Oligomeric State of Rhodopsin Within Rhodopsin–Transducin Complex Probed with Succinylated Concanavalin A

Article *in* Methods in molecular biology (Clifton, N.J.) · February 2015

DOI: 10.1007/978-1-4939-2330-4_15 · Source: PubMed

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Chapter 15

Oligomeric State of Rhodopsin Within Rhodopsin–Transducin Complex Probed with Succinylated Concanavalin A

Beata Jastrzebska

Abstract

Rhodopsin—a prototypical G protein-coupled receptor (GPCR)—is abundantly expressed in the eye and stabilized by its covalently bound chromophore 11-*cis*-retinal. The signal of light is amplified and transmitted through the binding of heterotrimeric G protein transducin (G_t) to photoactivated rhodopsin following downstream pathways activation leading to light sensing in the brain. As demonstrated by atomic force microscopy (AFM), rhodopsin exists in the native membrane of the rod outer segment disks as dimers highly organized in tightly packed oligomers. However, functional importance of this organization is still debated. To clarify the role of the rhodopsin dimer in signaling activation and thus the binding of transducin, the complex between rhodopsin and transducin can be formed, purified, and probed with succinylated concanavalin A. This method can be potentially applied to other GPCRs to verify their oligomeric state.

Key words Rhodopsin, Photoreceptor, Heterotrimeric G protein, Transducin, Succinylated concanavalin A, Membrane proteins, Purification, Transmission electron microscopy

1 Introduction

The GPCRs are the largest and diverse group of integral membrane proteins that in response to various stimuli via activation of specific G proteins help to control variety of biological processes. Undeniably they are key targets for pharmacological manipulations; thus complete understanding of their function, structure, and oligomeric organization is crucial for development of new, more specific medications and therapies. Despite this large diversity, GPCRs share a common architecture of seven transmembrane helices and several conserved motives critical for their function. Many GPCRs form dimers and higher-ordered oligomers in the membrane bilayers, and their importance has been recognized not only in allosteric modulation of ligand binding but also in signaling activation [1–3]. For example, the binding of a dimer to a single heterotrimeric G protein with formation of pentameric complexes

Beata Jastrzebska (ed.), *Rhodopsin: Methods and Protocols*, Methods in Molecular Biology, vol. 1271,
DOI 10.1007/978-1-4939-2330-4_15,

is supported by reconstitution experiments involving the leukotriene B4 receptor BLT1, serotonin 5-HT(4) receptor, and dopamine D2 receptor, each with their respective G proteins [4–6]. But the most compelling evidence for the functional importance of a GPCR dimer comes from in vivo studies on the luteinizing hormone receptor (LHR), which revealed intermolecular cooperation between a ligand-binding deficient GPCR and G protein activation defective mutants [7] resulting in rescue of receptor function. Predominate organization of opsin into dimeric clusters was recently observed in single cells by time-resolved pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) [8]. Dimeric organization of GPCR in "membrane-like" orientation has been detected also in the crystal structures of several family A GPCRs, including rhodopsin in several different activation forms, CXCR4, μ-opioid, and κ-opioid receptors [9–12]. Moreover, the presence of two distinct interfaces found on opposite sides of the μ-opioid receptor raises the possibility for formation of higher-ordered oligomers in a fashion similar to the packing observed by AFM for rhodopsin oligomers in native disk membranes [13]. Because structures of GPCRs, G proteins, and other GPCR-interacting proteins are highly homologous, general activation mechanisms are most likely shared among all GPCRs. Thus, if oligomers serve as functional units for some class A GPCRs [7], they likely do the same for most if not all GPCRs [14].

Here we demonstrate the method useful for the probing of the binding stoichiometry between rhodopsin and its cognate G protein transducin (G_t) within the rhodopsin–G_t complex stabilized by cross-linking and purified in conditions preventing disruption of the native rhodopsin dimer. Rhodopsin contains two sugar groups that modify the opsin structure through an asparagine linkage: mannose $(Man)_3$ attached to Asp^{15} and *N*-acetyl glucosamine $(GlcNAc)_3$ attached to Asp^2 [15]; thus the oligomeric state of rhodopsin in the rhodopsin–G_t complex can be probed by sugar-binding protein that binds only one kind of sugar. One of the possibilities is a dimeric form of concanavalin A (succinylated ConA) that binds only mannose [16] and provides two sugar-binding sites. Negatively stained particles of the triple complex formed between rhodopsin–G_t and succinylated ConA analyzed by transmission electron microscopy (TEM) unequivocally prove oligomeric state of rhodopsin [17]. This method can be potentially useful for studding binding stoichiometry of other GPCR–G protein complexes.

