Rhodopsin self-associates in asolectin liposomes

Steven E. Mansoor*, Krzysztof Palczewski†, and David L. Farrens‡

*Department of Biochemistry and Molecular Biology, Oregon Health & Science University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239-3098; and †Department of Pharmacology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106

Communicated by H. Gobind Khorana, Massachusetts Institute of Technology, Cambridge, MA, December 20, 2005 (received for review November 29, 2005)

We show that the photoreceptor rhodopsin (Rh) can exist in the membrane as a dimer or multimer using luminescence resonance energy transfer and FRET methods. Our approach looked for interactions between Rh molecules reconstituted into asolectin liposomes. The low receptor density used in the measurements ensured minimal receptor crowding and artificial association. The fluorescently labeled Rh molecules were fully functional, as measured by their ability to activate the G protein transducin. The luminescence resonance energy transfer measurements revealed a distance of 47–50 Å between Rh molecules. The measured efficiency of FRET between receptors was close to the theoretical maximum possible, indicating nearly quantitative Rh–Rh association. Together, these results provide compelling evidence that Rh spontaneously self-associates in membranes.

Preparation and Characterization of Rh Samples. Rh was labeled in the cytoplasmic face, as described in Supporting Text, which is published as supporting information on the PNAS web site. The labeling occurred at the uniquely reactive cysteine residues, C140 and C316 (17–21). The LRET studies used the label CS124-DTPA-EMCH-Tb3+ as the donor and CY3–maleimide as the acceptor. The FRET studies used CY3-maleimide as the donor and CY5–maleimide as the acceptor. These Cy-reactive fluorophores are well characterized (22–24), and their spectra are significantly red-shifted, resulting in minimal spectral overlap with the retinal chromophore in Rh (see Fig. 3B).

Rh was labeled with ~1.0 label per protein (data not shown). The C140 and C316 Cys residues labeled with roughly similar efficiencies, as assessed by V8 proteolysis and SDS/PAGE analysis (25), which produced two fragments (F1 and F2) with similar fluorescence intensity (Fig. 1A). Scanning electron microscopy indicated that the reconstituted Rh proteoliposomes ranged in size from 100 to 200 nm in diameter, with an approximate average diameter of 150 nm (Fig. 1B), consistent with previous measurements of asolectin liposomes (26).

The Labeled and Reconstituted Rh Is Preferentially Oriented Inside-Out and Is Fully Functional. Treatment with Asp-N protease increased the electrophoretic mobility of all liposome-bound Rh, as efficiently as for a detergent solubilized Rh control (Fig. 1C). This result indicates that the Rh is oriented inside-out in the liposomes (27), with the C-terminal tail of rhodopsin exposed to the Asp-N protease (28). Both labeled and unlabeled liposome-bound Rh samples showed essentially identical abilities to activate Go1, (initial activation rates ~ 1.3 pmol/min per pmol rhodopsin), demonstrating that the attached fluorophores do not affect rhodopsin function (Fig. 1D).

The Quantum Yield of Rh–CY3 Increases Upon Light Activation of Rhodopsin, Resulting in an Increase in the R0 Value Between Rh–CY3 and Rh–CY5. The quantum yield of reconstituted Rh–CY3 rose from 0.13 ± 0.01 in the dark state to 0.20 ± 0.01 after light activation of Rh, presumably because energy transfer from the CY3 label to the retinal chromophore was abolished. It is important to note this increase in Rh–CY3 quantum yield changes the R0 value for the Rh–CY3/Rh–CY5 FRET pair from 52 Å in the dark state to 56 Å when light activated.

Conflict of interest statement: No conflicts declared.

Abbreviations: GPCR, G protein-coupled receptor; Rh, rhodopsin; ROS, rod outer segment; LRET, luminescence resonance energy transfer.

§For simplicity, we have limited our interpretation to a dimeric interaction, as suggested by others (7–10, 16), but, formally, our data cannot discriminate between Rh dimers and other higher-order oligomers.

†To whom correspondence should be addressed. E-mail: farrens@ohsu.edu.

© 2006 by The National Academy of Sciences of the USA
LRET Measurements Show a Rh–Rh Distance of 47–50 Å in Liposomes. The LRET approach is illustrated in Fig. 2A. We first used the LRET method because it can accurately determine distances between two proteins, even in the presence of some labeled but noninteracting proteins (the latter are spectrally silent in LRET). Furthermore, LRET is not complicated by the false positives that often complicate FRET studies (23, 29–33). Most importantly, we could use the distances we obtained independently by LRET to quantitate the percentage of the total Rh proteins participating in the subsequent FRET studies.

Details on the LRET studies are given in the Supporting Text. Briefly, they involved exciting the Rh–Tb (donor) at 337 nm with a laser pulse and then measuring energy transfer to Rh–CY3 (the acceptor), as indicated by the “sensitized emission” given off from Rh–CY3 at 570 nm. The rate of transfer, $k$, is reflected in the lifetime of the sensitized emission ($\tau_{AD}$), because $k = 1/\tau_{AD}$. We used the $\tau_{AD}$ values and Eq. 4, to determine the efficiency of luminescence resonance energy transfer. From this efficiency, we calculated the donor-acceptor distance using Eq. 5.

