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Supplemental Information

Measurement of Slow Spontaneous Release of 11-*cis*-Retinal from Rhodopsin

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES (Tian *et al.*)

Preparation of the POPC/CHAPS bicelles buffer

The POPC lipids were dissolved in 20% (w/v) CHAPS solution at 1:1 POPC-to-CHAPS ratio (w/w) using repeated freeze-thaw cycles, and diluted with water to make a 10% (w/v) stock bicelle solution. Note that the concentration of amphiphiles (POPC and CHAPS) is twice that value of the percentage. For example, 10% POPC/CHAPS contain 100 mg/mL POPC and 100 mg/mL CHAPS. The solution was frozen in liquid nitrogen, then thawed and vortexed repeatedly to facilitate the dissolving of POPC. The 10% stock solution was aliquoted, kept at −20°C and thawed prior to use. For the retinal binding assay and chromophore exchange experiment, Buffer A (1% (w/v) POPC/CHAPS in 25 mM MES, 25 mM HEPES potassium salt, pH 6.7, 125 mM KCl) or Buffer B (1% (w/v) POPC/CHAPS in 25 mM MES, 25 mM HEPES hemisodium salt, pH 6.0, 137.5 mM NaCl, 0.25 mM EDTA) was prepared freshly by diluting 10% POPC/CHAPS stock solution with the corresponding concentrated buffer stocks.

Immunoaffinity Purification of ROS Rho for Trp-based FRET assay

Because Rho is light-sensitive, all the steps described below were performed in a dark room under dim red light, unless stated otherwise.

Native rod outer segment (ROS) membranes were isolated under dim red light from frozen bovine retinas (W. L. Lawson Co., Lincoln, NE) by gentle homogenization, differential centrifugation and density gradient centrifugation as described (Botelho *et al.*, 2002; Papermaster and Dreyer, 1974). The concentration of Rho in the ROS membrane preparations ranged from 140 to 180 μM. The aliquots of the ROS membranes were stored at −80°C.

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) for at least 1 hour at 4°C. To lyse cells, 1 mL of solubilization buffer was used for 10^7 cells. To lyse ROS membranes, the volume of solubilization buffer was not critical, as long as the final solubilization buffer contains 1% DM for rhodopsin concentrations up to 1 mg/mL. The lysate was cleared by centrifugation at 100,000·g for 30 min. The 1D4 resin was prepared as described before (Knepp et al., 2011; Oprian et al., 1987). The supernatant fraction was mixed with 1D4-Sepharose-2B resin (binding capacity by UV-Vis: 600 μg Rho/mL 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), which enabled efficient removal of buffer. For Trp fluorescence assay, 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 in 2 mM phosphate buffer, pH 6.0). The receptor was eluted with low-salt elution buffer (0.1% (w/v) DM and 0.33 mg/mL nonapeptide (sequence TETSQVAPA) in 2 mM phosphate buffer, pH 6.0, equal volume of the packed resin, incubated on ice for at least 1 hour). The purified receptor was collected in a clean 1.5-mL Eppendorf tube. Then 150 mM salt was supplemented into the sample to restore the ionic strength. This low-salt buffer serves to preferably elute from the resin the retinal-bound Rho, compared with the misfolded opsin (Ridge et al., 1995). In our experience, the fraction of misfolded receptor in the ROS membranes was negligible, and the low-salt buffer and saline buffer made little difference in the quality of the eluted sample. However, for Rho heterogeneously expressed in the HEK293F cells, the low-salt buffer improved the fraction of correctly folded, retinal-bound Rho. The unique property of Sepharose 2B resin results in strong compression of the resin bed during centrifugation filtration. The compressed resin restores its original volume upon "rehydration" with buffer. In this way the volume of Elution Buffer can be minimized to increase the concentration of receptor in the elution.

