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The Rhodopsin-Arrestin-1 Interaction in Bicelles

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Abstract

G-protein-coupled receptors (GPCRs) are essential mediators of information transfer in eukaryotic cells. Interactions between GPCRs and their binding partners modulate the signaling process. For example, the interaction between GPCR and cognate G protein initiates the signal, while the interaction with cognate arrestin terminates G-protein-mediated signaling. In visual signal transduction, arrestin-1 selectively binds to the phosphorylated light-activated GPCR rhodopsin to terminate rhodopsin signaling. Under physiological conditions, the rhodopsin-arrestin-1 interaction occurs in highly specialized disk membrane in which rhodopsin resides. This membrane is replaced with mimetics when working with purified proteins. While detergents are commonly used as membrane mimetics, most detergents denature arrestin-1, preventing biochemical studies of this interaction. In contrast, bicelles provide a suitable alternative medium. An advantage of bicelles is that they contain lipids, which have been shown to be necessary for normal rhodopsin-arrestin-1 interaction. Here we describe how to reconstitute rhodopsin into bicelles, and how bicelle properties affect the rhodopsin-arrestin-1 interaction.

Keywords

Arrestin; Bicelles; Lipid; Model membrane; Receptor; Rhodopsin

1 Introduction

The light receptor rhodopsin is located in the disk membranes of rod outer segments in the retina. When rhodopsin absorbs a photon of light, it initiates an electrical signal that allows dim light vision. Rhodopsin belongs to the largest membrane protein family: G-protein-coupled receptors (GPCRs). Rhodopsin signals through its cognate G protein transducin, and this signaling is quenched by visual arrestin-1. Light activation and subsequent phosphorylation by G-protein-receptor kinase-1 (GRK1, a.k.a. rhodopsin kinase) collectively promote arrestin-1 binding, which precludes any further coupling of activated rhodopsin to transducin (Fig. 1) [1, 2].

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Structure-function studies of both rhodopsin and arrestin-1 have advanced our understanding of the molecular mechanisms of vision. However, caution should be taken when applying these in vitro findings to the interpretation of the physiological process of vision. A major concern comes from the fact that most studies begin by extracting the receptor or the receptor complex from the lipid bilayer into detergent micelles, and thus the influence of the membrane is not accounted for. However, the lipid composition of the highly specialized disk membranes dramatically affects rhodopsin signaling (reviewed in [3, 4]). For this reason, several alternative membrane model systems have been explored that may be more native-like than micelles. Here we focus on the reconstitution of rhodopsin into bicelles and its consequences for rhodopsin signaling, particularly arrestin-1-mediated signal termination.

1.1 Introduction to the Role of Arrestin-1 in Rhodopsin Signaling

Arrestin-1 (a.k.a. S antigen, 48 kDa protein, rod or visual arrestin) mediates rhodopsin signal termination, which involves two steps. In the first step, light-activated rhodopsin (*Rh) is phosphorylated at multiple sites by GRK1. Arrestin-1 requires a minimum of three rhodopsin-attached phosphates for high-affinity binding [5, 6]. In the second step, arrestin-1 binds to active phosphorylated rhodopsin (p-*Rh) and blocks further transducin coupling by steric exclusion (Fig. 1) [2]. The high selectivity of arrestin-1 for p-*Rh (Fig. 2) ensures that signaling is terminated only when rhodopsin is in the activated and phosphorylated state [7–10].

The molecular mechanism underlying arrestin-1 selectivity has been extensively explored (reviewed in [11]). Arrestin-1 has two distinctly folded domains, termed the N- and C-domains (Fig. 3) [12–14]. In the basal, non-receptor-binding state, the distal C-terminal segment binds to the N-domain through two major interaction interfaces. One is the polar core comprising five charged residues in the N-domain (D30, R175), the C-domain (D296, D303), and the distal C-terminus (R382) (Fig. 3). The other is the hydrophobic three-element interaction between β-strand I (V11, I12, F13) and α-helix I (L103, L107, L111) in the N-domain and F375, V376, F377 in the C-terminus (Fig. 3) [12]. Receptor binding is proposed to disrupt the polar core and destabilize the three-element interaction [9, 15–17], which results in the release of the distal C-terminus [15, 18, 19]. Complementary studies employing site-directed spin labeling and electron paramagnetic resonance (EPR), functional mapping of arrestin-1 at single amino acid resolution, and X-ray crystallography of pre-activated arrestin-1 have also suggested that several arrestin-1 loops are involved in receptor binding [19–23]. In addition to these discrete conformational changes, recent evidence strongly suggests that arrestin-1 gains significant conformational flexibility upon binding to p-*Rh [15].

1.2 Introduction to Bicelles

Bicelles, first introduced by the Prestegard lab [24–27], have a central planar bilayer formed by long-chain lipids and edges shielded by either short-chain lipids or detergents (Fig. 4). The planar surface of bicelles and bilayered interior mimics the biological membrane much better than detergent micelles, while due to their small size and monodispersity, the attractive features of conventional micelles are retained. Indeed, bicelles have been

successfully used to crystallize several membrane proteins (reviewed in [26]), particularly the GPCR β2 adrenergic receptor [28], after decades of efforts using traditional detergent crystallization failed.

