Improvements in G protein-coupled receptor purification yield light stable rhodopsin crystals

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Abstract

G protein-coupled receptors (GPCRs) represent the largest family of transmembrane signaling proteins and are the target of approximately half of all therapeutic agents. Agonist ligands bind their cognate GPCRs stabilizing the active conformation that is competent to bind G proteins, thus initiating a cascade of intracellular signaling events leading to modification of the cell activity. Despite their biomedical importance, the only known GPCR crystal structures are those of inactive rhodopsin forms. In order to understand how GPCRs are able to transduce extracellular signals across the plasma membrane, it is critical to determine the structure of these receptors in their ligand-bound, active state. Here, we report a novel combination of purification procedures that allowed the crystallization of rhodopsin in two new crystal forms and can be applicable to the purification and crystallization of other membrane proteins. Importantly, these new crystals are stable upon photoactivation and the preliminary X-ray diffraction analysis of both photoactivated and ground state rhodopsin crystals are also reported.

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1. Introduction

Rhodopsin is the best characterized and most highly studied G protein-coupled receptor (GPCR) due in part to its stability in the dark state and the relative ease of purification of large quantities from retinal extracts (Filipek et al., 2003; Sakmar, 1998). Early electron microscopy and cryoelectron microscopy studies of rhodopsin revealed the seven transmembrane helices but were unable to clarify the mechanism by which photons are transformed into an intracellular signal (Edwards et al., 2004; Krebs et al., 2003; Schertler et al., 1993) (reviewed in (Schertler, 1999)). These innovative studies were further advanced when rhodopsin was crystallized and its molecular structure determined (Palczewski et al., 2000; Teller et al., 2001). Previous X-ray structural studies of rhodopsin relied on protein purified by one of two methods (reviewed in (Filipek et al., 2003; Palczewski, 2006)). The initial rhodopsin structure utilized the selective precipitation of opsins and other membrane proteins by Zn(OAc)2 treatment of alkyl-glucoside extractions of rod outer segments (ROS) which were isolated from dark adapted bovine retina (Papermaster, 1982).
These extractions leave purified rhodopsin and associated phospholipids in solution while precipitating opsins and other membrane proteins (Okada et al., 2000; Okada et al., 1998; Paleczewski et al., 2000). The other method by which rhodopsin was purified, (and consequently delipidated) and crystallized involved the solubilization of ROS in the detergent lauryldimethylamine-n-oxide, purification by concanavalin A affinity chromatography with further purification via anion exchange in the presence of the detergent C₈E₄ (Edwards et al., 2004). Both of these rhodopsin purification protocols for the most part are limited solely to rhodopsin as they rely on the high ratio of rhodopsin to other membrane protein expression in retinal tissue and thus cannot be extended to the purification of other GPCRs.

All of the crystal structures of rhodopsin that have been determined to date have been of ground state rhodopsin, and upon exposure to light, the crystals become disordered, prohibiting the determination of a photoactivated structure (Okada et al., 2000). In addition, one of the inactive intermediates of photoactivated rhodopsin, Meta I, has been structurally characterized in 2D crystals. However, the formation of the active state, Meta II, is blocked in that crystalline environment (Vogel et al., 2004). To address this shortcoming in the current structural data, we have developed a robust general system by which rhodopsin as well as recombinantly expressed GPCRs can be purified, concentrated, and screened for crystallization conditions. Recent advances in the expression of transgenic GPCRs in Xenopus laevis could utilize this procedure to generate the quality and purity of fully functional GPCRs needed for structural studies (Zhang et al., 2005). Rhodopsin purified utilizing this technique has resulted in the discovery of two new crystal forms which, unlike previous rhodopsin crystals, are stable upon exposure to light.

2. Results and discussion

To obtain new rhodopsin crystals that are stable to photoactivation, a novel combination of previously reported methods of purification was employed. In addition, an innovative approach to the concentration of membrane proteins was developed.

