Supporting Information

Kirchberg et al. 10.1073/pnas.1015461108

SI Text

Preparation of 11-*cis*-Retinal. 11-*cis*-Retinal was prepared as described in ref. 1. 100 mg all-*trans*-retinal were dissolved in 50 mL ethanol and illuminated with light ($\lambda > 435$ nm) for 2 h on ice. The sample was evaporated to dryness and redissolved in 25 mL hexane/6% diethyl ether. HPLC with a nucleosil 50-S column in hexane/8% diethyl ether was used to separate the various isomers.

Preparation of Rhodopsin Disk Membranes. Bovine retinae were purchased from W.L. Lawson Corp. (Lincoln, NE). Bovine rod outer segment disk membranes were prepared from bovine retinae at low ionic strength as described (2) and purified by discontinuous density-gradient centrifugation. Remaining soluble and membrane-associated proteins were removed by repetitive washes in low ionic strength buffer and an additional purification step by continuous sucrose-density centrifugation.

The disk membranes were stored at −80 °C.

Phosphorylation of Rhodopsin Disk Membranes. Multiphosphorylated rhodopsin was prepared according to ref. 3 by suspending disk membranes in buffer [70 mM potassium phosphate buffer (PPB) pH 6.8, 1 mM magnesium acetate (MgAc)] containing a twofold excess of GTP and a 300-fold excess of ATP (over rhodopsin). Phosphorylation of rhodopsin by the native rhodopsin kinase was initiated by illumination with light of $\lambda > 435$ nm. After 2 h the reaction was stopped by a fourfold dilution with buffer (70 mM PPB pH 6.8, 1 mM MgAc) and addition of 50 mM hydroxylamine and 2% bovine serum albumin (BSA). Membranes were washed with low ionic strength buffer to remove peripheral proteins and retinaloxime. A twofold excess of 11-*cis*-retinal was allowed to react with phosphorylated opsin overnight at 4 °C to regenerate rhodopsin. After regeneration excess 11-*cis*-retinal and retinaloxim were removed by multiple washing steps (70 mM PPB pH 6.8, 1 mM MgAc, with and without 1% BSA). A control sample, containing nonphosphorylated rhodopsin disk membranes, was prepared identically, except that no ATP was added during the phosphorylation procedure. More than 95% of the rhodopsin retained function after regeneration, as assessed by absorption spectroscopy.

Determination of the phosphorylation stoichiometry was performed in a spectroscopic assay (see *Flash Spectroscopy*) based on the stabilization of the Meta-II intermediate by phosphorylation. The concentration of Meta-II formed at low temperature and high pH increases by a factor of ∼2 for a phosphorylation stoichiometry of six phosphates/rhodopsin (4). Under our conditions this factor yields a lower bound for the phosphorylation stoichiometry. The phosphorylated disk membranes were stored at −80 °C.

Labeling of Rhodopsin Disk Membranes. Labeling of rhodopsin in disk membranes at rhodopsin position C316 with the fluorescent dyes 5-iodoacteamidofluorescein (5-IAF) and Alexa594-C_5-maleimide (Molecular Probes/Invitrogen) was carried out essentially as described (5). To achieve low (∼10%) and high (∼100%) labeling of rhodopsin a two or 20-fold excess, respectively, of the fluorescent dye was used. The incubation time was chosen accordingly with 15 and 60 min, respectively. The labeling stoichiometry was calculated using

$$\frac{c_{\text{Label}}}{c_{\text{Protein}}} = \left(\frac{\Delta A_L}{\varepsilon_L}\right)\left(\frac{\varepsilon_{\text{Protein}}}{A_{\text{Protein}}}\right) \qquad \textbf{[S1]}$$

with A_{Protein} being the absorbance of rhodopsin at 498 nm and $\varepsilon_{\text{Protein}}$ being the corresponding extinction coefficient with $\varepsilon_{498\text{ nm}} = 40{,}600\ \text{M}^{-1}\,\text{cm}^{-1}$. ΔA_L is the absorbance difference at the peak of the absorption band (λ_{max}) of the label to the absorption of the protein band. ε_L is the molar extinction coefficient of the label (IAF: $\varepsilon_{L(492\text{ nm})} = 77{,}000\ \text{M}^{-1}\,\text{cm}^{-1}$, Alexa594-maleimide: $\varepsilon_{L(590\text{ nm})} = 73{,}000\ \text{M}^{-1}\,\text{cm}^{-1}$). About 0.1 and 0.8 mol, respectively, of the label was incorporated per mol rhodopsin under these conditions. The covalent binding of the fluorescent label to rhodopsin-C316 and the removal of excess label was verified by the fluorescence of the rhodopsin band following SDS-PAGE. The rhodopsin samples were further characterized by their ability to form Meta-II upon illumination and the rates of their formation by flash photolysis.