The long-chain lipid determines the thickness of the lipid bilayer, while phospholipids with different head groups can tailor the charge characteristics of the surface and provide lipid composition versatility. Both the total phospholipid concentration (*c*) and ratio (*q*) of long-chain/short-chain phospholipid (or long-chain phospholipid/detergent) play an important role in shaping bicelles. Intuitively, a high *q* value indicates an extended bilayer, while bicelles with a low *q* value (e.g., $q < 1$) more closely resemble round isotropic spheres (Fig. 4). It is a matter of some debate as to whether bicelles with $q \leq 0.5$ retain any of the characteristics of ideal bicelles or whether they should be regarded as classical mixed micelles (*see*, e.g., [29, 30]). Interestingly, it has been reported that mixtures of n-Dodecyl β-D-maltoside (DDM) and cholesteryl hemisuccinate (CHS) have an architecture more reminiscent of bicelles than micelles. The modulation of micelle morphology was shown to contribute significantly to stabilizing GPCRs, such as opioid-like receptor ORL-1 [31]. In fact, it is now common practice to include 10 % CHS in detergent micelle solutions for the purification and structural characterization of GPCRs [32].

1.3 Introduction to Reconstitution of Rhodopsin into Bicelles

The idea of including phospholipids in detergent micelles was applied to the study of rhodopsin as early as 1995, although at that time, what are termed bicelles were simply called a “phospholipid-detergent mixture” [33]. In that study, the influence of lipids on the stability of opsin, the apoprotein of rhodopsin, was assessed. Opsin denatures rapidly in detergent solution, losing its ability to bind 11-*cis*-retinal and regenerate to rhodopsin [34]. However, the presence of asolectin, a mixture of phospholipids, significantly improved the stability of opsin such that the protein was functional following detergent extraction and subsequent purification [33]. Shortly thereafter, the Khorana group replaced asolectin with a single phospholipid, dimyristoylphosphatidylcholine (DMPC), while keeping the 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) as the detergent component [35]. The DMPC/CHAPS bicelles successfully extracted opsin from membranes, and opsin reconstituted into these bicelles retained its retinal binding activity [35]. The effects of DMPC/CHAPS and DMPC/dihexanoylphosphatidylcholine (DHPC) bicelles on rhodopsin and opsin were more extensively characterized in 2007 [36]. The authors showed that rhodopsin and opsin had significantly increased stability in both types of bicelles as compared to their stability in detergent micelles. The *q* value modulated the opsin stability, which suggests that the bicelle size impacts opsin stability. Interestingly, the authors also showed that inclusion of CHAPS rather than DHPC in the DMPC/ CHAPS bicelles further stabilized opsin [36]. This indicates that the detergent component of bicelles can affect protein stability either by directly interacting with the protein or by altering the bilayer properties. Due to high working lipid/protein ratios, bicelles usually contain monomeric GPCRs [15]. While this could create problems for constitutively dimeric class C GPCRs [37, 38], monomeric rhodopsin was shown to be sufficient for effective coupling to transducin [39, 40], efficient phosphorylation by GRK1 [6], and also arrestin-1 binding with physiologically relevant nanomolar affinity [6], which leads to the same conformational

rearrangements in bound arrestin-1 as its interaction with p-*Rh in native disk membranes [20]. Mutations that select for monomeric-only protomers of rhodopsin were also shown to be phosphorylated by GRK1 and bind arrestin-1 effectively [41, 42].

Bicelles not only serve as an excellent model membrane system for rhodopsin but also greatly facilitate structural and dynamics studies of the rhodopsin-arrestin-1 interaction. Indeed, prior to the application of bicelles to the rhodopsin-arrestin-1 interaction, studies of this complex in a defined, purified system were dramatically hindered since arrestin-1 binding to rhodopsin is severely diminished when the receptor is solubilized with commonly used detergents ([43] and unpublished results). Near-UV circular dichroism spectra indicate that detergents cause significant changes of arrestin-1 tertiary structure [15]. One possibility is that detergent penetration can potentially disrupt one of the two key interactions that hold arrestin-1 in the basal state (Fig. 3) and result in the “melting” of the arrestin-1 global structure. Interestingly, acidic phospholipids can restore the binding of arrestin-1 to rhodopsin purified in DDM. Moreover, arrestin-1 retains its selective binding to rhodopsin reconstituted in bicelles [43]. In addition to the fact that bicelles provide a milder environment than detergents, they also have several other clear advantages over detergent micelles in case of rhodopsin-arrestin interactions. First, the central planar bilayer of bicelles creates an extended native membrane-like surface, while detergent micelles might cover some receptor elements involved in arrestin-1 binding. Second, bicelles contain phospholipids that can regulate the rhodopsin-arrestin-1 interaction either via electrostatics or by directly binding to either rhodopsin or arrestin-1. It was found that acidic phospholipids significantly prolong the half-life of the rhodopsin-arrestin-1 complex and that 20–50 % lipids with negatively charged head group are essential to promote maximum binding [6, 43].

2 Materials

2.1 Preparation of Bicelles

1. Buffer A: 25 mM Bis–Tris, pH 6.5, 100 mM NaCl, 0.1 mM EDTA.
2. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG).
3. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC).
4. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC).

