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Supplemental Information

The Energetics of Chromophore Binding in the Visual Photoreceptor Rhodopsin

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Preparation of the POPC/CHAPS bicelles buffer and dynamic light scattering characterization

The POPC lipids were dissolved in 20% (weight/volume) CHAPS solution at 1:1 POPC(weight)-to-CHAPS (weight) ratio. The solution was frozen in liquid nitrogen, then thawed and vortexed repeatedly to facilitate the dissolving of POPC. The dissolved POPC/CHAPS solution was diluted with water to a final concentration of 10% (w/v). Here we refer to the concentration of the POPC/CHAPS bicelle buffer in term of the (w/v) percentage. For example, 1% POPC/CHAPS contain 10 mg/mLPOPC and 10 mg/mL CHAPS. Note that the concentration of amphiphiles (POPC and CHAPS) is twice that value of the percentage. The 10% stock solution was kept at −20°C and thawed prior to use. For dynamic light scattering experiment, 10% POPC/CHAPS stock solution was freshly diluted with Buffer A (25 mM MES, 25 mM HEPES, 125 mM KCl, 12.5 mM KOH, pH 6.0). Before the dilution, the POPC/CHAPS stock solution and the buffer were filtered with inorganic membrane filter (Whatman, Anotop 10, 0.02 μm pore size, 10 mm diameter) to remove any contamination of particles. Samples were added into a 384-well plate (60 μL each well, Greiner, black, clear bottom), and then the plate was sealed on top with an Oracal soft PVC film (Orafol) to prevent evaporation. All the samples in this DLS experiment was measured in triplicate at 28°C, on a DynaPro Plate Reader II (Wyatt).

Immunoaffinity Purification of Rho from ROS membrane.

All the procedures involving dark-state Rho were performed in a dark room under dim red light. Native rod outer segment (ROS) membranes were isolated from frozen bovine retinas (W. L. Lawson Co., Lincoln, NE) as described before (1, 2). The 1D4-Sepharose 2B resin was prepared from 1D4 monoclonal antibody and cyanogen bromide-activated Sepharose 2B as described before (3, 4).

The detailed protocol for immunopurifying ROS Rho has been described in full details in the preceding report (5). Briefly, bovine ROS were lysed with the solubilization buffer (1% (w/v) DM, 50 mM HEPES or Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM $CaCl_2$ with Complete EDTA-free Protease Inhibitor Cocktail, Roche). The lysate was cleared by centrifugation at 100,000×g. The supernatant was mixed with 1D4-Sepharose-2B resin and incubated overnight at 4°C. The resin was transferred in a centrifugal filter unit with a 0.45-μm microporous hydrophilic PVDF membrane (Ultrafree-CL; Millipore) to enable efficient removal of buffer. The resin was first washed with Wash Buffer (DPBS containing 0.1% (w/v) DM) for three times (30 minutes incubation each time), and then with a low-salt buffer (0.1% (w/v) DM, 2 mM phosphate buffer, pH 6.0). The receptor was eluted with low-salt elution buffer (0.1% (w/v) DM, 2 mM phosphate buffer, pH 6.0, 0.33 mg/mL nonapeptide (sequence TETSQVAPA)). Then 150 mM salt was supplemented into the sample to restore the ionic strength. The preparation of ROS membranes were aliquoted and stored at −80°C.

The purified product was characterized by UV-Vis spectroscopy. The dark-state absorption spectrum of Rho was recorded in a 50-μL micro-cuvette with 10-mm path length on a Lambda-800 spectrophotometer (PerkinElmer Life Sciences). To acquire the absorption spectra of the photobleached Rho, the sample was supplemented with NH_2OH and irradiated for 30 seconds with a 335-mW 505-nm LED light source (Thorlabs) placed on top of the cuvette.

Preparation of Alexa488-labeled Rho

The expression of azF-tagged Rho was done using HEK293F cells (Invitrogen). Before transfection, the cell culture was resuspended at a density of 10^6 cells/mL in a 30-mL culture supplemented with 1 mM azF. For amber codon suppression, HEK293F suspension cells were transfected with a mixture of plasmid DNAs (38.6 μg in total, 18.4 μg of pMT4.Rho containing the amber codon, 18.4 μg of pSVB.Yam, and 1.84 μg pcDNA.RS) that have been described before (6). To maximize the yield of azF-Rho mutants, the cells were harvested 96 hours post-transfection. The cells were then resuspended at a density of 10^7 cells/mL in DPBS and regenerated by overnight incubation with 5 μM 11CR at 4°C (7). The regenerated cells were centrifuged and frozen at −20°C (wrapped light-tight in aluminum foil).