Our protocol developed for the purification of bovine rhodopsin from frozen, dark-adapted retinas consists of four steps (Fig. 1A): (a) rod outer segment (ROS) isolation using a protocol based on sucrose density gradient centrifugation; (b) extraction of rhodopsin from ROS in the presence of nonyl-β-D-glucoside (NG) and Zn(OAc)₂; (c) 1D4 immunoaffinity chromatography; (d) rhodopsin concentration by (NH₄)₂SO₄-induced phase separation (Fig. 1B). All manipulations are performed in a dark room under dim red light.

Even though the steps a, b, and d were not strictly necessary to obtain purified rhodopsin competent to yield crystals, the inclusion of each additional purification step resulted in an increased quality of the rhodopsin crystals. Step d was essential for obtaining light stable crystals.

2.1. ROS isolation

The isolation of the rod outer segments (ROS) from dark-adapted bovine retina was essentially the established sucrose density gradient centrifugation procedure (Papamastor, 1982).

2.2. Nonyl-glucoside/Zn(OAc)₂ extraction of rhodopsin

Rhodopsin can be selectively extracted from ROS membrane preparations by utilizing a combination of alkyl(thio)glucosides and 2B series divalent cations (Okada et al., 1998), which solubilizes ground state rhodopsin but eliminates opsin and other protein contaminants.

ROS preparations were solubilized with NG to give final concentrations of 5–10 mg/ml of rhodopsin, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.3, 100 mM Zn(OAc)₂, and a ratio of NG/rhodopsin of 2.2 (w/w). Samples were incubated overnight at 4 °C, and precipitated proteins removed via centrifugation. Typically the ratio of A₂₈₀nm/A₅₀₀nm (a measure of rhodopsin purity (Wald and Brown, 1953)) of the supernatant was 1.8–2.0, indicating a purity of rhodopsin of 80–90%.

Fig. 1. (A) Flow chart summarizing the four steps for rhodopsin purification used in this work leading to crystallization. (B) Schematic depiction of the (NH₄)₂SO₄-induced concentration of rhodopsin.
Optimization of the ROS isolation and the NG/\text{Zn(OAc)}_2 extraction steps allowed one to obtain rhodopsin at $A_{280nm}/A_{500nm}$ near 1.6 (98\% purity) (Okada et al., 2000; Okada et al., 1998). However, this technique left the rhodopsin in mixed micelles containing lipids extracted from the membranes in addition to detergent so an extra chromatographic step was necessary to delipidate and further purify the rhodopsin.

### 2.3. Immunoaffinity purification

DEAE purified 1D4 antibody (an antibody that recognizes the 9 C-terminal amino acids of rhodopsin) was coupled to CNBr-activated Sepharose 4B (GE Healthcare). After coupling, binding capacity is $\sim 0.5 \text{mg rhodopsin/ml}$ resin.

Initial attempts to immunopurify rhodopsin directly in crystallization buffer based on previously described crystallization conditions (Fig. 2A) produced crystals. However, the crystals obtained in this manner “melt” or lose diffraction upon exposure to light. To remedy this shortcoming of the crystals, screening for crystallization conditions distinct from those previously described was necessary and a new scheme of purification was developed.

Typically, solutions for immunoaffinity purification were prepared in a Tris buffer (TB buffer: 50 mM Tris, pH 7.4, 280 mM NaCl, and 6 mM KCl) containing 50 mM NG. Immobilized 1D4 antibody gel was packed in a 20–50 cm long column, equilibrated with TB/NG, and the detergent-solubilized rhodopsin sample was loaded onto the column at a ratio of around 0.6 mg of rhodopsin/ml of 1D4 gel. Then, the column was washed at a linear flow rate of $\sim 18 \text{cm/h}$ with 10 column volumes of TB/NG, and eluted with 0.75 mg/ml of a competing peptide (TETSQVAPA) at 7 cm/h (Fig. 2B) and fractions were collected. The concentration of rhodopsin was determined spectrophotometrically by $A_{500nm}$. The sample (5–10 $\mu$l) used for the measurement was diluted with 1 ml of 10 mM Tris, pH 7.2, containing n-dodecyl-$\beta$-D-maltoside (DM) and NH$_4$OH. The most concentrated fractions contained about 5 mg/ml of rhodopsin, and the $A_{500nm}/A_{500nm}$ ratio of the fractions containing rhodopsin was $1.58 \pm 0.02$ (Fig. 2C) reflecting a purity of $\sim 99\%$ (Fig. 2C and D). In a typical preparation, $\sim 90\%$ of rhodopsin loaded onto the immunoaffinity
column was recovered. Similar results were obtained using 1.5% DM or n-octyl-β-D-glucoside (OG) instead of NG (not shown).