A representative result is shown in Fig. 2C. The data, measured from dark-state Rh reconstituted at the lowest receptor density (10,000 lipids per Rh), shows the decay of Rh–Tb donor alone ($\tau_D$; blue curve) and the “sensitized emission” decay of Rh–CY3 ($\tau_{AD}$; green curve). Two lifetime components were required to fit both the $\tau_D$ and $\tau_{AD}$ data, most likely because energy transfer from Rh–Tb to the retinal contributes to a short decay component. The quality of the data did not warrant a complex analysis; thus, we combined these values to calculate an “average” or amplitude-weighted lifetime, $\langle \tau \rangle = \alpha_1 \tau_1 + \alpha_2 \tau_2$, where $\alpha_1$ and $\alpha_2$ are the preexponential factors ($\alpha_1 + \alpha_2 = 1.0$) for $\tau_1$ and $\tau_2$, respectively. The complete set of amplitude-weighted LRET lifetimes, $\langle \tau_D \rangle$ and $\langle \tau_{AD} \rangle$, thus measured, are reported in Table 1.

These $\langle \tau_D \rangle$ and $\langle \tau_{AD} \rangle$ values yield a distance of 50 Å between Rh–Tb and Rh–CY3 in the dark state (Fig. 2D). Similar measurements were made for the samples immediately and 30 min after light activation (see Table 1). In each case, the distance between Rh proteins undergoing LRET was between 47 and 50 Å. Interestingly, this value is in good agreement with an expected Rh–Rh distance based on the known diameter of Rh (34).
reconstitution resulted in an identical amount of Rh in the liposomes. The results from these studies, shown in Fig. 3C, reveal substantial FRET, but only when the samples were combined and reconstituted together (green curves). The control samples show no FRET (red curves). The amount of FRET appears to increase immediately upon light activation of Rh and continues to increase over time (Fig. 3C Left), although we suspect this increase is mainly due to the inherent increase in the quantum yield for Rh–CY3 upon bleaching.

We quantified the amount of FRET observed in these excitation spectra by using standard approaches (35, 36). Our results showed a FRET efficiency of 32% in the dark, which increased to 38% efficiency immediately after light activation and up to 46% 30 min after light activation. Importantly, these donor- and acceptor-labeled ROS samples were prepared separately, their concentrations were determined and matched, and only then were the samples mixed for reconstitution. These stringent conditions ensured that a positive FRET signal unequivocally reflects intermolecular FRET occurring between at least two different receptors. We stress that the data in Fig. 3C are raw data that, aside from buffer subtraction, have not been manipulated or normalized in any way.

Table 2. FRET efficiencies calculated using steady-state excitation spectra and fluorescence lifetime decays of Rh reconstituted into liposomes

<table>
<thead>
<tr>
<th>Rh-CY3 + Rh-CY5 sample</th>
<th>FRET efficiency steady-state excitation data, %</th>
<th>FRET efficiency lifetime data, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark state</td>
<td>32 ± 1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>+hu, 0 min</td>
<td>38 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>+hu, 30 min</td>
<td>46 ± 1</td>
<td>32 ± 2</td>
</tr>
</tbody>
</table>

Measurements were carried out as described in Materials and Methods. See Supporting Text for more details.

FRET Efficiency at Different Receptor Densities. We determined the amount of nonspecific “background FRET” (due to receptor crowding) by measuring FRET for samples reconstituted at varying molar lipid/Rh ratios (10,000:1, 2,000:1, and 250:1). These conditions should theoretically produce receptor densities of ~20, ~100, and ~800 proteins per vesicle, respectively. We used these receptor densities, along with the concept of a reduced acceptor surface density (C_A), to calculate the amount of background FRET expected at each lipid/Rh concentration. The FRET results, and the “background FRET” expected for the different concentrations, are shown in Fig. 4A. Notice that the measured FRET signal is strongly dependent on the receptor density, and appears to be superimposed on the predicted background FRET at each concentration.

Experimentally Determined Receptor Density. The above calculation assumes a random distribution of Rh among the liposomes. We tested this assumption at the lowest Rh/lipid ratio (one Rh per 10,000 lipid molecules) by carrying out isopycnic density centrifugation analysis (38). These studies showed that Rh, in these preparations, is not uniformly distributed (Fig. 4B). Instead, ~90% of the Rh appears to be present in ~11% of the available vesicles. This interesting result (which may suggest that Rh is forming higher-order oligomers) indicates that in most vesicles containing Rh, the “true” receptor density is actually ~10× higher than predicted (~200 rhodopsin molecules per liposome). The correct C_A values are thus 0.039 in the dark state and 0.045 after light activation, yielding a more accurate assessment of the “background FRET” at this lipid/Rh ratio of ~8% in the dark and ~11% after light activation (see dotted curve in Fig. 4A).
Second, the of the labeled Rh can form donor–acceptor pairs (see Fig. 5). FRET, we had to take into account two factors. First, only half of the mixed samples can form donor, D, and acceptor, A, FRET pairs. The predicted FRET efficiencies (once the expected background FRET is subtracted; Fig. 4) are shown by the gray bars.