To label the azF-tagged Rho, the 11CR-regenerated cells were lysed with 1% DM, and the cleared lysate was incubated overnight at 4°C with 1D4-mAb-sepharose 2B (100 μL). The resin was transferred into a 1.5-mL Eppendorf tube, washed with Reaction Buffer (DPBS, pH 7.2, 0.1% (w/v) DM; 0.5 mL × 3, 30 minutes each time), and brought to a total volume of 300 μL. Alexa488-dibenzocyclooctyne (Molecular Probes/Thermo Fisher Scientific, 0.3 μL of 5 mM stock solution in DMSO) was added into the slurry to a final concentration of 5 μM. The reaction was allowed to proceed overnight (approximately 18 hours). The next day the reaction was stopped by centrifugation and removal of the supernatant fraction. The resin was transferred into a centrifugal filter with a 0.45-μm microporous hydrophilic PVDF membrane (Ultrafree-CL; Millipore) to facilitate washing. Alexa488-Rho was eluted as described for ROS Rho. The low-salt buffer elutes from the resin preferably the retinal-bound Rho, rather than the misfolded opsin (8). Based on our experience, the low-salt buffer was not essential for obtaining high-quality Rho from ROS membranes, in which the fraction of misfolded receptor was low. However, for wt or mutant Rho heterogeneously expressed in the HEK293F cells, the low-salt buffer served to increase the fraction of correctly folded, retinal-bound Rho. Purified samples were stored at −80°C and thawed on ice before use.

Immunoaffinity purification of opsin using 1D4-sepharose resin for the ITC experiment

In a typical experiment, an aliquot of ROS membranes (0.25 mL, ~1.4 mg Rho) was solubilized using Solubilization Buffer (2.5 mL; 7.5 mg/mL CHAPS, 1 mg/mL POPC, 137.5 mM NaCl, 25 mM MES, 25 mM hemisodium HEPES, 0.25 mM disodium EDTA, pH 6.0, supplemented with 0.14 mL 10% (w/v) CHAPS in water). The mixture was gently agitated for 30 minutes at 4°C. The insoluble fraction was removed by centrifugation for 30 minutes at 100,000×g. The supernatant fraction was incubated at 4°C for 3 hours under gentle agitation with 1.6 mL packed 1D4-Sepharose 2B resin (with 4 mg 1D4 mAb per mL packed resin) pre-equilibrated with Wash Buffer (10 mg/mL CHAPS, 10 mg/mL POPC, 137.5 mM NaCl, 25 mM MES, 25 mM hemi sodium HEPES, 0.25 mM disodium EDTA, pH 6.0). The resin was washed with Wash Buffer (6 mL × 4, 20 minutes each time), and then transferred into a 0.45-μm microporous centrifugal filtering unit, washed once with Wash Buffer (1 mL), and re-hydrated with 1 mL Wash Buffer supplemented with 50 mM hydroxylamine. The sample in the filtration unit was illuminated with a 500-nm long-pass filter equipped fiber optics coupled microscope illuminator (Dolan) for one hour with gentle head-over-head mixing. After the illumination, the resin was extensively washed with the POPC/CHAPS bicelle buffer (1.0 mL × 15). The large number of washing cycles ensured that the concentration of hydroxylamine is reduced below a level that could interfere with the ITC experiments. The Wash Buffer was removed by centrifugation and opsin was eluted (POPC/CHAPS bicelle buffer supplemented with 0.33 mg/mL nonapeptide, 1 mL × 2). Typically, the opsin concentration was in the range 9 ± 1 μM (corresponding to 0.36 ± 0.04 mg/mL opsin).

Determination of molar extinction coefficients of 11CR, opsin and Rho in POPC/CHAPS bicelles

The analysis of the results from titration calorimetry experiments in terms of molar binding enthalpies is critically dependent on a precise knowledge of the concentrations of ligand and protein solutions. Therefore it is necessary to determine the molar extinction coefficients under the experimental buffer conditions. The molar extinction of 11CR in ethanol was known ($\varepsilon_{376.5\ nm}$ = 24,935 M^{-1} cm^{-1}) (9). The buffer effect on the absorption spectrum of 11CR was determined by diluting 11CR ethanolic stock solution into POPC/CHAPS bicelles (Figure S2A). The absorption maximum of 11CR in POPC/CHAPS bicelle buffer was observed at 378 nm, similar to the maximum in ethanol. The linear serial dilution experiment showed that in POPC/CHAPS bicelles, the absorbance of 11CR increased by 2.6% compared with the value in ethanol. The molar extinction coefficient ($\varepsilon_{378\ nm}$) of 11CR in POPC/CHAPS bicelles was thus calculated to be 25,574 M^{-1} cm^{-1}. The A254/A378.5 ratio of the 11CR stock solution in ethanol was 0.73, slightly higher than the literature value of 0.69 (10) or 0.70 (9). This A254/A378.5 ratio indicated a high isomeric purity of the sample, as contamination of other retinoid species, such as ATR and 9CR, would lower this ratio. Reverse phase HPLC using an analytical C18 column demonstrated that the 11CR stock solution contained 2.4% impurities (Dr. Shixin Ye, personal communication).

To determine the molar extinction coefficients for opsin and Rho in POPC/CHAPS bicelles, a linear dilution series of 11CR (120 μL; 16 different concentrations ranging from 0 to 31.5 μM) were mixed with

purified opsin, as described for the ITC experiment (200 μL; 10.2 μM). The samples were incubated at room temperature for 4 hours in the dark. After the regeneration of opsin was complete, the UV-Vis spectra were recorded. The absorption of 11CR in buffer at 378 nm (corrected for a dilution factor $df = 0.375$) and the absorption of Rho at 499 nm yielded a perfectly linear correlation at sub-stoichiometric ratios (Figure S2B, C), giving the molar extinction coefficient of Rho at 499 nm ($\varepsilon_{499\,nm}$ = 42,742 M^{-1} cm^{-1}). The molar extinction coefficients of Rho and opsin at 279 nm were calculated using $\varepsilon_{499\,nm}$ of Rho as a reference.