2.4. Ammonium sulfate concentration

The addition of a high concentration of (NH₄)₂SO₄ induces phase separation in concentrated alkyl-glucoside solutions, yielding a top phase rich in detergent. We capitalized upon this phenomenon to concentrate the purified rhodopsin obtained by immunochromatography. By pooling the most concentrated fractions from the immunoaffinity purification and adding solid (NH₄)₂SO₄ at near saturated concentrations, the rhodopsin was concentrated by partitioning into the detergent rich upper phase (Fig. 3A). To perform this separation/concentration step, rhodopsin fractions were pooled together to achieve a rhodopsin concentration of 1–2 mg/ml. 0.25 volumes of 0.5 M MES, pH 6.3, were added and then 0.69 g of solid (NH₄)₂SO₄ per ml of total solution was dissolved by stirring at room temperature.

Samples became turbid immediately upon addition of (NH₄)₂SO₄ and were incubated on ice for 4–7 days to allow excess (NH₄)₂SO₄ to crystallize out of solution (Fig. 1B). Shorter incubation times allowed the growth of (NH₄)₂SO₄ crystals (Fig. 1B). The growth of (NH₄)₂SO₄ crystals together with rhodopsin crystals complicating crystallization trials. After a brief centrifugation at 7000g, the top, viscous rhodopsin/detergent phase was transferred to Eppendorf tubes using a wide bore pipette tip. Any remaining (NH₄)₂SO₄ phase was eliminated by pipetting the (NH₄)₂SO₄ solution out from underneath the rhodopsin phase with a gel loading tip after a brief centrifugation. Treatment of purified rhodopsin with solid (NH₄)₂SO₄ resulted in a 12–15-fold concentration of the protein. Alternatively, 20 volumes of saturated (NH₄)₂SO₄ can be added to the rhodopsin solution with similar results. The yield was significantly lower when the NG concentration during immunoaffinity chromatography was increased to 100 mM or when OG was used instead of NG (Fig. 3B).

2.5. Crystallization

Initial crystal trials with rhodopsin samples directly immunopurified in crystallization buffer based on the conditions for crystal growth described previously produced crystals (Fig. 4A). The final concentrations of the sitting drops were approximately 5 mg/ml rhodopsin, 50 mM NG, 30 mM MES, pH 6.3, 78 mM Zn(OAc)₂, 0.85 M (NH₄)₂SO₄, 0.8 mg/ml TETSQVAPA peptide, 6 mM β-mercaptoethanol, and 0.1% NaN₃. The drops were equilibrated against ~3.4 M (NH₄)₂SO₄ at 5°C. In 2–3 weeks triangular and rod-like crystals appeared, with maximal dimensions greater than 1 mm. However, these crystals diffracted to only low resolution (the best crystals, grown in the presence of 1,2,3-heptane triol, diffracted to ~9 Å) and lost integrity after photoactivation (Fig. 4A), much like the crystals described in (Okada et al., 2000). These conditions were not pursued further.