Lectin affinity purification of ROS Rho for chromophore exchange experiment

The immunopurified Rho samples contains the nonapeptide, which would interfere with a second purification step after the chromophore exchange. Therefore, concanavalin A (ConA) bound to a gel matrix was alternately employed to purify Rho from ROS membranes (Degrip, 1982; Litman, 1982). Briefly, an aliquot of ROS membranes (0.75 mL, ~4 mg Rho) was solubilized using a solubilization buffer (7.5 mL; 13

mg/mL CHAPS, 1 mg/mL POPC, 137.5 mM NaCl, 25 mM MES, 25 mM hemi sodium HEPES, 0.25 mM disodium EDTA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 6.0). The mixture was gently agitated for 1 hour at 4°C. The insoluble material was removed by centrifugation for 30 minutes at 100,000×g in a desktop ultracentrifuge (Beckman Optima Max). The supernatant was incubated at 4°C for 3 hours under gentle agitation with concanavalin A-sepharose conjugate affinity matrix (1.7 mL packed volume, Sigma). The concanavalin molecules of the affinity matrix were cross-linked by glutaraldehyde treatment to reduce leakage (Degrip, 1982). The resin was washed with Buffer OG (12.5 mL; 15 mg/mL *n*-octyl-*β*-D-glucoside, 137.5 mM NaCl, 25 mM MES, 25 mM hemi sodium HEPES, 0.25 mM disodium EDTA, pH 6.0; 4 times, 20 minutes each time) and separated from the wash buffer using a clinical centrifuge. The resin was transferred into a microporous centrifugal filter unit (Amicon Ultrafree-CL) to remove the Buffer OG. Rho was eluted with Buffer OG supplemented with 100 mM α-methyl-D-mannoside (1 mL × 3). The combined elution was concentrated at room temperature for about 10 min at 2,200×g to a volume of approximately 200 μL using a 30-kDa Amicon Ultra-4 centrifugal ultrafiltration device with a regenerated cellulose membrane (Millipore). The concentrated sample was applied to a small Sephadex G-50 column (5 cm × 0.7 cm) equilibrated the POPC/CHAPS bicelle Buffer B. The sample was eluted with the POPC/CHAPS bicelle Buffer B (200 μL × 5). The main Rho-containing fractions were identified by UV-Vis spectroscopy, pooled and diluted with the POPC/CHAPS bicelle Buffer B to 10 mL.

The detailed procedures of chromophore exchange in ROS Rho

Retinoid solution in benzene (approximately 0.3 mL, 30 mM) was transferred to nitric acid washed 4-mL glass vials. The solvent was evaporated under a stream of argon under red light. The sample was kept at high vacuum for 15 minutes. Then the retinoids were dissolved in pure ethanol (0.5 mL). The stock solution was diluted to about 2 mM. Lectin-affinity purified ROS Rho was diluted with the 1% POPC/CHAPS bicelle Buffer B to give a total volume of 6.74 mL (0.21 mg/mL, 5.2 μM). The sample was divided into five equal volumes and supplemented with the following: A) ethanol, B) 11CR, C) 9CR, D) ATR, and E) 50 mM NH_2OH. The final concentrations of retinoids were 49 μM. The samples were kept in 200-μL PCR tubes inside 1.5-mL brown micro centrifuge tubes. Each set of 5 tubes was put into large UZ tubes, wrapped in aluminum foil, and incubated in a water bath. To overcome buoyancy, large steel rods were added into the UZ tubes as weights. Each combination of Rho and retinoids were incubated under seven different conditions: 1) 28°C for 11.6 days (10^6 s), 2) 36°C for 11.6 days, 3) 44°C for 11.6 days, 4) 28°C for 2×11.6 days (2×10^6 s), 5) 36°C for 2×11.6 days, 6) 44°C for 2×11.6 days, 7) 28°C for 3×11.6 days (3×10^6 s). The samples were denoted as A1, A2, …A7, B1,… E6, E7. At the end of incubation, the samples were checked for their volume, and no loss was observed. The samples were then purified using 1D4-sepharose resin (100 μL each). In a centrifugal filter, the resin was washed with Wash Buffer (25 mM HEPES, adjusted with NaOH to pH 7.0, 150 mM NaCl 0.75% CHAPS , 0.1% POPC; 400 μL × 5). The mixture of Rho and isoRho was eluted with Elution Buffer (Wash Buffer + 0.33 mg/mL nonapeptide) and characterized by dark–light UV-Vis difference spectroscopy in presence of 50 mM hydroxylamine. For Rho incubated with ethanol, 11CR, and ATR, no spectral change was observed, and the receptor was completely recovered from the samples incubated at 28 and 36°C. Rho incubated with NH_2OH was irreversibly denatured due to the hydrolysis of Schiff base bond followed by thermal denaturation of opsin.