2.2 Reconstitution of Rhodopsin with Bicelles

1. Rod outer segment (ROS) membranes.
2. Buffer A: 25 mM Bis–Tris, pH 6.5, 100 mM NaCl, 0.1 mM EDTA.

2.3 Assessment of Structural Integrity of Arrestin by Near-UV Circular Dichroism (CD)

1. Buffer A: 25 mM Bis–Tris, pH 6.5, 100 mM NaCl, 0.1 mM EDTA.
2. Denaturing Buffer: 8 M urea in Buffer A.

2.4 Preparation of Radiolabeled Arrestin-1

1. Transcription mix [44]: 120 mM 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES), pH 7.5, 2 mM spermidine, 16 mM $MgCl_2$, 40 mM dithiothreitol (DTT), 3 mM each of adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), and uridine triphosphates (UTP), 2.5 U/ml inorganic pyrophosphatase, 200 U/ml RNasin, and 1,500 U/ml SP6 RNA polymerase.
2. 7.5 M LiCl.
3. 2.5 M LiCl.
4. 70 % (v/v) ethanol.
5. 3 M sodium acetate, pH 5.2.
6. 100 % ethanol.
7. Translation mix [7, 45]: 70 % rabbit reticulocyte lysate, 120 mM potassium acetate, 30 mM creatine phosphate, 200 μg/ml creatine kinase, 200 U/ml RNasin or Prime RNase inhibitor, 0.1 μg/ml pepstatin, 0.1 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 5 mM cAMP, 50 μM 19 unlabeled amino acids and 40–50 μM [^{14}C]-leucine (14,000–35,000 dpm/ml). When [^{3}H]-leucine is used, 800,000–1,000,000 dpm/ml is added along with [^{14}C]-leucine to a final concentration of 30–50 μM.
8. 40 mM ATP.
9. 40 mM GTP.
10. 10 % (w/v) trichloroacetic acid (TCA).
11. 5 % (w/v) trichloroacetic acid (TCA).
12. Scintillation fluid.
13. Buffer B: 50 mM Tris–HCl pH 7.5, 50 mM potassium acetate and 2 mM EDTA.
14. Buffer C: 10 mM Tris–HCl, pH 7.5, 100 mM NaCl.

2.5 Direct Binding Assay in Bicelles with Radiolabeled Arrestin-1

1. Binding Buffer: 50 mM Tris–HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 mM DTT, 100 mM potassium acetate.
2. Buffer C: 10 mM Tris–HCl, pH 7.5, 100 mM NaCl.
3. Scintillation fluid.

2.6 Expression of Isotopically Labeled Arrestin-1

1. BL21(DE3) cells.
2. LB agar plate with 100 mg/l ampicillin.
3. LB with 100 mg/l ampicillin (LB/A).

4. 1 l M9 minimal media prepared in D_2O supplement with 1 g/l of $^{15}NH_4Cl$: dissolve 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 4 g glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1 g of $^{15}NH_4Cl$ in D_2O.
5. 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG).
6. ^{15}N, ^{2}H-Bioexpress.
7. 1 l LB prepared in D_2O with 100 mg/l ampicillin: dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in 950 ml D_2O.
8. Glucose-D6, α-ketovaleric acid salt (3-methyl-^{13}C,3,4,4,4-D4), α-ketobutyric acid salt (methyl-^{13}C,3,3-D2).

2.7 NMR Study of Arrestin-1 Binding to Rhodopsin in Different States

1. Buffer D: 25 mM Bis–Tris, pH 6.5, 100 mM NaCl, 0.1 mM EDTA.
2. 1 M dithiothreitol (DTT).
3. D_2O.

3 Methods

3.1 Preparation of Bicelles

Two different methods of bicelle preparation are used, depending on both the stock solution concentration and the *q* value. For relatively low stock concentrations (<20 % w/v) or low *q* values ($q < 1$), we varied the temperature to induce phase transitions, as described in Subheading 3.1.1. The gel-to-liquid crystalline phase transition temperature for DMPG-DMPC mixtures is roughly 24 °C. For relatively high stock concentrations (>20 % w/v) or high *q* values ($q > 1$), we use a custom extrusion apparatus to mix bicelles in addition to varying the temperature change to induce phase transition, as described in Subheading 3.1.2.

3.1.1 Preparation of Bicelles at Low Stock Concentration or Low q Value

1. Calculate the amount of each component needed. For 1 ml 12.5 % w/v stock solution with $q = 0.33$ (molar ratio), this means 1 mol lipid (DMPC + DMPG) per 3 mol DHPC at a ratio of 1 mol DMPG per 4 mol DMPC: 32 mg of DMPC, 8 mg of DMPG, and 85 mg of DHPC (*see* **Note 1**).
2. Dissolve 85 mg of DHPC in 500 μl of Buffer A in one tube and mix 8 mg of DMPG and 32 mg of DMPC in 500 μl of Buffer A in another tube by vortexing. DHPC solution will become clear, while the DMPG and DMPC mixture remains cloudy.
3. Combine the solutions in these two tubes and mix them well by vortexing.

[1]We include DMPG in the DMPC/DHPC bicelles because DMPG contains the negatively charged head group, which is essential for the maximum binding of arrestin-1 to rhodopsin. Whether or not DMPG is added, the ratio of DMPG to DMPC can be adjusted for each individual study.

4. Cycle by incubating the mixture at 42 °C for 2 min and then at 0 °C (on ice) for 2 min. Repeat this cycle until the solution is completely clear (*see* **Note 2**).

5. Spin at 13,000 × *g* in a tabletop centrifuge for 10 min to remove of any insoluble material. The supernatant is the bicelle solution, now ready to use.

3.1.2 Preparation of Bicelles at a High Stock Concentration or a High q Value

1. Calculate the amount of each component needed. For 1 ml of 35 % w/v stock solution with $q = 3$ (molar ratio), that is 3 mol of lipid (DMPC + DMPG) per 1 mol of DHPC at a ratio of 1 mol of DMPG per 4 mol DMPC: 224 mg of DMPC, 57 mg of DMPG, and 66 mg of DHPC.

2. Dissolve 66 mg of DHPC in 1 ml of Buffer A.

3. Weigh 224 mg of DMPC and 57 mg of DMPG in one tube, add 500 μl of DHPC solution, mix by vortexing, and add it to one syringe (Fig. 5).

4. Add the remaining 500 μl DHPC solution to another syringe, which is then attached nose to nose with the other (lipid solution containing) syringe. Mix by extruding the solution back and forth between the syringes, effectively extruding the mixture through the narrow connecting tube.