**Fig. 4.** FRET signal as a function of receptor density. (A) Experimentally determined FRET efficiencies for Rh in the dark-state (DS), and after illumination (+hu), at different predicted receptor densities (Rh/liposome). The FRET signals are well above the nonspecific or “background FRET” predicted to occur due to molecular crowding with increasing receptor density (dotted lines) (46). (B) Isopycnic centrifugation at the lowest receptor concentration (10,000 × lipid/Rh). Open circles indicate the Rh–CY5 fluorescence, and filled circles indicate the NBD-labeled lipid fluorescence. The data indicate ~90% of Rh incorporates into ~11% of the total liposomes. Thus, Rh-containing vesicles have ~200 Rh per liposome, yielding a predicted background FRET signal of ~8% in the dark state and ~11% after light activation (note that these values are still well below the measured FRET signals in A).

**The Measured FRET Efficiencies Are Near the Theoretical Maximum Possible, Suggesting That the Majority of Rh Molecules Interact.** Our main postulate in this work is that quantitative Rh–Rh self-association is indicated if the measured FRET efficiencies equal the theoretically maximum FRET possible at the Rh–Rh distances measured from the LRET studies (Fig. 2D and Table 1).

We estimated the total amount of interacting Rh proteins as follows. We analyzed the excitation FRET spectra (35, 36) to determine the percentage of FRET in each sample (see Table 2). We then compared this value to the maximal FRET possible under these conditions. To calculate the theoretical maximum FRET, we had to take into account two factors. First, only half of the labeled Rh can form donor–acceptor pairs (see Fig. 5A). Second, the $R_0$ values for a specific donor–acceptor pair must be used. When these factors are accounted for, the following theoretically maximum FRET efficiencies are predicted: 28% for the Rh–CY3–Rh–CY5 pair in the dark state (distance = 50 Å), and 37% for the Rh–CY3–Rh–CY5 pair in the light-activated state (distance = 47 Å) (Fig. 5B).

Importantly, as shown in Fig. 5C, these predicted maximum possible FRET values are nearly identical to the measured FRET efficiencies (once the expected background FRET is subtracted; Fig. 4A). We conclude that, for this result to be possible, almost all of the Rh molecules must be within interacting distance.

**Implications of Rh–Rh Association.** Although the idea that visual Rh may self-associate in the membrane has been debated (39), our results clearly favor Rh dimerization and are consistent with evidence found for other GPCRs (1–4). In fact, our studies found no evidence for a substantial amount of monomeric Rh. Because Rh constitutes the majority of volume of the membrane in ROS, with a concentration as high as 3 mM (40), and only ~65 phospholipids solvating each Rh molecule (40), an interesting question may be “what could prevent Rh from interacting with itself?” The complete absence of any protein–protein interactions at such high concentrations would seem to require an extremely low affinity between Rh molecules, a possibility that is not supported by our present work or the accompanying work of Kota et al. (10).

Thinking about Rh as a dimer, instead of a monomer, is compelling when examining the architecture of various downstream signaling components in the visual pathway. For example, the interface surface area of Rh in a monomeric model of the Rh–transducin interaction is too small to cover all of the regions of transducin known to be critical for interaction with the receptor. However, subsequent modeling studies have demonstrated that the surface area of one transducin molecule is large enough to accommodate the docking of four rhodopsin molecules (40).

Similarly, the concept of a multimeric Rh may help explain Rh desensitization by visual arrestin. Arrestin acts by binding to activated, phosphorylated Rh, thus blocking further signaling (41). Interestingly, crystal structures of arrestin show a bilobed protein with two concave surfaces (42, 43). Both concave surfaces have been demonstrated by mutagenesis studies to be involved in Rh–arrestin interactions (44). Like transducin, the putative Rh-interaction surface on visual arrestin is highly striking: the two concave grooves can physically accommodate two molecules of rhodopsin (5). Although the specific reasons why Rh may function as a dimer remain to be established, it is clear a dimeric state may have a profound impact on the kinetics.
of Rh activation, signaling through transducin, and desensitization through arrestin.

Summary and Conclusions. We have found that Rh molecules in reconstituted asolectin liposomes are ~47–50 Å apart. Furthermore, the energy transfer between donor and acceptor-modified Rh is close to the theoretically possible maximum FRET efficiency, showing that most of the Rh molecules are in a dimeric state (if not higher-order oligomers). Finally, we anticipate the approach described here may prove generally useful for quantitatively studying GPCR self-association in membranes.

Materials and Methods

Materials. The origin of the materials used can be found in Supporting Text, which is published as supporting information on the PNAS web site.

Buffers. Buffer A (137 mM NaCl/8 mM Na2HPO4/2.7 mM KCl/1.5 mM KH2PO4, pH 7.2), buffer B [137 mM NaCl/8 mM Na2HPO4/2.7 mM KCl/1.5 mM KH2PO4/4% 1-O-n-octyl-β-D-glucoside (OG)/0.1% asolectin, pH 7.2], buffer C (137 mM NaCl/8 mM Na2HPO4/2.7 mM KCl/1.5 mM KH2PO4/1.46% OG/0.1% asolectin, pH 7.2), buffer D (137 mM NaCl/8 mM Na2HPO4/2.7 mM KCl/1.5 mM KH2PO4/1.46% OG/0.1% asolectin/0.3 M Methyl α-D-Mannopyranoside, pH 7.2), and Transducin Assay Buffer (10 mM Tris-HCl, pH 7.5/0.1 M NaCl/5 mM MgCl2/1 mM EDTA) were used.