SUPPLEMENTAL DATA ANALYSES

Calculation of bicelle concentration

We assumed that the bicelles took the shape of cylinder, with L as the length and d as the diameter. The head group area of phosphatidylcholine (area per lipid, A_{POPC}) is 0.71 nm^2 and lipid bilayer thickness (L) is 5 nm. CHAPS is a bean-like detergent and it is difficult to estimate its geometrical dimension. Here we assume that each CHAPS molecule stretches a length of 0.26 nm (d_s) along the circumference of the bicelle.

If bicelles took the shape of an equivalent sphere, the radius of the sphere (R_S) would be given by

$$\frac{4}{3}\pi R_S^3 = \frac{\pi d^2 L}{4} \qquad \text{Eq.(1)}$$

The dynamic light scattering experiments treated cylindrical bicelles as spheres. The correction factor of hydrodynamic radius of cylinder versus equivalent sphere is calculated by the following equation (11)

$$\frac{R_H}{R_S} = 1.0304 + 0.0193x + 0.06229x^2 + 0.00476x^3 + 0.00166x^4 + 2.663\times10^{-6}x^7 \qquad \text{Eq.(2)}$$

Where

$$x = \ln(p) \text{ and } p = L/d \qquad \text{Eq.(3)}$$

R_H is given by dynamic light scattering experiment. Substitute R_S with

$$R_S = \left(\frac{3}{16}d^2 L\right)^{1/3} \qquad \text{Eq.(4)}$$

d can be resolved numerically from the experimentally determined hydrodynamic radius (R_H) and estimated bicelle thickness (L). The bicelle diameter d yields an estimate for the number of POPC molecules (n_{POPC}) in one bicelle:

$$n_{POPC} = \frac{\pi d^2}{2A_{POPC}} = 2.21 nm^{-2} d^2 \qquad \text{Eq.(5)}$$

The concentration of bicelles can be given by

$$c_{bicelle} = \frac{c_{POPC}}{n_{POPC}} = \frac{0.452 nm^{-2} c_{POPC}}{d^2} \qquad \text{Eq.(6)}$$

In the experiments for deriving the energy diagram (*cf*. Figure 7A), the concentrations of POPC and CHAPS were both 1% (10 mg/mL), and their molar concentrations were 13 mM and 16 mM, respectively. CHAPS has a critical micelle concentration (CMC) of 6 to 10 mM. However, CMC is strictly valid only in the absence of any other binding partners. Even if the concentration of CHAPS is below its CMC, there is a small fraction of CHAPS available for stabilizing POPC. Because the ratio of available CHAPS to POPC

decreases dramatically, the size of the bicelles increases accordingly, and the concentration of bicelles also decreases.

In the experiment for measuring Rho regeneration kinetics (*cf*. Figure 5C), we used 1% (w/v) POPC/CHAPS (1:1), corresponding to 27 μM bicelles. In a typical Trp fluorescence-quenching experiment for purified ROS Rho, the concentration of 11CR was 1.5-2.0 μM, and the concentration of Rho was 0.2 μM. Therefore, the ratio of bicelle–retinal–Rho was approximately 140:8:1.

Comparing Trp-based and Alexa488-based FRET assays

In the Trp-based experiments, we observed varying degree of fluorescence quenching upon the addition of 11CR, which we attributed to: 1) the inner filter effect, 2) the dilution effect, and 3) the partitioning of retinal into the DM micelles or POPC/CHAPS bicelles. Here the slight change in the cuvette position due to pipetting or touching, and pipetting technique errors should be random and minor. Their contributions to the initial quenching are calculated as follows.

Inner filter effect. At 295nm 11CR has an extinction coefficient of 26,000 M^{-1} cm^{-1}. Based on the shape of the cuvette chamber, the effective path length for fluorescence excitation in the center of the cuvette is 0.5 cm, while the emission light path is negligible. The concentration of retinal is about 1.5~2.0 μM. The loss of excitation intensity at 295 nm is given by Beer-Lambert Law

$$A = \varepsilon_{295} c[W] l = 26000\ \mathrm{M^{-1}cm^{-1}} \times 2 \times 10^{-6}\ \mathrm{M} \times 0.5\ \mathrm{cm} = 0.026 \qquad \text{Eq.(7)}$$

The transmittance is given by

$$T = 10^{-A} = 0.942\ . \qquad \text{Eq.(8)}$$

Therefore, the absorbance (A) due to the inner filter effect reduces the transmission (*T*) in the middle of the cuvette (l = 0.5 cm) and accounts for 5.8% reduction in the Trp fluorescence. Experiments at a higher concentration of retinal would cause greater inner filter effects in the Trp fluorescence-quenching experiment.

Dilution effect. Adding retinal working solution (20 μL) into the assay buffer (480 μL) resulted in 4% reduction of the fluorescence signal.

Partitioning effect. The inner filter effect and the dilution effect together cause a reduction of the Trp signal by 9.6% (=1–0.942×0.96). In the 1% POPC/CHAPS bicelles (*cf*. Figure 4D), we observed 7.7% loss of signal, slighter smaller than the expected 9.6%, but within the error range of pipetting technique. The absence of the initial fast quenching step was not surprising, if we consider that in a typical experiment the concentration of bicelles (27 μM) was much higher than that of Rho (0.25-0.30 μM). In the case of 0.1% (w/v) DM (*cf*. Figure 4E), we observed a 44% decrease in Trp fluorescence, while in 2% (w/v) DM (*cf*. Figure 4E: inset) we only observed 15% loss of signal. After correcting for inner filter and dilution effects, the quenching in 0.1% (w/v) DM was 38% and in 2% (w/v) DM it was 6%.