Subsequent crystallization trials produced crystals under the following conditions: (NH₄)₂SO₄ concentrated rhodopsin samples were diluted with one volume of crystallization buffer, consisting of 100 mM MES, pH 6.3, containing 100 mM NG, 0.1% NaN₃, and 12 mM β-mercaptopetethanol, and then 3–5 μl sitting drops of the protein solution were equilibrated against 3.1–3.3 M (NH₄)₂SO₄ at 5°C. Samples containing ~12 mg/ml rhodopsin formed trigonal crystals with maximal dimensions of ~0.1 mm when equilibrated against 3.1 M (NH₄)₂SO₄, whereas lower concentrations (~6 mg/ml) typically required ~3.3 M (NH₄)₂SO₄ in the reservoir. Most importantly, these crystals were not destroyed upon exposure to light (Fig. 4B), but their X-ray diffraction patterns were poor. A battery of crystallization additives was tested to increase the resolution of the crystals, including

Fig. 3. Ammonium sulfate-induced rhodopsin concentration. (A) Varying amounts of solid (NH₄)₂SO₄ were added to 1 mg/ml purified rhodopsin, dissolved with magnetic stirring at room temperature, and the sample was incubated overnight on ice. The next day the samples were centrifuged and an aliquot of the bottom phase was used to determine rhodopsin concentration. In samples treated with more than 0.6 g (NH₄)₂SO₄/ml of sample, rhodopsin partitioned into a thin layer on top of the (NH₄)₂SO₄ phase. For the two samples with higher (NH₄)₂SO₄ concentration, salt crystals were observed after centrifugation. [Rho]bottom corresponds to the concentration of rhodopsin found in the lower (NH₄)₂SO₄ phase. (B) (NH₄)₂SO₄-induced concentration of purified rhodopsin by addition of solid (NH₄)₂SO₄. [Rho]pool corresponds to the concentration of rhodopsin prior to treatment with solid (NH₄)₂SO₄ and [Rho]pool corresponds to the final concentration of rhodopsin after (NH₄)₂SO₄-induced phase concentration. Rhodopsin was in 50 mM NG (■) or 50 mM OG (○). In another series of experiments, 20 volumes of saturated (NH₄)₂SO₄ were added to purified rhodopsin in 100 mM NG (□). Scattering of the points in the plot is mainly due to the difficulty of accurately pipetting small volumes of the viscous rhodopsin solution.
buffers of different pHs, salts, lipids, detergents and amphiphiles, carbohydrates, reducing agents, and organic solvents. Rhodopsin samples were often diluted with one volume or more of water or additives, and this dilution had no apparent adverse effect on crystal formation. The addition of the polyoxyethylene surfactants Merpol HCS or Merpol DA clearly resulted in improved crystal shape and resolution (Fig. 4C).

2.6. Cryoprotection of rhodopsin crystals

The effects of various cryoprotectants upon diffraction resolution for the rhodopsin crystals were screened. Carbohydrates (sucrose, glucose, xylitol, and trehalose) dissolved in (NH₄)₂SO₄ solutions (at concentrations similar to that of the reservoir) were able to maintain crystal integrity. After adding the cryoprotectant solution, a phase separation was observed (Fig. 4C), and the crystals partitioned into the top, detergent-rich phase. Crystals could also be cryoprotected by dipping in paraffin oil without losing resolution. However, due to the difficulty of removing the viscous mother liquor surrounding the crystal, cryoprotectant solutions were sometimes ineffective in preventing the formation of ice rings in the diffraction pattern.

Because high concentrations of (NH₄)₂SO₄ were often sufficient for cryoprotection, it was possible to freeze crystals directly in their mother liquor. However, we routinely added 10 µl of ∼3.3 M buffered (NH₄)₂SO₄ to the drops containing the crystals in order to modulate the pH during photoactivation. Crystals were harvested with a cryoloop (Hampton Research), and excess viscous phase removed. The crystals were immediately frozen by plunging into liquid nitrogen. Some crystals were exposed to light prior to freezing and exhibited a color change from red to yellow.