Nomenclature. Here, rhodopsin is abbreviated as Rh. Abbreviations for rhodopsin derivatives are identified by Rh, followed by the fluorophore used in the labeling. For example, Rh–CY5 stands for Rh labeled with CY5-maleimide and Rh–Tb stands for Rh labeled with the Tb3+ chelator.

Purification and Fluorescent Labeling of Rh. Rh was purified from ROS membranes and modified with the appropriate fluorophore. V8 proteolysis was used to assess the sites of fluorescent labeling. Specific details on these procedures are provided in Supporting Text.

Reconstitution of Purified, Fluorescently Labeled Rh. Reconstitution of fluorescently labeled Rh into asolectin liposomes was performed in the dark under dim red light, as described (27). Different receptor densities were achieved by reconstituting samples with varying molar ratios of asolectin and Rh. The lipid/Rh ratios, 250:1, 2,000:1, or 10,000:1, should theoretically produce receptor densities of ~800, 100, and 20 Rh protein molecules per liposome, respectively. More details are provided in Supporting Text.

Analysis of Rh-Reconstituted Proteoliposomes Using Electron Microscopy, Asp-N Proteolysis, and Transducin Activation Assays. After reconstitution, the size of the proteoliposomes was determined by electron microscopy. Asp-N proteolysis was used to determine the relative orientation of Rh in the liposomes (27). The effect of the fluorescent labels on Rh function was measured by performing transducin activation assays (45). For details, see Supporting Text.

Determination of the Quantum Yield of Rh–CY3 Donor and R0 Value for CY3–CY5 FRET Pair. The quantum yield for Rh–CY3 reconstituted into liposomes was measured and used to determine the overlap integral (R0) for the Rh–CY3 and Rh–CY5 FRET pair. Details are given in Supporting Text.

Isopycnic Density Centrifugation. Isopycnic density centrifugation analysis was carried out on Rh–CY3 and Rh–CY5 reconstituted into asolectin liposomes. The liposomes were supplemented with NBD-labeled phosphatidyl serine (0.4% of the total lipid content) to enable independent fluorescent monitoring of the lipid fractions. The proteoliposomes were subjected to discontinuous flotation gradients (38), and fractions were analyzed for Rh and lipid content. See Supporting Text for more details.

Predicted Receptor Density. The number of Rh molecules per liposome was calculated as follows. The average radius of the asolectin liposomes was ~75 nm (see our EM data, Fig. 1B, and ref. 26) thus producing a liposome surface area of ~7,000,000 Å2. Assuming the surface area of one lipid molecule to be 70 Å2, and the vesicle membranes is a bilayer, yields ~200,000 lipids per vesicle. If equally distributed, the number of Rh molecules per liposome is

\[
\text{#Rh/liposome} = \frac{\text{#Lipids/Liposome} \times \text{Lipid Ratio}}{\text{Rh/Lipid Ratio}},
\]

where Rh/Lipid Ratio is the inverse of the lipid/Rh ratio used during the reconstitution.

Calculation of Reduced Acceptor Surface Density (C_A). The reduced acceptor surface density (C_A) is equal to the R0 of the FRET pair multiplied by the surface density of acceptor-labeled proteins (46). For the CY3–CY5 FRET pair on Rh, we measured an R0 of ~52 Å in the dark state and 56 Å after light activation. These values are similar to those published in refs. 22 and 24. We then calculated the C_A at each lipid/Rh ratio using the following relationship

\[
C_A = \frac{(R_0)^2 \times \frac{\text{no. of Acceptors}}{\text{Liposome Surface Area}}}{(56.1 \, \text{Å})^2 \times \frac{10}{7,000,000 \, \text{Å}^2}} = 0.0045.
\]

Note that the change in the R0 value between dark- and light-activated Rh–CY3 and Rh–CY5 requires the C_A values to be calculated for each state.

Calculation of Expected Random Energy Transfer (E_random) Based on the Reduced Acceptor Surface Density (C_A). The C_A values (described above) were used to assess the amount of random “background” energy transfer expected under the different reconstitution conditions, as follows (46–48)

\[
E_{\text{random}} = 1 - \left( 1 + \left[ \frac{\pi \times C_A \times \left( \frac{R_0}{r} \right)^4}{2} \right] \right)^{-1},
\]

where E_random is the amount of random energy transfer expected, C_A is the reduced acceptor surface density, and r is the distance of closest approach of the donors and acceptors (which can be approximated by the protein diameter) (46–52). The value of r for rhodopsin was approximated to be ~48 Å, the diameter across the face of an ellipsoid shape observed for rhodopsin from the crystal structure (34).

Measurement of the Rate of LRET Between Labeled Rh Samples in Asolectin Proteoliposomes. LRET studies were made by using a PTI LaserStrobe phosphorescence lifetime system. The Rh–Tb samples were excited with a 337-nm laser pulse, and the emission was monitored at 545 nm to obtain the lifetime of the Rh–Tb donor alone (τ_D), and at 570 nm, when Rh–Tb and Rh–CY3 were reconstituted together, to obtain the sensitized emission lifetime (τ_AD) from Rh–CY3. For further details, see Supporting Text.
Determination of Distance from LRET Measurements. The lifetime of Rh–Tb luminescence ($\tau_D$) and the Rh–CY3 sensitized emission ($\tau_{AD}$) were used to calculate the efficiency of energy transfer ($E$)

$$E = 1 - \frac{\tau_{AD}}{\tau_D}$$  \[4\]

This efficiency was then used to calculate the distance between the two probes (35, 53)

$$R^6 = \frac{R_0^6 (1 - E)}{E}$$  \[5\]

where $E$ is the efficiency of energy transfer, $R$ is the distance between the probes, and $R_0$ is the distance at which the energy transfer is 50%. Eqs. 4 and 5 were used to plot $\tau_{AD}$ as a function of $R$. This plot yields the appropriate distance for any experimentally measured $\tau_{AD}$ (see Fig. 2D). An $R_0 = 61.2 \text{ Å}$ was used for the Rh–Tb and Rh–CY3 pair (23).