By comparison, the Alexa488 signal from the engineered S144-Alexa488 Rho only suffered a 4% loss of signal due to the dilution effect. The Alexa488-based assay is more compatible with a high concentration of retinal and more suitable for measuring the ligand binding kinetics for "slow" mutants that would require much higher retinal concentrations to drive regeneration to completion.

Comparing regeneration-independent quenching in bicelles and micelles

We have estimated that the concentration of bicelles for 1% POPC/CHAPS (27 μM) is one order of magnitude higher than the concentration of retinal (1.5–2 μM). The chance that retinal randomly co-inhabits the same bicelle as opsin was 7%. Therefore, the maximal regeneration-independent quenching of Trp fluorescence by retinal should be 7%.

The critical energy-transfer distance is dependent on the wavelength of the donor (Trp) and the acceptor (retinal). In the case of Trp fluorescence (ex. 295 nm, em. 330 nm), the critical distance is short, typically less than 30 Å based on literature values (12). Our DLS experiment showed that the hydrodynamic radius of 1% POPC/CHAPS bicelles is 64.7 ± 0.5 Å. We observed an apparent 7.7% initial fast quenching (Figure 4D). Our calculation showed that the inner filter effect and the dilution effect together could cause a reduction of the Trp signal by 9.6%, which is even slightly higher than the observed 7.7%, but within the error range of pipetting. These numbers indicated that the energy transfer efficiency between retinal and Trp residues within in one POPC/CHAPS bicelle should be far below 100%, practically negligible.

In the 0.1% DM micelles we observed 44% initial fast quenching in 0.1% (w/v) DM, and after correcting for the inner filter and dilution effects it was 34%. We calculated the aggregation number of DM micelles based on a study that used small-angle X-ray scattering to determine the size and shape of detergent micelles (13). Based on these published parameters, 0.1% DM corresponds to DM micelles is approximately 20 to 25 μM micelles, about the same concentration of bicelles in our 1% POPC/1%CHAPS system. Therefore, the probability that retinal randomly co-inhabits the same micelle as opsin was similar to the case of 1% POPC/CHAPS bicelles. However, in 0.1% DM, the regeneration-independent quenching of Trp is at least one order of magnitude higher than in 1% POPC/CHAPS. We infer that retinal in DM micelles does associate with opsin more closely, possibly as non-covalent complexes. In this case, retinal will have a higher probability residing in the same micelle or bicelle as the opsin. The remaining question is where retinal is bound in these non-covalent complexes, whether 11CR is docked into the ligand-binding pocket or it is associated with an external, secondary binding site at the outer surface of opsin. As we ruled out with the Alexa488 assay the formation of the covalent complex of the fully regenerated mature pigment, it would be hard to understand why a non-covalent complex with 11CR inside the ligand-binding pocket would not progress to the normal covalent Schiff base. Unless the conformation of the receptor would be very different from that of the rhodopsin ground state or the non-covalent binding site is outside of the orthosteric binding pocket.

In 2% DM, the fast initial drop in fluorescence indicates a quenching efficiency between opsin and retinal of 6% (after correcting the dilution and inner filter effect). The simplest explanation would be that the non-covalent opsin/retinal complex also formed in 2% DM, but since the local concentration of retinal in 2% DM decreases, the equilibrium shifts away from the non-covalent complex.

Deriving the values in the energy diagram

Strategy for deriving the energy diagram (*c.f.* Figure 7)

In order to draw the energy diagram for a ligand-receptor binding reaction with a single transition state (TS), minimally two out of three of the following measurements need to be made: 1) the activation energy of the forward reaction ($\Delta^{\ddagger}G_{on}$), 2) the activation energy of the reverse reaction ($\Delta^{\ddagger}G_{off}$), and 3) the energy difference between the reactants and the products under a particular state ($\Delta G°$). Based on the transition state theory:

$$k = \frac{k_B T}{h} e^{-\frac{\Delta^{\ddagger}G}{RT}} = \frac{k_B T}{h} e^{-\frac{\Delta^{\ddagger}H - T\Delta^{\ddagger}S}{RT}} \qquad \text{Eq.(11)}$$

Where $\Delta^{\ddagger}G$ is the Gibbs energy of activation, $\Delta^{\ddagger}H$ is the enthalpy of activation, $\Delta^{\ddagger}S$ is the entropy of activation, k_B is Boltzmann's constant, and h is Planck's constant. $\Delta^{\ddagger}G_{on}$ and $\Delta^{\ddagger}G_{off}$ need to be obtained by kinetic measurements, while $G°$ requires a method to measure the energy difference between the reactants and the products.

Based on the linear form of the Eyring-Polanyi equation:

$$\ln\frac{k}{T} = -\frac{\Delta^{\ddagger}H}{R}\cdot\frac{1}{T} + \ln\frac{k_B}{h} + \frac{\Delta^{\ddagger}S}{R} \qquad \text{Eq.(12)}$$

By measuring the temperature-dependent reaction kinetics, it is possible to separate the enthalpic and entropic contribution to free energy.