5. Cycle the bicelle mixture through its phase transition temperature (24 °C) four times by incubating the entire apparatus at 4 and 55 °C; homogenize between cycles.

6. Dispense the bicelle mixture to a tube on ice and centrifuge at 13,000 × *g* for 10 min to remove bubbles and insoluble material. The supernatant is the bicelle solution, now ready for use.

3.2 Reconstitution of Rhodopsin into Bicelles

1. All steps are performed in the dark under dim red light at 4 °C.

2. Measure the rhodopsin 500 nm absorption in the dark (extinction coefficient: 40,600 M^{-1} cm^{-1}) to determine the rhodopsin concentration.

3. Pellet ROS membranes by centrifugation at 13,000 × *g* for 30 min, discard supernatant, and wash the pellet twice with Buffer A.

4. Pellet ROS membranes after washing and estimate the volume of the membrane pellet by weighing in a pre-weighted 1.5 ml eppendorf tube (*see* **Note 3**). Add equal volume of 11.25 % stock of bicelles to the membranes, pipette up and down to dissolve the membranes, and incubate on a shaker in dark cold room for 30 min (*see* **Note 4**).

5. Centrifuge as above to remove insoluble material and save the supernatant.

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[2]Typically, it takes about 3–4 iterations to get the solution clear. Homogenizing the DMPG and DMPC mixture greatly assists this process.

[3]We estimate the volume of membrane by weight, assuming 1 mg is approximately 1 μl.

[4]The solution should become clear after the bicelles are added and the sample is mixed. If the solution is cloudy, more bicelles should be added.

6. Determine the concentration of rhodopsin in the supernatant (using absorption at 500 nm) and calculate the bicelle concentration using $\frac{11.25\% \times V1}{V2}$ (V1 is the added bicelle volume and V2 is the final volume) (*see* **Note 5**).

7. Typically, 70 μl of 11.25 % bicelle stock are added to ROS membranes containing 1 mg of rhodopsin, which yields a final rhodopsin concentration around 8 mg/ml and bicelle concentration around 8 %. The lipid from ROS membrane is also dissolved in bicelles and the concentration is around 8 mg/ml.

3.3 Assessment of the Structural Integrity of Arrestin-1 by Near-UV Circular Dichroism (CD) Spectroscopy

1. Prepare 500 μl arrestin-1 protein samples at the concentration of 50 μM in Buffer A, Denaturing Buffer, and the model membrane conditions of choice: for example, 0.2 % DDM micelles or 4 % bicelle mixtures prepared as described in Subheading 3.1. Incubate arrestin-1 in these conditions at room temperature overnight, ~12 h (*see* **Note 6**).

2. Transfer the samples to a masked cell (1 cm pathlength) with a minimum volume of 500 μl, and collect the near-UV CD spectra over the wavelength range of 250–320 nm using the CD spectropolarimeter with the bandwidth of 1 nm. Average five scans for each sample to achieve reasonable signal to noise. For each protein sample, collect the near-UV CD spectrum of the same buffer without protein, which yields the blank signal that should be subtracted from the spectrum of protein sample to reveal the signal from protein only.

3. The raw data from Jasco J-810 is given in ellipticity (measured in millidegrees). Apply the following equation: $[\theta] = \frac{100 \times (\text{signal})}{c \times n \times l}$ (*c* is the protein concentration in mM, *n* is the total number of amino acid residues, and *l* is the cell path-length in cm) to convert the output to units of mean residue ellipticity (degrees squared centimeters per decimole) (Fig. 6).

3.4 Preparation of Radiolabeled Arrestin-1

1. Purify and linearize plasmid DNA where arrestin-1 coding sequence is under control of a SP6 promoter [44, 46].

2. Incubate 10 μg of linearized DNA in 300 μl of the transcription mix at 38 °C for 90 min.

3. Add 150 μl of 7.5 M LiCl, incubate on ice for 10 min, and centrifuge for 10 min at 16,100 × *g* and 4 °C to pellet mRNA.

4. Wash the pellet with 1 ml of 2.5 M LiCl at 4 °C.

5. Wash the pellet with 1 ml of 70 % (v/v) ethanol at room temperature.

[5]Measure the final volume with a pipette.
[6]The incubation time and temperature is case dependent.

6. Let the pellet dry for 5–7 min or until it is completely dry and then dissolve it in 300 μl of ultrapure distilled water (we use a volume equal to that of the transcription reaction). Remove an aliquot to measure the amount of mRNA via absorption at 260 nm.

7. Add 30 μl of 3 M sodium acetate, pH 5.2, and 960 μl of ethanol. Then vortex and incubate on ice for 10 min (*see* **Note 7**).

8. Before translation, pellet the necessary amount (~24 μg for 0.2 ml translation) of mRNA from this suspension, wash with 70 % ethanol, dry for 5–7 min, and dissolve in 16 μl of ultra-pure distilled water.

9. Incubate mRNA with 184 μl translation mix [7, 45] for 2 h at 22.5 °C.

10. Add 4 μl of 40 mM ATP and 4 μl of 40 mM GTP (1 mM final concentrations) and incubate at 37 °C for 7 min (ribosome runoff).

11. Cool the samples on ice and centrifuge at 600,000 × *g* for 60 min at 4 °C (in TLA 120.1 rotor, Beckman TLA tabletop ultracentrifuge) to pellet ribosomes and aggregated proteins. The supernatant contains [^{14}C]- and [^{3}H]-labeled arrestin-1 and free [^{14}C]- and [^{3}H]-leucine.