2 Materials

2.1 Extraction and Purification of Transducin (G_t)

1. ROS membranes isolated from 200 retinas according to [18] (*see* **Notes 1** and **2**).
2. Pentyl–agarose beads.

3. Superdex S200 size exclusion column (two connected in tandem) (GE Healthcare Life Sciences).
4. Amicon Centricon 30,000 MWCO (Millipore).
5. Beckman J-20 rotor.
6. High-speed 50 ml (Nalgene) centrifuge tubes.
7. Isotonic extraction buffer: 20 mM 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES), pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT (*see* **Note 3**).
8. Hypotonic extraction buffer: 5 mM HEPES, pH 7.5, 0.1 mM EDTA, 1 mM DTT.
9. 1 M $MgCl_2$ stock solution.
10. 1 M HEPES, pH 7.5 stock solution.
11. Equilibration buffer: 10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT.
12. Washing buffer: 10 mM HEPES, pH 7.5, 75 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT.
13. Elution buffer: 10 mM HEPES, pH 7.5, 400 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT.
14. Cleaning buffer: 10 mM HEPES, pH 7.5, 2 M NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.01 % sodium azide.
15. Gel filtration buffer: 10 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT.

2.2 Extraction of Bovine Rhodopsin from Dark-Adapted Rod Outer Segments (ROS)

1. ROS membranes isolated from 200 retinas (*see* **Notes 1** and **2**).
2. Glass–glass homogenizer.
3. Beckman J-20 rotor.
4. High-speed 50 ml (Nalgene) centrifuge tubes.
5. Membrane washing buffer: 5 mM Bis–Tris-propane (BTP), pH 7.5.
6. 0.5 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.3.
7. 1 M $ZnCl_2$ stock solution.
8. 1 M DTT stock solution.
9. 10 % NaN_3 stock solution.
10. 1 M hydroxylamine pH 7.5 stock solution (*see* **Note 4**).
11. Resuspension buffer: 50 mM BTP pH 7.5 and 100 mM NaCl.
12. Solubilization buffer: 10 mM BTP pH 7.5, 100 mM NaCl, 20 mM DDM.
13. UV buffer: 10 mM BTP pH 7.5, 100 mM NaCl, 2 mM DDM, 1 mM hydroxylamine.
14. Dialysis buffer: 10 mM (BTP), pH 7.5, 100 mM NaCl, 0.02 mM DDM.

2.3 Preparation of Rhodopsin–Transducin Complex

1. Succinylated concanavalin A (sConA) (Vector Laboratories).
2. CNBr-activated agarose (Santa Cruz Biotechnology Inc.).
3. SRT™ SEC-300 size exclusion column (SEPAX-Technologies Inc.).
4. 150 W fiber light (Dolan-Jenner Industries Inc.).
5. 480–520 band-pass filter (Chroma Technology).
6. Amicon Centricon 30,000 MWCO (Millipore).
7. Bradford ULTRA (Novexin).
8. Disuccinimidyl glutarate (DSG) cross-linker.
9. Equilibration buffer A: 20 mM BTP, pH 6.9, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.5 mM dodecyl-β-D-maltoside (DDM).
10. Elution buffer: 20 mM BTP, pH 6.9, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.5 mM DDM, 200 mM α-methyl-D-mannoside.
11. Regeneration buffer: 10 mM BTP, pH 6.9, 500 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 % Triton, 1 mM DTT.
12. Storage buffer: 10 mM BTP, pH 6.9, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$.
13. Gel filtration buffer A: 20 mM BTP, pH 6.9, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1 mM DDM.
14. 10 mM lauryl maltose neopentyl glycol (LMNG) stock solution.
15. Gel filtration buffer B: 20 mM BTP, pH 6.9, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 0.05 mM LMNG.