In our case, the kinetics of the forward reaction, *i.e.*, the recombination reaction between opsin and 11CR, was obtained using FRET-based assays. The kinetics of the reverse reaction, *i.e.*, the dissociation reaction of 11CR from Rho, was obtained by monitoring the exchange rate of the bound 11-*cis*-retinylene chromophore with 9CR. The enthalpy of reaction was measured by isothermal titration calorimetry (ITC).

Note that all the following calculations are done with 28°C data.

The free energy (ΔG)

1. The free energy of activation of the forward reaction ($\Delta^{\ddagger}G_{on} = 13.4 \pm 0.02$ kcal mol^{-1}) was obtained from the retinal binding kinetics ($k_2 = (1.08 \pm 0.04)\times10^3$ M^{-1} s^{-1}) with a standard state ligand concentration ($c°$) of 1 m using $k_{on} = c^{\circ} k_2$:

$$\Delta^{\ddagger}G_{on} = -RT \ln \frac{k_{on} h}{k_B T} = 13.4 \text{ kcal mol}^{-1} \qquad \text{Eq.(13)}$$

The error was given by:

$$\sigma\left(\Delta^{\ddagger}G_{on}\right) \approx RT \frac{\sigma\left(k_{on}\right)}{k_{on}} = 0.02 \text{ kcal mol}^{-1} \qquad \text{Eq.(14)}$$

2. The activation energy of the reverse reaction ($\Delta^{\ddagger}G_{off} = 27.9 \pm 0.03$ kcal mol^{-1}) was obtained from the chromophore exchange kinetics ($k_{off} = (0.328 \pm 0.016)\times10^{-7}$ s^{-1})

$$\Delta^{\ddagger}G_{off} = -RT \ln \frac{k_{off} h}{k_B T} = 27.9 \text{ kcal mol}^{-1} \qquad \text{Eq.(15)}$$

The error was given by:

$$\sigma\left(\Delta^{\ddagger}G_{off}\right) \approx RT \frac{\sigma\left(k_{off}\right)}{k_{off}} = 0.03 \text{ kcal mol}^{-1} \qquad \text{Eq.(16)}$$

3. The free energy difference between the reactants and the products under a particular state ($\Delta G° = -14.5 \pm 0.04$ kcal mol^{-1}) was obtained by dissociation constant:

$$K_d = k_{off} / k_{on} = 30 \text{ pM} \qquad \text{Eq.(17)}$$

$$\Delta G^{\circ} = RT \ln K_d = -14.5 \text{ kcal mol}^{-1} \qquad \text{Eq.(18)}$$

The error is given by:

$$\sigma\left(K_d\right) = K_d \left\{ \left[\frac{\sigma\left(k_{off}\right)}{k_{off}} \right]^2 + \left[\frac{\sigma\left(k_{on}\right)}{k_{on}} \right]^2 \right\}^{1/2} = 0.5 \text{ pM} \qquad \text{Eq.(19)}$$

$$\sigma\left(\Delta G°\right) \approx RT \frac{\sigma\left(K_d\right)}{K_d} = 0.04 \text{ kcal mol}^{-1} \qquad \text{Eq.(20)}$$

In the energy diagram (*cf.* Figure 7A) we only indicated $\Delta^{\ddagger}G_{on}$ and $\Delta G°$.

The enthalpic term (ΔH)

4. The activation enthalpy of the forward reaction ($\Delta^{\ddagger}H_{on}$ = 20.2 ± 0.9 kcal mol^{-1}) was obtained from the Eyring plot of the retinal binding kinetics (*cf.* Table S2). The error was derived from the fitting error.

$$\ln\frac{k_{on}}{T} = -\frac{\Delta^{\ddagger}H_{on}}{R}\frac{1}{T} + \ln\frac{k_B}{h} + \frac{\Delta^{\ddagger}S_{on}}{R} \qquad \text{Eq.(21)}$$

5. The activation enthalpy of the reverse reaction ($\Delta^{\ddagger}H_{off}$ = 38.4 ± 4.4 kcal mol^{-1}) was obtained from the Eyring plot of the chromophore exchange kinetics (*cf.* Table S3). The error was derived from the fitting error.

$$\ln\frac{k_{off}}{T} = -\frac{\Delta^{\ddagger}H_{off}}{R}\frac{1}{T} + \ln\frac{k_B}{h} + \frac{\Delta^{\ddagger}S_{off}}{R} \qquad \text{Eq.(22)}$$

6. The reaction enthalpy ($\Delta H°$ = –21.6 ± 1.3 kcal mol^{-1}) was directly measured by the ITC experiment (*cf.* Figure 5). The error for the reaction enthalpy was obtained based on three independent measurements.

Ideally the difference between the activation enthalpy of the forward and reverse reactions should have the same value as the enthalpy of formation:

$$\Delta^{\ddagger}H_{on} - \Delta^{\ddagger}H_{off} - \Delta H° = 0 \qquad \text{Eq.(23)}$$

Insert the values above, we obtain:

$$\Delta^{\ddagger}H_{on} - \Delta^{\ddagger}H_{off} - \Delta H° = 3.4 \pm 4.7 \text{ kcal mol}^{-1} \qquad \text{Eq.(24)}$$

Therefore, the apparent discrepancy of the reaction enthalpy from isothermal titration calorimetry and from kinetic transition state theory analysis is with 3.4 ± 4.7 kcal mol^{-1} statistically indistinguishable from zero considering the experimental errors.