To assess the maximum possible error in the LRET measurements due to uncertainties in the orientation factor ($\kappa^2$), the steady-state anisotropies of Rh–CY3 and Rh–CY5 in the liposomes were measured. Details are given in Supporting Text.

FRET Steady-State and Lifetime Measurements. The steady-state fluorescence measurements were carried out on Rh–CY3 and Rh–CY5 samples reconstituted individually and together. FRET was measured in two ways, first by measuring the emission spectrum of the acceptor while exciting the donor and then by measuring the excitation spectrum of the donor while collecting emission from the acceptor. Fluorescence lifetimes were measured on the samples reconstituted at the lowest receptor density. Further details are given in Supporting Text.

FRET Efficiency Calculated From Steady-State Fluorescence Intensity and Fluorescence Lifetimes. FRET efficiency was determined by measuring the sensitized emission from steady-state excitation spectra using standard analysis procedures (35, 36). The FRET efficiency was also determined from the fluorescence lifetimes of the donor (Rh–CY3) in the presence and absence of the acceptor (Rh–CY5) (35). See Supporting Text for details.

We thank Mr. J. Fay for technical assistance on the transducin activation assays, Dr. E. Barklis for performing the electron microscopy analysis, and Dr. T. Huber for suggesting the isopycnic density centrifugation analysis. We thank Dr. T. Heyduk for providing lanthanide reagents for initial LRET trials, and Drs. T. Heyduk and S. Lutsenko for valuable comments on the manuscript. Finally, we thank Drs. I. Sokal and G. Jang for help in initiating this project. This work was supported by National Institute of Drug Abuse Grants DA14896 (to D.L.F.) and F30DA15584 (to S.E.M.) and in part by U.S. Public Health Service Grant EY08081 from the National Eye Institute, National Institutes of Health, Bethesda (to K.P.).

Supporting Text

Materials. CY3-maleimide and CY5-maleimide were purchased from Amersham Pharmacia Biochemicals. The Tb\(^{3+}\) chelate, CS124-DTPA-EMCH•Tb\(^{3+}\), was purchased from Panvera. Asolectin soybean lipid was purchased from Fluka. Sephadex G15 size-exclusion beads were purchased from Sigma and 10,000 molecular weight cut-off concentrators were purchased from Millipore. NBD-labeled Phosphatidyl Serine (NBD-PS) was purchased from Avanti Polar Lipids.

Purification of Rh. Rod outer segment (ROS) membranes were prepared from bovine retina, as described in ref. 1, and then 1 ml of ~1.0-2.0 mg/ml Rh in the membranes was solubilized in 10 ml of buffer B by nutating at 4°C in the dark for 90 min. After solubilization, the sample was centrifuged at 30,000 \(\times\) g for 30 min. The supernatant was then removed and added to Con A beads previously equilibrated with buffer B and allowed to bind at 4°C for 90-120 min. Next, the binding slurry was added to a small, disposable polystyrene column. The beads, bound with protein, were then washed with 30 ml of buffer C before being eluted in buffer D (350- to 400-µl elution fractions collected after 20- to 30-min incubations). The concentration of protein in each elution fraction was determined using a Shimadzu 1601 UV/VIS spectrophotometer and an extinction coefficient for Rh of \(\varepsilon_{500} = 40,600\ \text{L} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}\) (2).

Fluorescent Labeling of Purified Rh. Immediately after purification, Rh in buffer D was labeled overnight (~16 h) at 4°C with either CY3-maleimide or CY5-maleimide (at a 7-fold molar excess) or CS-124-DTPA-EMCH•Tb\(^{3+}\) (at a 1.5-fold molar excess). Free label was removed by concentrating the solutions to 100 µl by using a Millipore 10,000 molecular weight cut-off concentrator and then passing the solutions over a Sephadex G15 size-exclusion column previously equilibrated with buffer C. This procedure was repeated three to four times on each sample to ensure complete removal of free label. The fluorophore labeling efficiency for each sample was determined by diluting an aliquot of sample into buffer C containing 50 mM hydroxylamine and then taking the absorption
spectrum before and after photo-bleaching using a 150-watt light source from a Techni-Quip Corporation filtered through a >500-nm long pass filter. The Rh concentration was determined by subtracting the dark state spectrum from the light-activated spectrum to give a difference spectrum. Fluorophore concentrations were calculated from the bleached spectrum using extinction coefficients of $\varepsilon_{554} = 150,000 \text{ L} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}$ for CY3-maleimide, $\varepsilon_{652} = 250,000 \text{ L} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}$ for CY5-maleimide, and $\varepsilon_{343} = 10,560 \text{ L} \cdot \text{cm}^{-1} \cdot \text{mol}^{-1}$ for CS-124-DTPA-EMCH.