2.4 Visualization of Rhodopsin–G_t and sConA–Rhodopsin–G_t Complexes by Transmission Electron Microscope (TEM)

1. 400 mesh, carbon-coated grids (Quantifoil Micro Tools GmBH).
2. 0.22 μm syringe filter (Millipore).
3. FEI TF20 microscope.
4. Tobacco mosaic virus (TMV) (*see* **Note 5**).
5. Dilution buffer: 20 mM BTP, pH 6.9, 120 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, and specified detergent (0.05 mM LMNG or 1 mM DDM) (*see* **Note 6**).

3 Methods

3.1 Extraction and Purification of Transducin

1. All steps must be performed under dim red light and on ice.
2. If using frozen ROS membranes, thaw them on ice. Resuspend them in 40 ml of the isotonic extraction buffer, transfer to the centrifuge 50 ml Beckman tubes, and centrifuge at 18,000 × *g* in Beckman J-20 rotor at 4 °C for 15 min.
3. Discard supernatant 1 and resuspend pellet in 40 ml of the hypotonic extraction buffer.

4. Extract membrane-associated proteins by homogenizing them 4–5 times in glass–glass homogenizer and then pellet homogenized membranes by centrifugation at 25,000 × *g* in Beckman J-20 rotor at 4 °C for 30 min.
5. Collect supernatant 2 and keep it on ice. Repeat extraction step. Collect supernatant 3 and combine it with supernatant 2 (*see* **Note 7**).
6. Spin combined supernatants at 25,000 × *g* in Beckman J-20 rotor at 4 °C for 45 min to pellet residual membrane contaminants.
7. To the resulted supernatant, add HEPES, pH 7.5 to a final concentration 10 mM and $MgCl_2$ to a final concentration of 2 mM, each from 1 M stock solution, and centrifuge the sample again using the above conditions (Fig. 1a).
8. The resulting supernatant applies at a flow rate of 15 ml/h to the 10 × 100 mm column containing 5 ml of pentyl-agarose pre-equilibrated with the equilibration buffer.
9. Wash the column with 10 column volumes of the same equilibration buffer following 30–50 ml of the washing buffer.

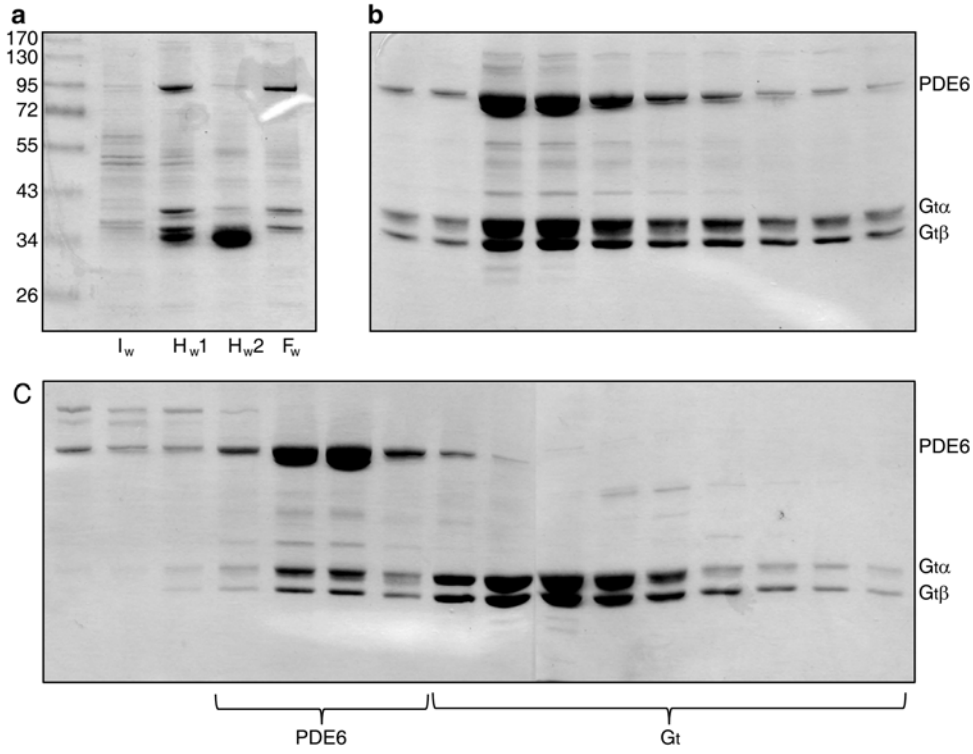

Fig. 1 Extraction and purification of G protein from rod outer segments. (**a**) SDS-PAGE gel electrophoresis demonstrating protein composition in subsequent washes of ROS membranes. I_w isotonic wash, H_w1 hypotonic wash 1, H_w2 hypotonic wash 2, F_w final wash used for purification of G_t. (**b**) SDS-PAGE gel electrophoresis demonstrating protein composition in the fractions eluted from pentyl–agarose column. (**c**) SDS-PAGE gel electrophoresis of protein fractions eluted from gel filtration column