During the unusually long reaction time of the chromophore exchange experiment, opsin denaturation could compete with 9CR binding, resulting in underestimation for the exchange kinetics, particularly at high temperatures. Therefore, we performed a correction for the effect of opsin denaturation. We assume that retinal dissociation was the rate-limiting step, and the resulting opsin either followed the thermal denaturation pathway, or recombined with 9CR. The amount of the denatured opsin could be quantified by UV-Vis spectroscopy. At 44°C, the recovery of isoRho and Rho was 80.4% for 1×10^6 s and 45.6% for 2×10^6 s. For samples incubated at 28 and 36 °C the recovery was ~100% for up to 3×10^6 s incubation.

Let the total recovery of receptor be a

$$a = \frac{[\text{isoRho, recovered}]+[\text{Rho, recovered}]}{[\text{Rho, initial}]} \qquad \text{Eq.(1)}$$

And the fraction of isoRho be b:

$$b = \frac{[\text{isoRho, recovered}]}{[\text{isoRho, recovered}]+[\text{Rho, recovered}]} \qquad \text{Eq.(2)}$$

The fraction of retinal dissociation is $(1-a+ab)$ (Table S3).

The rate constant was obtained by fitting the fraction of retinal dissociation with a mono-exponential model.

Table S1. The fraction of retinal dissociation and the dissociation rate constant (k_{off})

	Temperature (°C)			
	28	36	44 (uncorrected)	44 (corrected)
$t = 1\times10^6$ s	0.0345	0.125	0.475	0.578
$t = 2\times10^6$ s	0.0708	0.225	0.658	0.844
$t = 3\times10^6$ s	0.0880	n.d.	n.d.	n.d.
$k_{off}(10^{-7}\ s^{-1})$	0.328 ± 0.016	1.29 ± 0.02	5.82 ± 0.38	8.89 ± 0.22

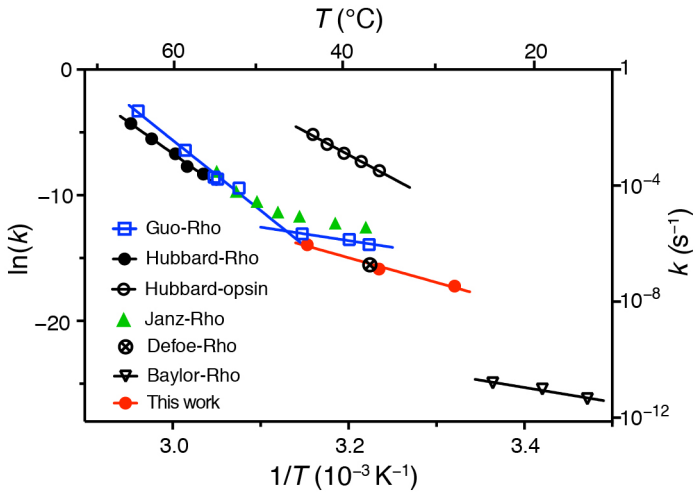

Figure S1. Comparison of the Arrhenius plots for the thermal decay rates of Rho or opsin. The Eyring analyses of the same sets of data are shown in the main text, Figure 5. *Empty blue square*: the thermal decay of Rho in 0.1% DM (Guo et al., 2014). *Solid black circle*: Rho in 2% digitonin (Hubbard, 1958) *Empty black circle*: opsin in 2% digitonin (Hubbard, 1958). *Solid green triangle*: Rho in 0.05% DM (Janz and Farrens, 2004). *Crossed empty circle*: chromophore exchange of Rho in mouse retina measured by radioactive tracers *in vivo* (Defoe and Bok, 1983). *Inverted empty triangle*: spontaneous isomerization and activation of Rho in toad retina measured electrophysiological recording (Baylor et al., 1980). *Red solid circle*: Rho chromophore release and exchange with 9CR in 1% POPC/CHAPS bicelles in the present study.