2.7. Specific conditions for crystal growth and photoactivation

During the course of this work we analyzed ~3000 crystals, and in this article we report preliminary X-ray data analysis of three rhodopsin crystals (Table 1); two in the ground state and another one in a photoactivated state. The two ground state crystals came from the same preparative purification. After the (NH₄)₂SO₄ concentration step, the concentration of rhodopsin was 16.5 mg/ml.

To obtain the R32 crystal, the concentrated rhodopsin sample was diluted with one volume of 80 mM MES, pH 6.3, containing 110 mM NG, 200 mM Zn(OAc)₂, 16 mM β-mercaptoethanol, 0.4% Merpol DA, and 0.1% NaN₃. The sitting drop, consisting of 7 µl of this sample plus 4 µl of water, was equilibrated for 4.5 months against 1 ml of 3.0 M (NH₄)₂SO₄ in 10 mM MES, pH 6.3, at 5 °C in the dark. The crystal was harvested under dim red light and cryoprotected with paraffin oil prior to freezing in liquid nitrogen.

To prepare the ground state P3₁2 crystal, rhodopsin at 16.5 mg/ml was diluted with one volume of 80 mM MES, pH 6.3, containing 110 mM NG, 200 mM Zn(OAc)₂, 16 mM β-mercaptoethanol, 2.6% Merpol HCS, and 0.1% NaN₃. The sitting drop consisted of 7 µl of diluted sample plus 8 µl of 63 mM Na₃ citrate, pH 5.6, and 63 mM NaCl, and was equilibrated for 2 months against 1 ml of 3.2 M (NH₄)₂SO₄ in 10mM MES, pH 6.3, at 5°C in the dark. The crystal was transferred to 10 µl of 10 mM MES, pH 6.3, 7.5% glucose, 0.45% Merpol HCS, and 3.3 M (NH₄)₂SO₄ for ~1 min, harvested, and frozen.
To obtain the photoactivated P3\(_{12}\) crystal, rhodopsin was purified following the four purification steps described in this work. After affinity chromatography in the presence of 50 mM NG and (NH\(_4\))\(_2\)SO\(_4\)-induced concentration, the rhodopsin (at 23 mg/ml) was diluted with one volume of 100 mM MES, pH 6.3, containing 100 mM NG, 12 mM β-mercaptoethanol, 4.5% Merpol HCS, and 0.1% NaN\(_3\). The sitting drop contained 3 μl of diluted sample plus 6 μl water, and was equilibrated for 1 month against 1 ml of 3.1 M (NH\(_4\))\(_2\)SO\(_4\) in 10 mM MES, pH 6.3, at 5°C in the dark. Then, 10 μl of 3.4 M (NH\(_4\))\(_2\)SO\(_4\) in 200 mM Tris, pH 7.2, was added to the drop containing multiple crystals of ground state rhodopsin. After 1 h at room temperature, the drop was exposed to ~300 cd/m\(^2\) of white light for 200 min at room temperature. After exposure to light, the crystals were harvested and frozen in liquid nitrogen.

2.8. Two crystal forms of ground state rhodopsin that withstand photoactivation

Crystals grown in the presence of Merpol DA yielded rod-like rhombohedral crystals (space group R32) that diffracted to 3.8 Å (Table 1). Alternatively, when Merpol HCS was included as a crystallization additive, trigonal protein crystals of space group P3\(_{12}\) grew. Crystals exposed to light did not appear to undergo a large change in unit cell dimensions as unit cell constants were consistent between light exposed and unexposed crystals. Both the trigonal and rhombohedral crystals exhibit a color change from red to yellow after exposure to white or 500 nm light. However, the resolution of the diffraction resolution decreased in a manner dependent on photoactivation temperature, pH, light intensity, crystal size, and the presence of additives. Crystals 0.1 mm or larger have a propensity to crack parallel to the triangular face (perpendicular to the unique axis) upon exposure to light, but after considerable optimization we were able to collect several data sets of photoactivated crystals below 5 Å.