10. Elute bound proteins with ~50 ml of the elution buffer, collecting 1 ml fractions.
11. Check the content of every other fraction by SDS-PAGE gel electrophoresis (*see* **Note 8**) (Fig. 1b).
12. Pool all fractions containing G_t and concentrate with 30,000 NMWL Centricon device to ~400 μl (*see* **Notes 9** and **10**).
13. Clean pentyl–agarose resin with 5–10 column volumes of the cleaning buffer (*see* **Note 11**).
14. To purify G_t to homogeneity, apply 200 μl (half) of the sample prepared in **step 12** on tandem S200 gel filtration column equilibrated with the gel filtration buffer at a flow rate of 0.4 ml/min and collect 0.5 ml fractions.
15. Purify second half of G_t.
16. Check the content of every other fraction by SDS-PAGE gel (Fig. 1c).
17. Pool all fractions containing G_t and concentrate with 30,000 NMWL Centricon device to ~10 mg protein/ml.
18. Determine protein concentration by the Bradford assay [19].

3.2 Extraction of Bovine Rhodopsin from Dark-Adapted Rod Outer Segments (ROS)

1. Use G_t-extracted ROS membranes (*see* **Note 7**). Thaw them on ice, resuspend in 50 ml of membrane washing buffer, and gently homogenize 4–5 times by using glass–glass homogenizer.
2. Transfer homogenized membranes to the centrifuge 50 ml Beckman tubes and pellet them by centrifugation at 25,000 × *g* in Beckman J-20 rotor at 4 °C for 30 min.
3. Repeat membrane wash in membrane washing buffer 2–3 times (*see* **Note 12**).
4. Then spin wash ROS in the resuspension buffer. Resuspend final ROS pellet in small volume (3–4 ml) of the above buffer and measure rhodopsin concentration.
5. To measure rhodopsin concentration, transfer an aliquot (10–20 μl) of ROS to the 1 ml of solubilization buffer and incubate 10–15 min at room temperature mixing on the rotating platform followed by sample centrifugation at 16,000 × *g* for 5 min at 4 °C in the Eppendorf top bench centrifuge. Measure UV-visible absorption spectrum between 260 and 650 nm in the resulted supernatant. Use the absorbance at 498 nm, along with appropriate dilution and molar extinction coefficient $\varepsilon_{498\text{nm}} = 40.600/\text{M/cm}$ to estimate initial rhodopsin concentration.
6. To extract rhodopsin pipette ROS membranes containing about 1 mg of rhodopsin to 2 ml Eppendorf tube and pellet by centrifugation at 16,000 × *g* for 15 min at 4 °C in the Eppendorf top bench centrifuge.

7. Descant residual liquid and to the ROS pellet, add 1 M stock solutions of the following components to reach final concentrations of 55 mM MES, pH 6.3, 100 mM $ZnCl_2$, 1 mM DTT, 0.02 % sodium aside and DDM powder in 4–5 w/w of rhodopsin.
8. Incubate overnight at room temperature mixing on the rotating platform.
9. Remove precipitated proteins by ultracentrifugation at 100,000 × *g* in Beckman TLA-55 rotor at 4 °C for 30 min (*see* **Notes 13** and **14**).
10. Collect supernatant containing solubilized rhodopsin and reduce concentration of $ZnCl_2$ by dialysis against the dialysis buffer for at least 24 h at 4 °C and in the dark (*see* **Note 15**).
11. Measure rhodopsin concentration. Typically it should reach between 8 and 13 mg/ml. The A_{280nm}/A_{498nm} ratio should be about 1.6–1.8 indicating rhodopsin purity about 90 % (Fig. 2).
12. Aliquot rhodopsin sample, wrap in aluminum foil, and freeze to −20 °C for storage.