V8 Proteolysis of Fluorescently Labeled Rh to Assess Labeling Stochiometry.
Fluorescently labeled Rh was incubated in the dark at room temperature with V8 protease at a V8:Rh molar ratio of 1:30. After incubating for 1.5 h, additional V8 was added to bring the V8:Rh ratio to 1:15 and the reaction was allowed to proceed for an additional 1.5 h. Following incubation, the reactions were analyzed by SDS-PAGE. The fluorescent fragments, F1 (~27 kDa containing Cysteine 140) and F2 (~13 kDa containing Cysteine 316) (3), were visualized by using an Alpha Innotech FluorChem 5500 imaging system.

Reconstitution of Labeled, Purified Rh. Reconstitution of Rh into asolectin liposomes was performed in the dark under dim red light, following a published protocol (4). Reconstitution was carried out by first incubating together equal moles of protein samples for ~ 16 h at 4°C in buffer C according to the following scheme: (1) donor-labeled Rh with equimolar unlabeled Rh, (2) acceptor-labeled Rh with equimolar unlabeled Rh, (3) donor-labeled Rh with equimolar acceptor-labeled Rh, and (4) buffer only – reconstituted lipid but no protein. Importantly, all of the reconstitutions were set up such that equal molar total protein was used in each of the above conditions.

After 16 h, the reconstitution procedure was continued by the addition of a defined amount of asolectin lipid. This involved adding the appropriate volume of buffer A containing 1 mg/ml asolectin lipids (MW ~ 760 g/mol) plus 1.46% OG such that the molar ratio of asolectin/Rh was 250:1, 2000:1, or 10,000:1. These lipid/protein ratios should theoretically produce ~800, 100, and 20 total Rh proteins per liposome, or dark-state reduced acceptor surface density ($C_A$) values of 0.156, 0.020, and 0.0039, and light
activated $C_A$ values of 0.180, 0.023, and 0.0045, respectively. By keeping the amount of lipid at 1 mg/ml in 1.46% OG and altering the volume of this lipid/detergent solution added to the protein, the lipid/protein ratio was altered without changing the lipid/detergent ratio during the reconstitution procedure. The samples were then dialyzed for 36-48 h against buffer A (~1,000 volume fold excess) at 4°C in the dark with buffer exchanges every 6-8 h. The proteoliposome suspensions were then pelleted at $140,000 \times g$ for 60 min at 4°C and resuspended in buffer A for experiments and storage in the dark at 4°C.

**Electron Microscopy of Asolectin Proteoliposomes.** Proteoliposomes were lifted onto carbon-coated copper grids for 1 min, wicked, rinsed in water for 1 min, wicked, stained 1 min in 1.33% uranyl acetate, wicked and dried. Samples were imaged at 100 kV on a Philips CM120/Biotwin transmission electron microscope (EM) at magnifications of $\times 11,000$ to $\times 37,000$ and photographed on a Gatan 794 charge-coupled device (CCD) multiscan camera at 0.524-1.76 nm/pixel. Images in Gatan DM3 format were converted to TIFF format with GATAN DIGITAL MICROGRAPH software.

**Orientation of Rhodopsin in Liposomes as Determined by Proteolysis.** The orientation of the receptor in the liposome was determined using the endoprotease Asp-N (4), which specifically cleaves between Gly-329 and Asp-330 in the C terminus of rhodopsin (5). Briefly, 1.5 μg of each rhodopsin sample in 10 μl was combined with Asp-N at a molar ratio of 1:4 (Asp-N/rhodopsin) in the dark at room temperature for 4 h. The reaction was stopped by the addition of SDS/PAGE loading buffer and the samples then subjected to SDS/PAGE. The resultant fragments were analyzed both by fluorescence imaging of the CY3 label and by Coomassie stain using an Alpha Innotech FluorChem 5500 imager. Unfortunately, the FluorChem 5500 was unable to image CY5 fluorescence.

**Determination of the Quantum Yield of Rh-CY3 Donor and $R_0$ Value for CY3-CY5 FRET Pair.** The quantum yield for reconstituted Rh-CY3 in the dark state and following light-activation was measured by using the following relation (6, 7):
\[
\Phi_x = \Phi_{st} \times \frac{F_x}{F_{st}} \times \frac{OD_{st}}{OD_x} \quad (S1)
\]

where subscripts \(st\) and \(x\) refer to the standard and unknown solutions, respectively, \(\Phi\) is the quantum yield, \(F\) is the relative integrated fluorescence intensity, and \(OD\) is the optical density at the exciting wavelength. Rhodamine-6-G (quantum yield equal to 0.94 in ethanol; ref. 8) was chosen as the standard. Emission spectra were performed at 22°C using 520-nm excitation (1/4-nm bandpass) while collecting from 529-800 nm (8-nm bandpass). The buffer intensity was subtracted from each sample and from the standard before integration.

