original spectrum of t-rhodopsin (curve 2).

Purified t-Acid Metarhodopsin activates Octopus G_q

To test whether the purified t-Acid Metarhodopsin is able to activate a retinal G-protein in the dark, we conducted GTPγS-binding experiments. Fig. 4B shows the time course of GTPγS-binding to G_q in the presence of t-Acid Metarhodopsin. GTPγS-binding to G_q occurred in the presence of t-Acid Metarhodopsin in the dark. When t-Acid Metarhodopsin was illuminated with orange light to revert it back to t-rhodopsin, the rate of GTPγS-binding to G_q decreased markedly. These results suggest that the stable, purified t-Acid Metarhodopsin we prepared activated G_q in the dark. As anticipated, the photoregenerated t-rhodopsin is an inactive form of the GPCR, not able to cause GTPγS-binding to G_q.

Previously we reported that a precursor of Acid Metarhodopsin (Transient Acid Metarhodopsin) activates G_q (Nakagawa *et al*., 1998) more readily than the final, stable form of Acid Metarhodopsin. This conclusion was derived from experiments where octopus rhodopsin alone was incubated in the dark after illumination. The photoproduct's ability to stimulate GTPγS-binding by added G_q decreased in a time-dependent manner (minute to hour). The life time of Transient Acid Metarhodopsin is so fast (180 μs) (Nishioku *et al*., 2002), that it probably is not a factor in our experiments but it is possible that it is in equilibrium with t-Acid Metarhodopsin. Since the measured differences between the two types of Acid Metarhodopsin are small (Zhang *et al*., 1997), even if the transient species is important we believe it must have a structure close to that of t-Acid Metarhodopsin.

In conclusion, we succeeded in preparing a stable, pure active form of a GPCR, octopus t-Acid Metarhodopsin. The t-Acid Metarhodopsin activated G_q in the dark and G-protein activation was inhibited by irradiation with orange light, which converted the active species back to rhodopsin.

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