3.3 Preparation of Rhodopsin–Transducin Complex (Fig. 3)

Rhodopsin–G_t complex should be prepared freshly from $ZnCl_2$-extracted rhodopsin and freshly purified G_t. It is important to control temperature (keep it 4 °C) during complex purification since room temperature promotes complex dissociation.

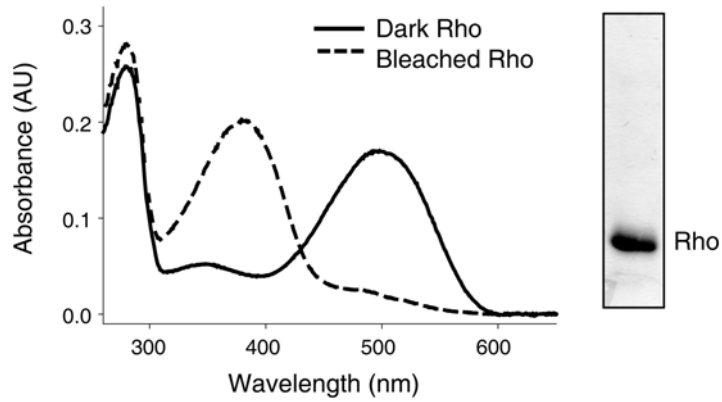

Fig. 2 Absorption spectrum of Zn^{2+}-extracted rhodopsin. *Solid line*, spectrum of dark rhodopsin; *broken line*, spectrum of rhodopsin sample bleached for 5 min through band pass 480–520 nm. SDS-PAGE gel indicates more than 95 % purity of rhodopsin sample

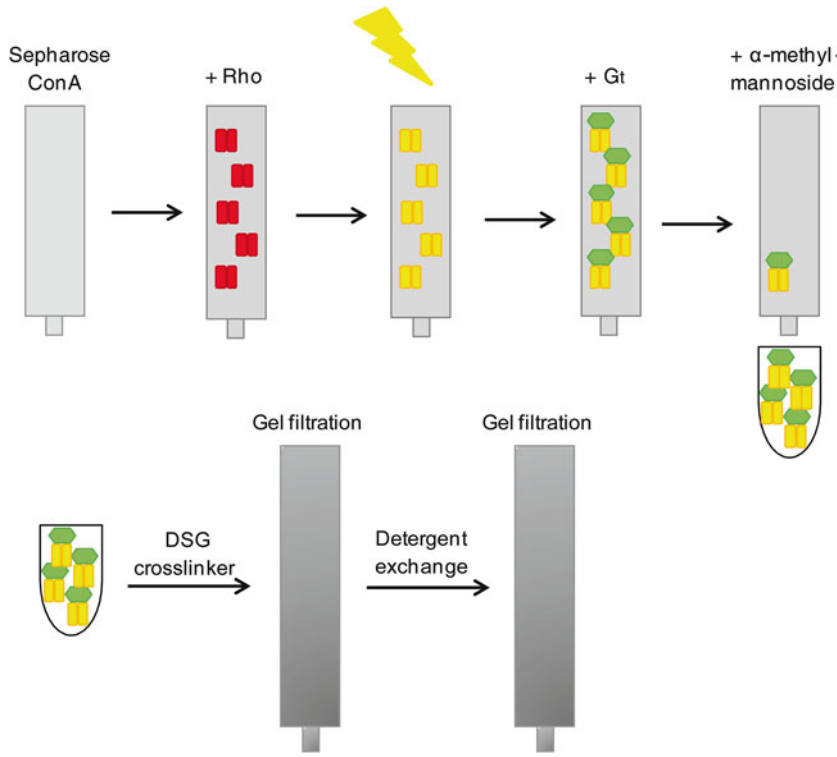

Fig. 3 Purification scheme of sConA-rhodopsin-G_t (sConA-Rho*–G_t) complex. Zn^{2+}-extracted rhodopsin was bound to succinylated concanavalin A (sConA) affinity resin in the dark. After wash rhodopsin bound to the resin was illuminated for 7 min and G_t was immediately loaded on the column. After wash Rho*–G_t complex was eluted with elution buffer containing competing sugar α-methyl-mannopyranoside. Rho*–G_t complex was immediately cross-linked with DSG cross-linker and purified by size exclusion chromatography. To exchange detergent Rho*–G_t complex was passed through the same gel filtration column the second time. To prepare triple sConA–Rho*–G_t complex, cross-linked Rho*–G_t was incubated with sConA and then separated by gel filtration