The entropic term (−TΔS)

7. The activation entropy of the forward reaction ($\Delta^{\ddagger}S_{on}$ = 22 ± 3 cal mol^{-1} K^{-1}) was obtained from the Eyring plot of the retinal binding kinetics (Eq.(21)). The entropic term ($-T\Delta^{\ddagger}S_{on}$ = − 6.7 ± 0.9 kcal mol^{-1}) was calculated for 28°C. The error was derived from the fitting error.

8. The activation entropy of the reverse reaction ($\Delta^{\ddagger}S_{off}$ = 34 ± 14 cal mol^{-1} K^{-1}) was obtained from the Eyring plot of the chromophore exchange kinetics (Eq.(22)). The entropic term ($-T\Delta^{\ddagger}S_{off}$ = −10.4 ± 4.4 kcal mol^{-1}) was calculated for 28°C. The error was derived from the fitting error.

9. The reaction entropy was calculated by

$$-T\Delta S° = \Delta G° - \Delta H° = 7.3 \text{ kcal mol}^{-1} \qquad \text{Eq.(25)}$$

The error was derived by:

$$\sigma(T\Delta S°') = \left[\sigma(\Delta G°)^2 + \sigma(\Delta H°)^2\right]^{1/2} = 1.3 \text{ kcal mol}^{-1} \qquad \text{Eq.(26)}$$

10. The change of reaction equilibrium constant can be calculated from Van't Hoff equation assuming a temperature-independent reaction enthalpy:

$$\ln\left(\frac{K_2}{K_1}\right) = \frac{-\Delta H}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \qquad \text{Eq.(27)}$$

Assuming the reaction enthalpy measured at 28°C remains a constant between 25°C and 37°C, for the formation reaction $\Delta H°$ = − 21.6 ± 1.3 kcal mol^{-1}, then the K_d at 25°C and 37°C are 21 pM and 86 pM, respectively.

The equilibrium constant can also be estimated by extrapolating the reaction kinetics. At 25°C, k_2 is 6.9×10^2 M^{-1} s^{-1}, and k_{off} is 1.7×10^{-8} s^{-1}. At 37°C, k_2 is 2.7×10^3 M^{-1} s^{-1} and k_{off} is 2.2×10^{-7} s^{-1}. Thus K_d at 25°C and 37°C are 25 pM and 82 pM, respectively.

11. The effect of lipids on the dissociation constant

The regeneration reaction is bimolecular and its kinetics (k_2) is inversely correlated to concentration of lipids. The k_2 extrapolated from our experimental results would be 235 M^{-1} s^{-1} at 37°C, slightly smaller than the value measured in ROS membrane ($k_2 = 3.0\sim 4.0\times10^2$ M^{-1} s^{-1} at 35°C) (14). In the calculation below, we assume a k_2 of 300 M^{-1} s^{-1} at 37°C. The dissociation reaction is unimolecular, and the rate ($k_{off} = 2.2\times10^{-7}$ s^{-1} at 37°C) should be independent of lipids. Because the dissociation constant $K_d = k_{off}/k_2$, then in ROS membrane

$$K_{d,\,ROS} = (2.2\times10^{-7}\ s^{-1}) / (300\ M^{-1}\ s^{-1}) = 0.73\ nM \qquad \text{Eq.(28)}$$

Therefore, the K_d in ROS membrane is 9-fold larger than that in 1% (w/v) POPC/CHAPS bicelles, corresponding to a difference of 1.3 kcal in $\Delta G°$. On the energy diagram, the barrier for the forward reaction would be higher by 1.3 kcal, and for the reverse reaction unchanged.