Table S2. The thermodynamic parameters derived from the Eyring plots in Figure 5 [a]

Source of data	Guo, Rho		Hubbard, Rho	Hubbard, opsin	Baylor, Rho	This work, Rho
Temperature (°C)	52.0 ~ 64.6	37.1 ~ 44.6	56.4 ~ 65.6	35.9 ~ 43.3	14.9 ~ 24.1	28.0 ~ 44.0
$\Delta^{\ddagger}H$ (kcal mol^{-1})	110 ± 7	20.6 ± 4.5	98.2 ± 4.7	73.7 ± 3.0	23.0 ± 3.8	38.5 ± 4.4
$\Delta^{\ddagger}S$ (cal mol^{-1}K^{-1})	261 ±15	-20 ± 9	222 ± 9	164 ± 6	−19 ± 7	35 ± 9

[a] We did not include the thermal decay data by Janz & Farrens in Table S2, because their results exhibited a concave Eyring plot. Therefore, the linear fitting assuming a single activation barrier would be unsuitable. There were too few data points for the high temperature regime to fit a two-process model with separate sets of thermodynamic parameters for the high- and low-temperature regimes, respectively.

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SUPPLEMENTAL REFERENCES

Baylor, D.A., Matthews, G., and Yau, K.W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. J. Physiol. *309*, 591-621.

Botelho, A.V., Gibson, N.J., Thurmond, R.L., Wang, Y., and Brown, M.F. (2002). Conformational energetics of rhodopsin modulated by nonlamellar-forming lipids. Biochemistry *41*, 6354-6368.

Defoe, D.M., and Bok, D. (1983). Rhodopsin chromophore exchanges among opsin molecules in the dark. Invest. Ophthalmol. Vis. Sci. *24*, 1211-1226.

Degrip, W.J. (1982). Thermal stability of rhodopsin and opsin in some novel detergents. Method Enzymol. *81*, 256-265.

Guo, Y., Sekharan, S., Liu, J., Batista, V.S., Tully, J.C., and Yan, E.C. (2014). Unusual kinetics of thermal decay of dim-light photoreceptors in vertebrate vision. Proc. Natl. Acad. Sci. USA *111*, 10438-10443.

Hubbard, R. (1958). The thermal stability of rhodopsin and opsin. J. Gen. Physiol. *42*, 259-280.

Janz, J.M., and Farrens, D.L. (2004). Role of the retinal hydrogen bond network in rhodopsin Schiff base stability and hydrolysis. J. Biol. Chem. *279*, 55886-55894.

Knepp, A.M., Grunbeck, A., Banerjee, S., Sakmar, T.P., and Huber, T. (2011). Direct measurement of thermal stability of expressed CCR5 and stabilization by small molecule ligands. Biochemistry *50*, 502-511.

Litman, B.J. (1982). Purification of rhodopsin by concanavalin A affinity chromatography. Method Enzymol. *81*, 150-153.

Oprian, D.D., Molday, R.S., Kaufman, R.J., and Khorana, H.G. (1987). Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. Proc. Natl. Acad. Sci. USA *84*, 8874-8878.

Papermaster, D.S., and Dreyer, W.J. (1974). Rhodopsin content in the outer segment membranes of bovine and frog retinal rods. Biochemistry *13*, 2438-2444.

Ridge, K.D., Lu, Z., Liu, X., and Khorana, H.G. (1995). Structure and function in rhodopsin. Separation and characterization of the correctly folded and misfolded opsins produced on expression of an opsin mutant gene containing only the native intradiscal cysteine codons. Biochemistry *34*, 3261-3267.