12. Take a 2 μl aliquot, add it to 18 μl water (tenfold dilution), and spot 5 μl of diluted sample onto Whatman 3MM paper (1 cm × 1 cm square); incubate the paper in ice-cold 10 % (w/v) TCA for >10 min (to wash away free radiolabeled leucine) and then in boiling 5 % TCA for exactly 10 min (hydrolyzes aminoacyl-tRNA and removes radiolabeled leucine attached to tRNA). Then, let the paper dry and add each square to a separate scintillation vial, let the protein dissolve in 0.5 ml of Buffer B, then add 5 ml of scintillation fluid, briefly shake, and quantify protein-incorporated radioactivity using scintillation counter capable of quantifying ^{3}H and ^{14}C separately.

13. Measure the radioactivity of the control sample (translation mix without mRNA).

14. Calculate protein yield based on specific activity of the radiolabeled leucine used. Dividing the total protein-incorporated radioactivity (dpm per microliter of translation mix with the value from the control sample subtracted) by the specific activity (dpm/fmol) of the arrestin-1 gives the yield in fmol/μl (*see* [44] for details).

15. To separate the free [^{14}C]- and [^{3}H]-leucine from [^{14}C]- and [^{3}H]-labeled arrestin-1, load the supernatant onto a 2 ml Sephadex G-75 column equilibrated with Buffer C. Add 100 μl of Buffer C and collect the eluted 100 μl buffer. Repeat this for 15 times. For the last elution, add 500 μl of Buffer C and collect the eluted 500 μl buffer.

16. Take 2 μl of each elution and add it to 18 μl H_2O, add 5 ml of scintillation fluid, and determine the radioactivity in each elution fraction to get the elution profile and pool the fractions containing arrestin-1 together. This is translated arrestin-1 ready to use.

[7] mRNAs in this suspension can be stored at −80 °C for several years.

3.5 Direct Binding Assay in Bicelles with Radiolabeled Arrestin-1

1. Incubate 100 fmol of radiolabeled arrestin-1 with the 7.5 pmol of p-*Rh in bicelles in a final volume of 50 μl of Binding Buffer at 30 °C under ambient light.
2. Cool the samples on ice and load them on 2 ml Sephadex G-75 columns equilibrated with Buffer C (*see* **Note 8**).
3. Wash the column with 100 μl and then 500 μl Buffer C.
4. Elute with 600 μl Buffer C into scintillation vials, add 5 ml of scintillation fluid, and count the radioactivity from bound arrestin-1 as total binding.
5. Determine the nonspecific binding in the presence of equal amount of empty bicelles.
6. Subtract the nonspecific binding from total binding to obtain the specific binding. Dividing specific binding (dpm) by arrestin-1-specific activity (dpm/fmol) gives the fmol of arrestin bound to the p-*Rh (*see* Fig. 7).

3.6 Expression of NMR Isotopically Labeled Arrestin-1

3.6.1 Preparation of ^{2}H, ^{15}N-Labeled Arrestin-1

1. Transform a pTrc-based plasmid encoding arrestin-1 [44] to BL21(DE3) cells and plate on LB agar with 100 mg/l ampicillin.
2. Start a 10 ml small culture from a single colony in LB with 100 mg/l ampicillin (LB/A) at 30 °C overnight.
3. Centrifuge the 10 ml small culture for 3–5 min at 2,000 rpm (800 × *g*). Resuspend the cell pellet in 1 l M9 minimal media prepared in D_2O supplemented with 1 g/l of $^{15}NH_4Cl$.
4. Incubate the culture at 30 °C with vigorous shaking at 250 rpm until OD reaches 0.8.
5. Add 250 μl of 100 mM IPTG to 1 l culture. The final IPTG concentration is 25 μM. Continue shaking for another 18 h (*see* **Note 9**).

3.6.2 Preparation of ^{13}C, ^{1}H-Methyl-Labeled Perdeuterated Arrestin-1

1. Transform a pTrc-based plasmid encoding arrestin-1 to BL21 (*DE3*) cells and plate on LB agar with 100 mg/l ampicillin.
2. Start a 5 ml small culture from a single colony in a LB prepared in D_2O with 100 mg/l ampicillin at 30 °C overnight.
3. Transfer 50 μl of overnight cultures prepared in **step 2** into 5 ml M9 minimal medium prepared in D_2O and contains 4 g/l glucose-D6.

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[8]Different columns are used to separate rhodopsin-bound or free arrestin-1. For rhodopsin in native disk membranes, we use Sepharose 2B, while for rhodopsin reconstituted in bicelles or nanodisks, we use Sephadex G-75.

[9]Optional: 5–10 ml of ^{15}N, ^{2}H-Bioexpress can be added to the M9 minimal media. This can significantly shorten the cell growth time in **step 4** from 3 days to 1 day.

4. Inoculate 5 ml of cultures in **step 3** to 500 ml of M9 minimal medium that is prepared in D_2O and contains 4 g/l glucose-D6, 37.5 mg/l 3-methyl-^{13}C, 3,4,4,4-D_4-α-ketovaleric acid salt, and 22.5 mg/l methyl-^{13}C, 3,3-D_2-α-ketobutyric acid salt.
5. Shake the cultures at 30 °C until OD600 reaches 0.6. Add additional 3-methyl-^{13}C, 3,4,4,4-D_4-α-ketovaleric acid salt and methyl-^{13}C, 3,3-D_2-α-ketobutyric acid salt to the culture to make the final concentration 75 and 45 mg/l, respectively.
6. Continue shaking for 30 more min.
7. Add 250 μl of 100 mM IPTG to 1 l culture. The final IPTG concentration is 25 μM.
8. Harvest the cells by centrifugation after induction for 18 h.
9. Purify the proteins as described in [44].