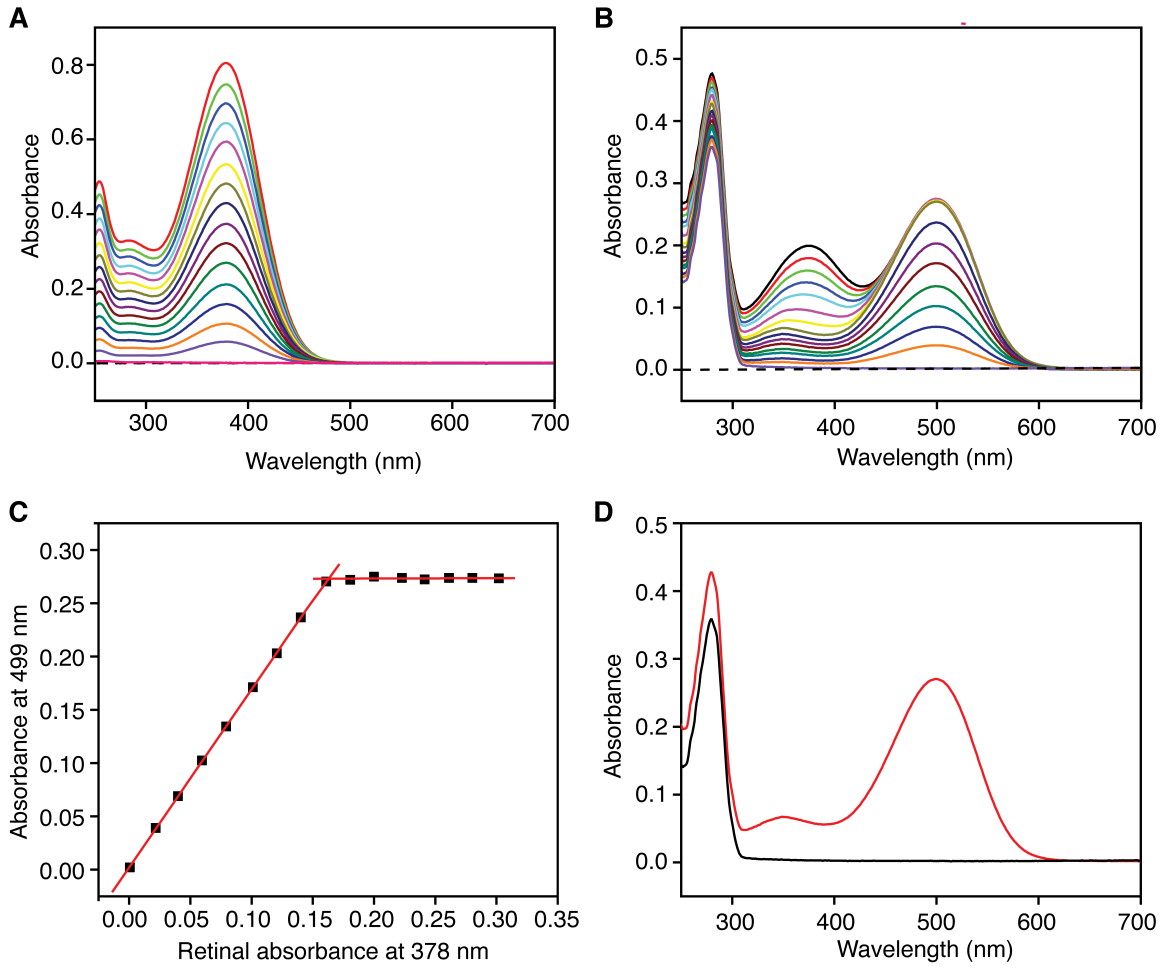

Figure S1. Characterization of the recombination product between opsin and 11CR. (A) 11CR ethanolic stock solution was diluted in POPC/CHAPS bicelles to evaluate the buffer effect on 11CR absorption. The extinction coefficient of 11CR in POPC/CHAPS bicelle buffer ($\varepsilon_{378\ nm}$) was determined to be 25,574 M^{-1} cm^{-1}. (B) Aliquots of 11CR (120 µL, linear dilution series with 16 concentrations in the range from 0 to 31.5 µM) was mixed with aliquots of purified opsin (200 µL, 10.2 µM). The UV-Vis spectrum of each sample was recorded after the binding reaction was complete. (C) The 378-nm absorbance of 11CR (Panel A, corrected for a dilution factor of 0.375) was plotted against the 499-nm absorbance of Rho (Panel B). The perfect linear correlation before the saturation of the retinal-binding site yielded the molar extinction coefficient of Rho at 499 nm ($\varepsilon_{499\ nm}$ = 42,742 M^{-1} cm^{-1}). (D) The absorption spectra of opsin (*black*) and Rho (*red*) are compared. The resulting A279/A499 ratio of Rho at the equivalence point is 1.58 ± 0.01, which indicates the recombination product between opsin and 11CR has a slightly higher purity than previously best results obtained by column chromatography: 1.65–1.75 (15), and 1.60–1.70 (16). Thus opsin in POPC/CHAPS bicelles is fully regenerable. The extinction coefficients of Rho and opsin at 279 nm were calculated by using the 499-nm absorbance of Rho as the reference.

Table S1. Molar extinction coefficient of opsin and Rho in POPC/CHAPS bicelles.

	$\varepsilon_{279\ nm}$, $M^{-1}\ cm^{-1}$	$\varepsilon_{499\ nm}$, $M^{-1}\ cm^{-1}$
Opsin	56,057 ± 184	–
Rho	67,532 ± 221	42,742