The \(R_0\) value for the CY3-CY5 FRET pair was calculated using the relationship:

\[
R_0 = [8.8 \times 10^{-23} \times K^2 \times n^{-4} \times \Phi_D \times J(\lambda)]^{1/6} \text{ Å} \quad (S2)
\]

where \(K^2\) is the orientation factor (equal to 2/3), \(n\) is the refractive index (equal to 1.3), \(\Phi_D\) is the quantum yield of the donor, and \(J(\lambda)\) is the spectral overlap integral between the emission spectrum of Rh-CY3 and the absorbance spectrum of Rh-CY5.

**Rhodopsin Function Assessed by Transducin Activation Assays.** The final reaction mixture contained 10 mM Tris·HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, ~275 pmol of transducin, 0.5 pmol of rhodopsin, and 4.5 μM \([^{35}\text{S}]\text{GTP}[\gamma\text{S}]\) (32 nCi/mol). The samples were assayed as follows. \([^{35}\text{S}]\text{GTP}[\gamma\text{S}]\) was added in the dark at room temperature, then the samples were exposed to yellow light for 30 s, and 10 μl aliquots subsequently removed at different time points, spotted onto filter paper in a modified Brandell M-24 cell harvester and washed three times with 4 ml of transducin assay buffer. Each filter paper was then removed and assayed for \([^{35}\text{S}]\text{GTP}[\gamma\text{S}]\) content bound to transducin. As a negative control, two dark state time points were assayed for \([^{35}\text{S}]\text{GTP}[\gamma\text{S}]\) content: one immediately before light activation and one after the last light-state time point.
**Isopycnic Density Centrifugation.** Rh was analyzed by isopycnic density centrifugation using purified Rh, labeled with CY3- and CY5-, and reconstituted into asolectin liposomes which have been supplemented with NBD-labeled Phosphatidyl Serine (0.4% of the total lipid content) to enable fluorescent monitoring of the lipid fractions. After reconstitution, proteoliposomes were subjected to discontinuous flotation gradients in the absence of light, using a slight modification from a previously published protocol (9).

Briefly, this procedure involved mixing 250 $\mu$l of the proteoliposome mixture (described above) with 3.0 ml of 60% sucrose (wt/wt) in buffer A and 2.75 ml of buffer A supplemented with 0.05% Triton X-100. The resulting 6 ml solution of proteoliposomes in 30% sucrose was added to the bottom of a 35 ml centrifuge tube. Subsequently, 6 ml each of 24%, 18%, 12%, and 6% sucrose (wt/wt) in buffer A were successively layered onto the sample, followed by 3 ml of buffer A, and the discontinuous gradients were then centrifuged overnight at 110,000 $g$ in a Beckman SW 28 rotor. Fractions were collected in the dark in 550 $\mu$l aliquots from the top of the gradient down to the bottom and analyzed for rhodopsin and lipid content. Rh content was assessed by following the CY5 fluorescence emission (650 nm excitation; emission collected from 656-800 nm). Lipid content was determined following NBD fluorescence (360 nm excitation; emission measured from 366-800 nm).

**Measurement of the Rate of Lanthanide Resonance Energy Transfer (LRET) Between Labeled Rh Samples in Asolectin Proteoliposomes.** LRET studies were performed on 1 $\mu$M of Rh-Tb (donor) and Rh-CY3 (acceptor) reconstituted in asolectin liposomes at 10,000 lipids per Rh. The measurements were made on a PTI LaserStrobe phosphorescence lifetime system, exciting the samples with a 337 nm laser pulse. The emission was either monitored at 545 nm (10-nm bandpass), to obtain the lifetime of the donor alone ($\tau_D$) when Rh-Tb and unlabeled Rh were reconstituted together, or at 570 nm (10-nm bandpass), to obtain the sensitized emission lifetime ($\tau_{AD}$) of Rh-CY3 when both Rh-Tb and Rh-CY3 were reconstituted together. To reduce light scatter from the liposomes, a 298-435-nm bandpass filter was also used on the excitation beam and three
>500 nm long pass filters were used with the emission monochromator. Measurements were performed at 22°C. Each data point represents two averages of five laser shots collected linearly in time over 350 total channels. To eliminate possible artifacts due to tube ringing after the laser pulse (10), the first 75 µs of the decay after the peak channel of the instrument response function was excluded from the data analysis. This treatment only improved the quality of the fits and did not substantially affect the resulting calculated lifetimes.

**Steady-State Fluorescence Measurements for FRET Measured in Asolectin Proteoliposomes.** Before FRET measurements, four samples were set up to incubate at room temperature overnight (~16 h): one containing donor-labeled Rh reconstituted with unlabeled Rh, one containing acceptor-labeled Rh reconstituted with unlabeled Rh, one containing donor-labeled Rh reconstituted with acceptor-labeled Rh, and finally a control sample containing only liposomes (no protein).