2.9. Effects of additives and photoactivation time on diffraction resolution

2.9.1. Exposure time to light

Analysis of multiple crystals from single crystallization drops indicate that resolution decreases during the first minutes of illumination from 4–5 to 8–10 Å. After 1–2 h of light exposure at room temperature, resolution improves. After prolonged illumination, no diffraction was observed.

2.9.2. Additives

Hundreds of additives were screened to improve diffraction resolution. A high percentage of additives screened were compatible with crystal growth but their effect on diffraction resolution was not obvious. Detergents and amphiphiles had major effects on space group and crystal morphology. The detergent Merpol HCS clearly improved resolution and time stability under light (Fig. 5). When Anapoe X-114 (Anatrace) was used in combination with Merpol HCS, diffraction resolution decreased with exposure to light (Fig. 5). Although Merpol DA resulted in crystals that diffracted to 3.8 Å in the ground state, these crystals lost diffraction resolution upon photoactivation.

3. Conclusions

Rhodopsin has previously been crystallized after purification by either alkylglucoside/Zn(OAc)\(_2\) extraction or from a combination of concanavalin A/anion-exchange chromatography.
While the latter conditions result in rhodopsin that is partially or completely delipidated (Edwards et al., 2004; Li et al., 2004), the former yields rhodopsin in mixed lipid/detergent micelles. Recently, crystals diffracting to 2.2 Å resolution have been generated with rhodopsin purified in heptyl-thioglycoside/Zn(OAc)2 extractions (Okada et al., 2004). Unfortunately, all 2D and 3D rhodopsin crystal structures solved to date are of ground state rhodopsin or can be (stably) photoactivated only to the Meta I rhodopsin state (Vogel et al., 2004). Activation to the Meta II state results in the destruction of the crystals (Okada et al., 2000).

In this work, we took advantage of existing purification methods to produce delipidated, homogenously pure rhodopsin and with the addition of the (NH4)2SO4 phase separation/concentration step, we determined crystallization conditions that produce two new crystal forms that withstand photoactivation. The R32 crystal forms diffracted to $\Delta$ spacings of 3.8 Å and the P312 crystals diffracted to spacings of 4.1 Å; however, both crystal forms diffract to lower resolution upon photoactivation. Optimization of the crystallization and photoactivation conditions for the P312 crystals enabled the collection of both dark-state and photoactivated datasets that exhibit diffraction to similar maximum resolutions ($\sim$4.1 Å).

It has been observed in a recent crystal structure of the lactose permease (LacY) from *Escherichia coli*, that the ratio of phospholipids to membrane proteins is an important parameter to be screened for crystallization (Guan et al., 2006). In fact, under different phospholipid concentrations, LacY crystals grew in different crystal forms with different crystal packing and protomer contacts within the crystals. Similarly, the two previously described rhodopsin purifications resulted in different ratios of rhodopsin to phospholipid and exhibit different crystal packing and intermolecular contacts (Li et al., 2004; Palczewski et al., 2000). By developing this protocol to procure delipidated membrane proteins, we have the flexibility to adjust phospholipids as a parameter during crystallization screening.

Herein, we describe a new method to concentrate purified membrane protein from detergent solutions using (NH4)2SO4. This step was critical in obtaining photoactivatable rhodopsin crystals and the residual (NH4)2SO4 most likely acts as a precipitant. This method can be extended to other membrane proteins, as it is often difficult to concentrate membrane proteins without over-concentration of the solubilizing detergent. In addition to high concentration and purity, post-translational homogeneity of the protein population is usually advantageous when attempting to crystallize a protein. In this respect, the expression of 1D4-tagged GPCRs in the retinas of transgenic *Xenopus laevis* represents a promising approach for the expression of recombinant GPCRs in rod cells, where they are homogeneously processed and transported to ROS, much like rhodopsin (Zhang et al., 2005). These tagged GPCRs can then be purified and likely concentrated using the immunoaffinity and (NH4)2SO4 concentration steps described in this work.

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