3.3.1 Formation and Purification of Rhodopsin–Transducin Complex by sConA Affinity Chromatography

1. First prepare sConA affinity resin by coupling of sConA to CNBr-activated agarose at a density of 8 mg sConA/ml of resin according to standard protocol.
2. Pack ~6–7 ml of sConA affinity resin to 5 × 200 mm long, thin column and equilibrate the resin with the equilibration buffer.
3. Dilute solubilized rhodopsin with the same equilibration buffer to the final concentration ~0.2 mg/ml and load it at a flow rate of 0.5 ml/min onto the sConA column (*see* **Note 16**).
4. Wash the column with 5 column volumes of the same ice-cold buffer.
5. Then illuminate the column for 10 min with a 150 W fiber light covered with 480–520 nm band-pass filter.
6. Immediately after light exposure load purified and diluted in the equilibration buffer A to ~0.2 mg/ml native G_t at a flow rate of 0.5 ml/min.
7. Wash the column with 10 column volumes of the above buffer.

8. Elute the complex with the elution buffer containing 200 mM α-methyl-D-mannoside at a flow rate of 0.2 ml/min (*see* **Note 17**). Collect 1 ml fractions.
9. Take 20 µl aliquot and check the content of each fraction by SDS-PAGE gel.
10. Perform chemical cross-linking in all fractions containing rhodopsin–G_t complex.

3.3.2 Chemical Cross-Linking of Rhodopsin–Transducin Complex

1. To each fraction eluted from the sConA resin and containing rhodopsin–G_t complex, add 100 mM DSG to the final concentration of 1 mM and incubate the samples 2 h on ice.
2. Stop cross-linking reaction by adding 1 M Tris, pH 8.0 to the final concentration of 50 mM and incubate for 15 min on ice.
3. Take 20 µl aliquot and run SDS-PAGE gel to check efficiency of the cross-linking reaction (Fig. 4a).