3.7 NMR Study of Arrestin-1 Binding to Rhodopsin in Different States

3.7.1 Sample Preparation

1. Buffer exchange arrestin-1 to Buffer D using the Amicon Ultra centrifugal filters with 30 kDa molecular-weight cutoff.
2. Concentrate arrestin-1 sample to the desired concentration. Ideally the concentration should be at least double the concentration in final working solution (*see* **Note 10**).
3. Mix arrestin-1 and rhodopsin reconstituted into bicelles at the molar ratios of 1:1, 1:3, and 1: 5 in dark. The final volume is 200 μl. Add 1 μl of 1 M DTT and 10 μl of D_2O. Adjust the final bicelle concentration to 4 % with the bicelle stock. The solution should contain 30 μM arrestin-1; 30, 90, or 150 μM rhodopsin; 5 mM DTT; 5 % D_2O; and 4 % bicelle.
4. To investigate arrestin-1 binding to activated rhodopsin or phosphorylated rhodopsin, light activate the sample on ice for 30 min or until the A500 nm absorption decreases to the baseline. The preparation of phosphorylated rhodopsin is described in [5].
5. To investigate arrestin-1 binding to rhodopsin or phosphorylated rhodopsin in an inactive state, everything should be kept in the dark during sample preparation, sample transfer, and data collection.
6. Add 200 μl sample to a 5 mm Shigemi NMR or to a 3 mm conventional tube. It is ready for NMR experiments.

3.7.2 NMR Titration Experiments

1. All NMR data are obtained in Bruker Avance spectrometers with ^{1}H resonance frequency of either 800 MHz or 600 MHz at 308 K. Collect the two-dimensional ^{1}H–^{15}N correlated spectra using sensitivity-enhanced, phase-sensitive

[10]The arrestin-1F85/197A mutant used in the NMR study can be concentrated up to 640 μM without visible precipitation.

transverse relaxation optimized spectroscopy (TROSY) pulse sequence. It is important to use a version of this sequence that filters out all extraneous signal (from protons not directly attached to an ^{15}N) using pulsed field gradients rather than via phase cycling [47] (*see* **Note 11**). Key NMR parameters include the ^{1}H and ^{15}N 90° pulse widths and the relaxation recovery delay between scans, which are kept the same during the same series of titration points.

2. Two-dimensional methyl-TROSY spectra were obtained using heteronuclear multiple-quantum correlated spectroscopy (^{1}H, ^{13}C-HMQC) pulse sequence (reviewed in [48]). Set the ^{1}H and ^{13}C spectra widths at 14 and 22 ppm, respectively, with the ^{13}C carrier frequency corresponding to 20 ppm (*see* **Note 12**).

3. Collect one-dimensional TROSY spectra before and after each titration point to monitor the decaying of rhodopsin in different states. Process ^{1}H,^{15}N-TROSY data using a program such as nmrPipe [49] with zero filling, Gaussian apodization, and linear prediction in indirect dimension (^{15}N) before Fourier transformation. For ^{1}H,^{13}C-methyl-TROSY spectra, process the data with one-time zero filling and squared sine-bell function. Visualize and analyze the spectra using programs such as nmrDraw, NMRview, and Sparky [50, 51].

4. Plot the titration curve as arrestin NMR peak chemical shift against the concentration of rhodopsin. The calculation of K_d from the concentration dependence of NMR resonance chemical shift changes has been described previously in [15].

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[11]Typical TROSY spectra for NMR titration experiments are obtained using 1,024 × 128 complex points with 200 scans per increment, which requires total acquisition time of 20 h for one spectrum.

[12]A typical HMQC spectrum contains 1,024 × 256 complex points with 128 scans per increment, which requires total acquisition time of 20 h for one spectrum.

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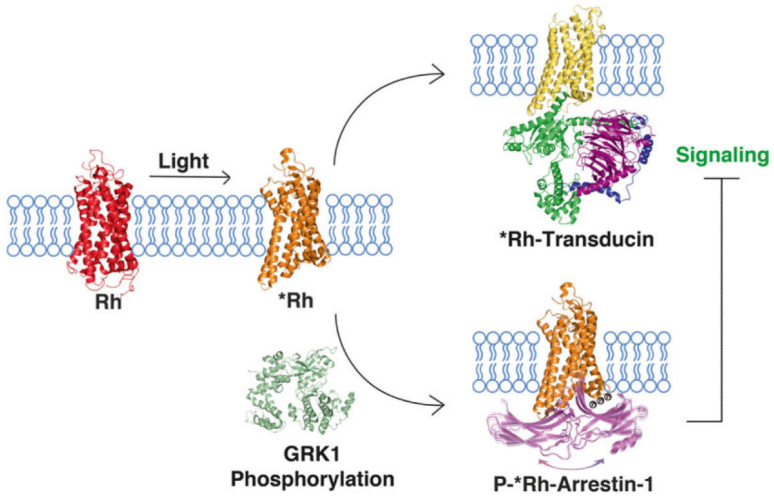

Fig. 1.