The FRET measurements, performed on 1 µM reconstituted Rh at 22°C, compared the individual emission and excitation spectra from separately reconstituted Rh-CY3 and Rh-CY5 to the spectra where Rh-CY3 and Rh-CY5 were mixed together before reconstitution. The amount of FRET was measured for each sample in two ways. First, the emission spectrum of the acceptor was monitored while exciting the donor, and then the excitation spectrum of the donor was measured while collecting emission from the acceptor. Emission spectra were measured by exciting the donor at 520 nm (1/4-nm bandpass) while scanning the fluorescence intensity of the acceptor from 529-800 nm (8-nm bandpass). The excitation spectra were performed by collecting emission from the acceptor at 670 nm (8-nm bandpass) while scanning the excitation spectrum of the donor from 400-661 nm (1/4-nm bandpass). All measurements were performed in the dark state, immediately after light activation, and 30 min after light activation using a step size of 1 nm, 0.2-s integration time, and two averages. Under these conditions, Rh was found to undergo less than 5% bleaching (as measured by following the loss of absorbance at 500 nm).
Fluorescence Lifetime Measurements of FRET Efficiency Measured in Asolectin Liposomes. Fluorescence lifetimes of the fluorescently labeled Rh samples reconstituted at the lowest receptor density were measured using a PTI LaserStrobe fluorescence lifetime instrument. Measurements used magic angle conditions (vertically polarized excitation and an emission polarizer oriented 54.7° from the vertical (8)), and 520 nm excitation pulses (fwhm ~ 1.5 ns), while monitoring the emission with a monochromator at 580 nm (20-nm bandpass) and three long pass filters (> 550 nm). Measurements used 100 µl of 1 µM reconstituted samples and represent two averages of five laser shots per channel, collected in an arithmetic progression over 100 channels. The instrument response function (IRF) was determined by measuring scatter from a solution of Ludox. The measurements were performed at 22°C in the dark state, immediately after light activation, and 30 min after light activation. Under these conditions, Rh was found to undergo less than 15% bleaching (as measured by following the loss of absorbance at 500 nm).

Steady-State Anisotropy Measurements. The steady-state fluorescence anisotropy (r) of the labeled Rh samples reconstituted into liposomes was measured using the PTI steady-state fluorometer, and the data analyzed as:

\[ r = \frac{I_\parallel - G I_\perp}{I_\parallel + 2G I_\perp} \quad (S3) \]

where I_\parallel and I_\perp refer to the intensity of fluorescence emission parallel and perpendicular to the plane of excitation light, respectively. The G-factor was determined before measuring the anisotropy of each sample to correct for any bias in the monochromator.

Anisotropy measurements of Rh-CY3 used 520 nm excitation (2-nm bandpass) and emission collected at 570 nm (8-nm bandpass). The anisotropy of Rh-CY5 used excitation at 650 nm (2-nm bandpass) and emission collected at 670 nm (8-nm bandpass). Anisotropy measurements were performed on 1 µM samples at 22°C in the dark state, immediately after light activation and 30 min after light activation. The measurements at
each time point were done in duplicate and the average steady-state anisotropies were recorded.

**Anisotropy Correction of LRET Distance Measurements.** The error in FRET and LRET measurements associated with changes in $\kappa^2$, the orientation factor, were calculated from the maximum and minimum range of $\kappa^2$ using the following equations (11, 12):

$$\kappa^2_{\text{max}} = \frac{2}{3}(1 + 2.5A_d + 2.5A_a) \quad (S4)$$

$$\kappa^2_{\text{min}} = \frac{2}{3}\left(1 - \frac{2.5A_d + 2.5A_a}{2}\right) \quad (S5)$$

where $A_d$ is the anisotropy of the donor and $A_a$ is the anisotropy of the acceptor, measured as described above. For the LRET experiments, the anisotropy of the terbium is $A_d = 0$ (11). The complete list of anisotropy values can be found in Table 3 of Supporting Text. It is important to note that because these errors are the absolute extreme values that arise only if the fluorophore dipoles are all oriented at fixed, perpendicular angles relative to one another, the actual errors are likely much less significant (11).

**Determination of FRET Efficiency.** FRET efficiency was determined by assessing the extent of sensitized emission from the excitation spectra using the following equation (13, 14):

$$E = \left[\frac{G(\lambda_2)}{G(\lambda_1)} - \frac{\varepsilon_A(\lambda_2)}{\varepsilon_A(\lambda_1)}\right] \frac{\varepsilon_A(\lambda_1)}{\varepsilon_D(\lambda_2)} \quad (S6)$$

The acceptor excitation spectra ($G$) and the extinction coefficients ($\varepsilon$) were used to choose $\lambda_1$ (650 nm), where the donor ($D$) has minimal absorption, and $\lambda_2$ (554 nm), where the extinction coefficient of the donor is large relative to that of the acceptor ($A$).
The extinction coefficients used for the donor and acceptor fluorophores at the chosen wavelengths were $\varepsilon_A(\lambda_2) = 18,243 \text{ Lcm}^{-1}\text{mol}^{-1}$, $\varepsilon_A(\lambda_1) = 247,766 \text{ Lcm}^{-1}\text{mol}^{-1}$, and $\varepsilon_D(\lambda_2) = 150,000 \text{ Lcm}^{-1}\text{mol}^{-1}$, determined from the absorbance spectra of Rh-CY3 and Rh-CY5.

The FRET efficiency was also determined from the fluorescence lifetimes of the donor (Rh-CY3) in the presence and absence of the acceptor (Rh-CY5) using the following equation:

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$ (S7)

where $\tau_D$ is the lifetime of the donor in the absence of the acceptor and $\tau_{DA}$ is the lifetime of the donor in the presence of the acceptor (13).


A. General Scheme:

- Purify and label rhodopsin
- Reconstitute into liposomes
- Measure:
  - LRET: distance between rhodopsins
  - FRET: fraction rhodopsin dimers

B. CS-124-DTPA-EMCH•Tb

C. CY3-, CY5- maleimide