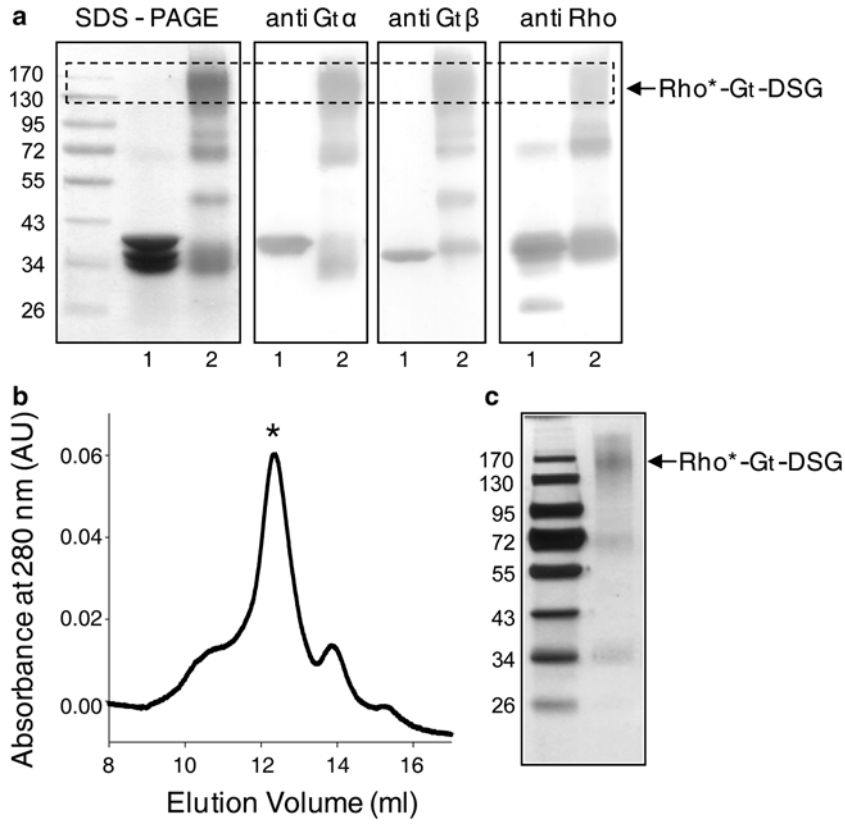

Fig. 4 Preparation and purification of rhodopsin-G_t (Rho*-G_t) complex. (**a**) DSG cross-linking of the Rho*–G_t complex purified by sConA affinity chromatography. SDS-PAGE gel, stained with Coomassie blue and immunoblot analyses with antibody against $G_t\alpha$, $G_t\beta$, and rhodopsin, indicated formation of intact Rho*–G_t complex after treatment with DSG (lanes 1, non-cross-linked complex and lanes 2, DSG-cross-linked complex). (**b**) Gel filtration elution profile of DSG-cross-linked Rho*–G_t complex. (**c**) Silver-stained SDS-PAGE gel analysis of the peak fraction indicated with a *star*

4. Pool all fractions containing cross-linked rhodopsin–G_t complex and concentrate to 200 µl with 30,000 NMWL Centricon device. Use this sample for size exclusion chromatography.
5. Regenerate sConA affinity resin by washing with 5–10 column volumes of the regeneration buffer at a slow rate of 0.2 ml/min.
6. Then wash the column with 5 column volumes of the storage buffer and store it at 4 °C (*see* **Note 18**).

3.3.3 Isolation of Cross-Linked Rhodopsin–Transducin Complex by Gel Filtration

1. Load 200 µl of cross-linked rhodopsin–G_t complex onto a SRT™ SEC-300 gel filtration column equilibrated with the gel filtration buffer A at a flow rate of 0.4 ml/min. Fractions containing cross-linked complex will separate from free rhodopsin and free G_t (*see* **Note 19**) (Fig. 4b, c).
2. Pool fractions containing cross-linked rhodopsin–G_t complex, concentrate to 200 µl, and process on the same gel filtration to increase sample homogeneity.
3. If the exchange of the detergent (from DDM to LMNG) is desired, pool fractions containing rhodopsin–G_t complex and concentrate to 100 µl. Then add 100 µl of 10 mM LMNG stock solution dissolved in the gel filtration buffer lucking detergent and incubate for 1 h on ice.
4. Gel filtrate this sample using the same column (SRT™ SEC-300) equilibrated with the gel filtration buffer B.
5. Determine protein concentration in the peak fractions by the Bradford assay.
6. Use peak fractions containing rhodopsin–G_t complex for negative staining and analysis by transmission electron microscopy (TEM).

3.3.4 Labeling of Rhodopsin–Transducin Complex with sConA

1. Concentrate fractions containing rhodopsin–G_t–LMNG complex to 100 µl and incubate at 4 °C with the excess of sConA in the gel filtration buffer B at least 1 h (*see* **Notes 20** and **21**).
2. Purify triple sConA–rhodopsin–G_t complex by the same gel filtration column (SRT™ SEC-300) equilibrated with the gel filtration buffer B (Fig. 5a).
3. Determine protein concentration in the peak fractions by the Bradford assay.
4. Use peak fractions containing rhodopsin–G_t complex for negative staining and analysis by TEM.

3.4 Visualization of Rhodopsin–G_t and sConA–Rhodopsin–G_t Complexes by TEM

1. Dilute all purified complexes to ~20 µg/ml with the dilution buffer (*see* **Note 5**).
2. Glow discharge carbon-coated 400 mesh grids for 1 min just before adsorption of protein complexes.