Role of arrestin-1 in rhodopsin signaling. Rhodopsin is located in the disk membranes of the outer segments of rod photoreceptor cells. Upon light activation, dark rhodopsin (Rh PDB: 1F88) [52] undergoes conformational change to the active state (*Rh PDB: 3PQR) [53]. Its cognate G protein, transducin, binds to the active rhodopsin and initiates the downstream signaling (structure from molecular simulation) [54]. Active rhodopsin also recruits and activates GRK1 (PDB: 3C50) [55]. *Rh is phosphorylated at multiple sites by GRK1. Arrestin-1 binds to active phosphorylated rhodopsin (p-*Rh), blocking further transducin activation by steric exclusion. The displayed p-*Rh-arrestin-1 complex is the assembly of *Rh (PDB: 3PQR) [53] and pre-activated arrestin-1 (PDB: 4J2Q) [14] in Pymol. Arrestin-1 gains conformational flexibility in the receptor bound state

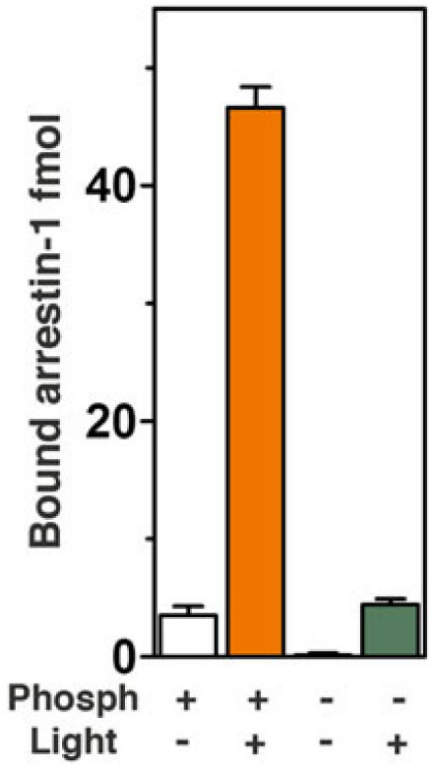

Fig. 2.

Arrestin-1 has very high selectivity for phosphorylated active rhodopsin. Its binding to phosphorylated active rhodopsin is about 10- to 20-fold higher than to only-activated or only-phosphorylated rhodopsin, while its binding to dark rhodopsin is barely detectable

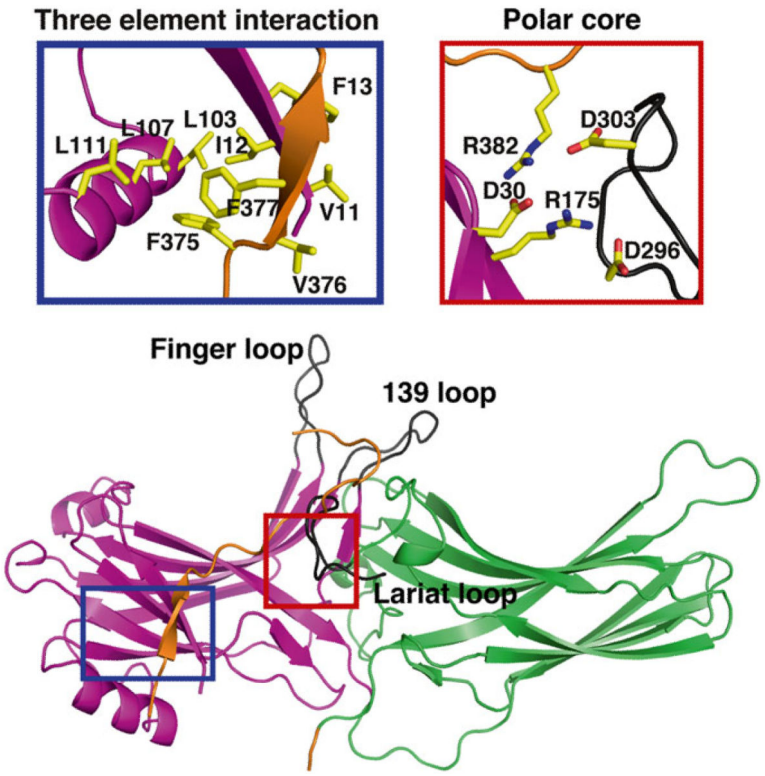

Fig. 3.

The crystal structure of arrestin-1 (PDB: 1CF1) [12]. The N-domain is colored *magenta*, the C-domain is colored *green*, and the C-terminus is colored *orange*. Three loops have been reported to be involved in receptor binding: the finger loop, the 139 loop (referred to as the middle loop for nonvisual arrestins), and the lariat loop are shown in *dark gray*. Two major interactions that stabilize arrestin-1 in the basal state are displayed in details. The polar core comprises five charged residues distributed between the N-domain (D30, R175), the C-domain (D296, D303), and the C-terminus (R382). The hydrophobic three-element interaction is between β-strand I (V11, I12, F13) and α-helix I (L103, L107, L111) in the N-domain and F375, V376, F377 in the C-terminus

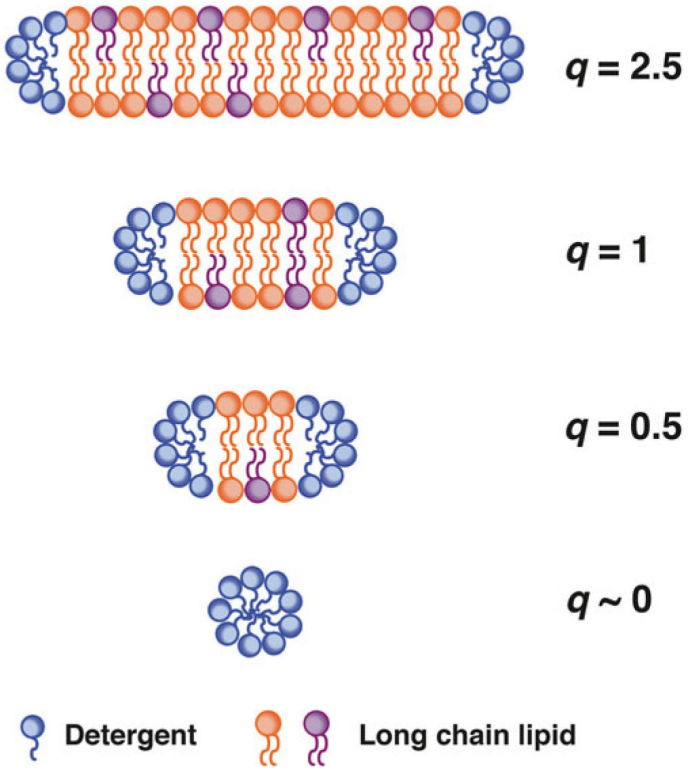

Fig. 4.