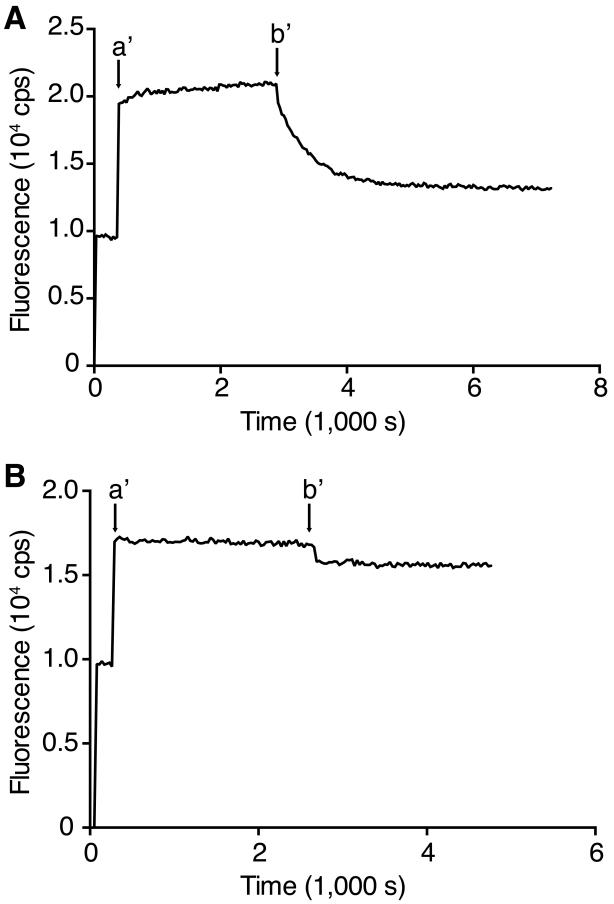

Figure S2. The regeneration of micelle-solubilized Rho after transfer into bicelles, measured by the Trp fluorescence-quenching assay. (A) Dark-state Rho solubilized in 0.1% DM (30 μL) was photobleached to form the active Meta-II state. The sample was incubated at 28°C for 40 min (not shown), 60 min (Panel A) or 13 hours (Panel B). Then the sample was added into the POPC/CHAPS bicelle buffer (450 μL, arrow a′). Exogenous 11CR solution (20 μL) was added later (arrow b′). (B) No regeneration can be observed for the 13-hour sample, indicating complete denaturation of opsin. For the 40-min sample and 60-min sample, the second-order rate constant for Rho regeneration (k_2) at 28°C was $1.0 \pm 0.2 \times 10^3$ M^{-1} s^{-1}. This value is in agreement with the results obtained for Rho reconstituted in bicelles ($1.08 \pm 0.04 \times 10^3$ M^{-1} s^{-1}, at 28°C).

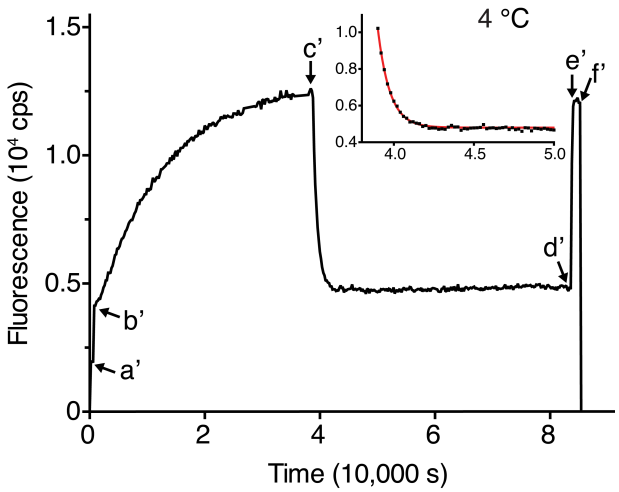

Figure S3. The regeneration of micelle-solubilized Rho at 4°C, measured by the Trp fluorescence-quenching assay. The sample compartment was cooled to 4°C by water bath. Dry air was blown through the sample compartment to prevent condensation on the cuvette. Dark-state Rho solubilized in 0.1% DM (30 μL) was added into the 1% DM micelle buffer (450 μL, arrow a′). 11 hours after photobleaching (arrow b'), exogenous 11CR solution (20 μL) was added (arrow c′). After overnight regeneration, 50 mm NH_2OH was added (arrow d'), and then the sample was photobleached again (arrow e'). The increase in Trp fluorescence in response to illumination indicates formation of mature pigment. At the end of acquisition, the shutter was closed to record the dark count (arrow f'). Inset: the change of Trp fluorescence signal after the addition of 11CR was fitted with a mono-exponential model. The second-order rate constant for Rho regeneration in 1% DM at 4°C was 1.4×10^3 M^{-1} s^{-1}.

Table S2. Second-order rate constants (k_2) for the recombination reaction between opsin and 11CR, and the thermodynamic parameters calculated from Eyring and Arrhenius plot.

	ROS Rho [a]	PMT4 Rho [a]	S144-Alexa488 Rho [b]
k_2 (36°C, 10^3 M^{-1} s^{-1})	2.2 ± 0.2	2.4 ± 0.6	2.8 ± 0.5
k_2 (28°C, 10^3 M^{-1} s^{-1})	1.08 ± 0.04	0.91 ± 0.31	1.2 ± 0.2
k_2 (20°C,10^3 M^{-1} s^{-1})	0.37 ± 0.03	0.28 ± 0.06	0.57 ± 0.13
k_2 (12°C, 10^3 M^{-1} s^{-1})	0.13 ± 0.3	0.13 ± 0.2	0.19 ± 0.4
$\Delta^{\ddagger}H_{on}$ (kcal mol^{-1})	20.2 ± 0.9	21.3 ± 1.3	18.8 ± 1.0
$-T\Delta^{\ddagger}S_{on}$ (28°C, kcal mol^{-1})	−6.7 ± 0.9	−7.7 ± 1.3	−5.4 ± 1.0
$\Delta^{\ddagger}G_{on}$ (28°C, kcal mol^{-1})	13.4 ± 0.02	13.5 ± 0.2	13.4 ± 0.1
$E_{a,on}$ (kcal mol^{-1})	20.8 ± 0.9	21.9 ± 1.3	19.4 ± 1.0
ln(A/(M^{-1}s^{-1}))	41.6 ± 1.6	43.4 ± 2.2	39.5 ± 1.7

[a]Measured by Trp fluorescence-quenching assay

[b]Measured by Alexa488 fluorescence-quenching assay

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