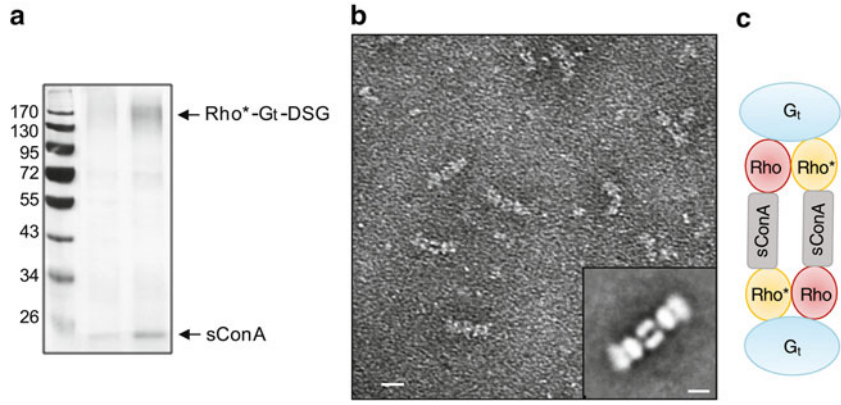

Fig. 5 Purification and EM analysis of sConA-decorated Rho*–G_t complex. (**a**) Silver-stained SDS-PAGE gel analysis of protein fractions eluted from gel filtration column, containing triple sConA–Rho*–G_t complex. (**b**) TEM image of negatively stained sConA–Rho*–G_t complex. Scale bar 20 nm. *Inset*, 2-dimensional average of multiple sConA–Rho*–G_t particles. (**c**) Scheme of sConA–Rho*–G_t composition indicating stoichiometry of the Rho*–G_t complex

3. Load 4 μl of protein on the grid and incubate for 1 min.
4. Adsorb the excess of protein solution with the filter paper, and immediately wash grids with four drops of distilled H_2O (*see* **Note 22**).
5. Negatively stain protein complexes with 2 % (w/v) uranyl acetate (*see* **Note 23**).
6. Image the sample with available TEM (Fig. 5b, c).

4 Notes

1. Fresh bovine retinas can be isolated from fresh bovine eyes obtained from local slaughter house. Frozen retinas can be purchased from W L Lawson Company LLC.
2. Bovine rod outer segment (ROS) membranes can be isolated by step sucrose gradient centrifugations described in detailed previously in ref. 18 and in Chapter 2 of this book.
3. All buffers that contain DTT should be prepared freshly. Stock solution of 1 M DTT can be prepared in advance and stored at −20 °C.
4. Hydroxylamine solution is unstable; therefore, it should be prepared freshly or kept −20 °C in small aliquots.
5. Tobacco mosaic virus (TMV) can be added to the protein sample before imaging by TEM as an internal standard in the concentration of ~10 μg/ml. It forms larger elongated structures and can be helpful in recognizing good area on the grid covered with the appropriate thickness of stain (2 % uranyl acetate).

6. Prepare 10 mM stock solution of LMNG in the buffer of interest. Solubilization of this detergent may take a few (1–3) hours.
7. Membrane pellet stripped off G_t can be stored at −80 °C and used for rhodopsin extraction.
8. Typically 12 or 10 % SDS-PAGE gels should be used for G_t separation.
9. After pentyl–agarose column, the sample contains mixture of G_t and PDE6. Thanks to the large enough difference in molecular masses (G_t, 86 kDa and PDE6, 218 kDa), these proteins can be further separated by size exclusion chromatography.
10. At this step purification can be stopped. Keep the sample on ice until the next day.
11. Store pentyl–agarose column in the cleaning buffer at 4 °C.
12. In the hypotonic buffer, ROS membranes are very loose; thus carefully descant the supernatant with 5 ml pipette.
13. To spin the sample in the bench top ultracentrifuge in TLA-55 rotor, transfer the sample to the Eppendorf Beckman tubes for ultracentrifuge.
14. Up to 50 % of rhodopsin can be lost during this step.
15. Change dialysis buffer at least twice.
16. The entire preparation of the rhodopsin–G_t complex is performed in the dark room, except of rhodopsin light activation step.
17. To elute the rhodopsin–G_t complex from the sConA affinity resin, first apply ~3–4 ml of the elution buffer at a flow rate of 0.5 ml/min, then close the column, and incubate at 4 °C for at least 30 min.
18. sConA affinity resin can be used several times but after each use it loses its binding capacity.
19. The rhodopsin–G_t complex is light sensitive. Perform gel filtration experiment under dim red light.
20. sConA is purchased as a powder. Reconstitute this protein at least 1 h before the binding with rhodopsin–G_t complex in the buffer composed of 10 mM BTP, pH 6.9, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$.
21. Binding of sConA to the rhodopsin–G_t complex can be performed overnight if needed.
22. In the standard protocol of negative staining, two drops of distilled H_2O are used [20]. However for membrane proteins, we recommend to use more wash to wash out the excess of detergent.
23. To prepare uranyl acetate dissolves 1 g stain in 50 ml of distilled water in 50 ml conical tube wrapped in aluminum foil. Place on the rotating platform and mix for 1 h. Filtrate this solution through the 0.22 μm syringe filter. This solution can be stored at room temperature or aliquot and kept in −20 °C.

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