Bicelles. Bicelles have a central planar bilayer formed by long-chain lipids (*orange*) with the edges stabilized by either short-chain lipids or detergents (*blue*). Phospholipids with different head groups (*purple*) can be used to tailor the charge characteristics of the surface and provide lipid composition versatility. The bicelles with a higher q value (e.g., $q = 2.5$) have more extended lipid bilayers than ones with a lower q value (e.g., $q = 0.5$). In this sense, detergent micelles can be viewed as bicelles with the q value equal to 0

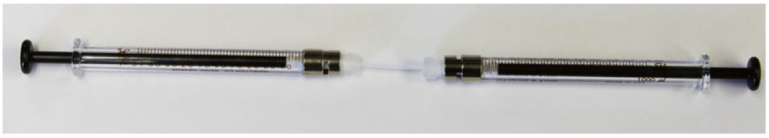

Fig. 5.

Apparatus to make bicelles. Two Hampton syringes are connected by a plastic coupler for extrusion. For example, to make 1,000 μl 35 % (w/v) bicelle stock, add 500 μl of DHPC solution to one syringe and 500 μl of DHPC solution homogenized with 224 mg of DMPC and 57 mg of DMPG to another syringe. The bicelles are homogenized by passing the contents back and forth between syringes multiple times. For this process, the entire apparatus is incubated at 4 and 55 °C and homogenized after equilibration at each temperature

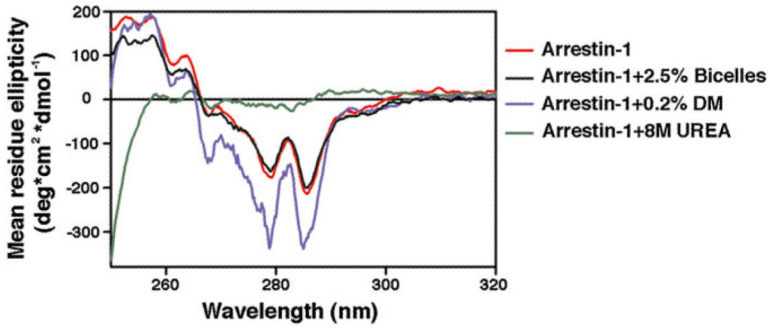

Fig. 6.

Near-UV CD spectra of arrestin-1 in the presence of model membranes. Arrestin-1 (50 μM) samples were prepared in detergent micelles composed of DM (*blue*) and DMPC/DHPC bicelles (*black*). The reference spectrum for native conformation of arrestin-1 in Buffer A is shown in *red*. The spectrum of fully denatured arrestin-1 in 8 M urea is shown in *green*

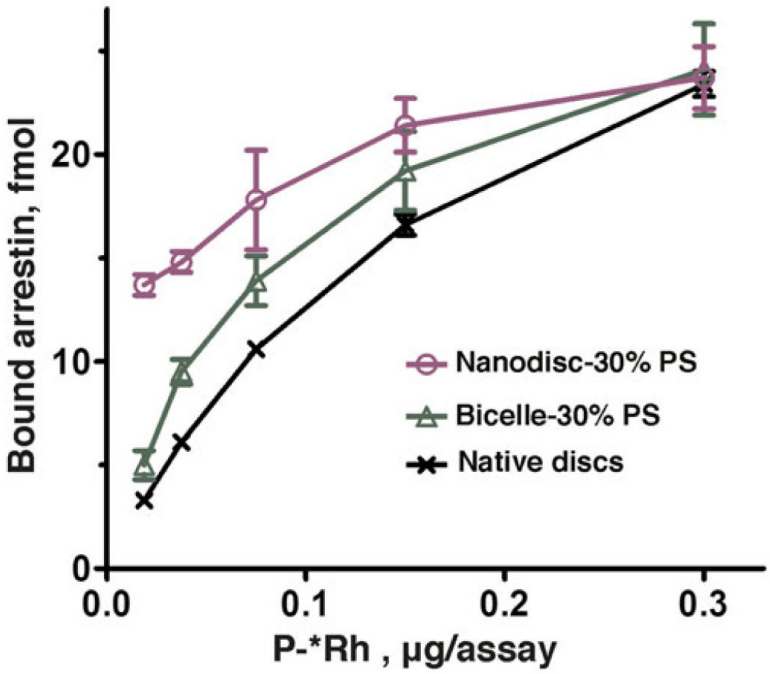

Fig. 7.

Arrestin-1 binding to P-*Rh. P-Rh from the same batch (0.3 µg) in native disk membranes (*black crosses*), or solubilized and reconstituted in DMPC bicelles with 30 % DMPS (*green triangles*), or solubilized and reconstituted in POPC nanodisks with 30 % POPS (*purple circles*) was incubated with radiolabeled arrestin-1 (100 fmol) in 50 µl at 30 °C for 5 min. The samples were light activated and cooled on ice, and bound and free arrestin-1 was separated by gel filtration on Sephadex G-75 (bicelles and nanodisks) or Sepharose 2B (native disk membranes). Means ± SD from three experiments performed in duplicate are shown