Expression and Purification of Recombinant Arrestin. Construction of recombinant arrestin and cysteine mutants in the cysteineless base arrestin mutant (ArrCA) are described in ref. 6. In the cysteineless arrestin all three wild-type cysteines were exchanged towards alanines. Large-scale expression of arrestin was performed as described (6). Briefly, a culture of F11α transformants in medium containing 0.67% yeast nitrogen base without amino acids (wt/vol), 2% galactose (wt/vol) and 0.1% drop out mix without leucine (wt/vol) was grown at 30 °C with vigorous shaking to an OD_{600} of 4–6. 0.5 mM $CuSO_4$ was added to the culture to induce protein expression. After 5 h, the cells were harvested. Cells were lysed and arrestin purification was done by affinity chromatography using the N-terminal Strep-tag II on a Strep-Tactin sepharose column (IBA, Göttingen). Purified samples were stored at −80 °C.

Labeling of Arrestin. The single-cysteine arrestin mutants CA-S106C and CA-S60C (6) were labeled with a 10-fold excess of Lucifer yellow (LY) iodoacetamide (Molecular Probes/Invitrogen) or Atto647N-maleimide (Atto-Tec) in 50 mM potassium phosphate buffer pH 7.5 for 2 h at room temperature. Unbound dye was removed by gel filtration using Sephadex G-25 fine (Amersham). The labeling stoichiometry was calculated using Eq. **S1** with A_{Protein} as the absorbance of arrestin at 297 nm and and $\varepsilon_{\text{Protein}} = 31{,}860\ \text{Mol}^{-1}\,\text{cm}^{-1}$ as the corresponding extinction coefficient. The extinction coefficient of LY at 426 nm is $\varepsilon_L = 11{,}000\ \text{Mol}^{-1}\,\text{cm}^{1}$. The extinction coefficient of Atto647N at 644 nm is $\varepsilon_L = 150{,}000\ \text{Mol}^{-1}\,\text{cm}^{-1}$. A labeling stoichiometry of 90–100% was achieved for LY covalently bound to position 60 and 106. For Atto647N-maleimide bound to position 60 a labeling stoichiometry of ∼80% was achieved using this procedure. The covalent binding of the fluorescent label to S106C and S60C and the removal of excess label was verified by the fluorescence of the arrestin band following SDS-PAGE. The arrestin samples were characterized by their ability to bind to rhodopsin (see *Flash Spectroscopy*).

Flash Spectroscopy. Flash spectroscopy and data analysis with a sum of exponentials were performed as described (5). Meta-II formation was measured in a single flash experiment as the time trace of rhodopsin absorbance changes at 380 nm. The excitation was with 10 ns pulses of ∼10 mJ of energy at 493 nm. Under these conditions about 30–40% of rhodopsin molecules are excited.

The binding of arrestin (1 μM) to rhodopsin (3 μM) was monitored using flash photolysis of rhodopsin, as arrestin binding stabilizes Meta-II at low temperature (3 °C) and higher pH (potassium phosphate buffer pH 8.0, 150 mM NaCl). To charac-

terize the binding affinity of arrestin to fluorescently labeled rhodopsins we used rhodopsin samples with high labeling stoichiometry.

Time-Resolved Fluorescence Spectroscopy. Fluorescence lifetime and anisotropy decays were measured employing a tunable Ti: Sapphire laser/microchannel plate based single-photon counting apparatus with picosecond time resolution equipped with a TCSPC Module (SPC830, B&H GmbH) (5, 7). The fluorescence pump-probe experiments were performed using the setup described in ref. 7. To obtain light-activated rhodopsin for the anisotropy measurements, Rh and P-Rh were illuminated with light ($\lambda > 435$ nm) for 60 s at room temperature. For fluorescence pump-probe experiments rhodopsin activation was triggered by a short flash of light.

The fluorescence decay profiles ($I_{||}(t)$ and $I_{\perp}(t)$) and the time-resolved anisotropy as given by the following equation,

$$r(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)} \qquad \textbf{[S2]}$$

were analyzed using the software package Global Unlimited V2.2. The time course of the fluorescence was fitted with a sum of exponentials.

The anisotropy decay was fitted with the model function (Eq. **S3**):

$$r(t) = \sum_{i=1}^{n} \beta_i e^{-t/\Phi_i} + r_{\infty} \qquad \textbf{[S3]}$$

with β_i the amplitude and ϕ_i the correlation time of the ith decay component.

Because the rotational correlation time due to the tumbling of the rhodopsin membranes is in the ms time range and thus far out of the fluorescence lifetime scale of the label used (fluorescein, $\tau \sim 4$ ns) the amplitude of this component (r_{∞}) is not time dependent on the ns time scale of our measurement. The measurements were performed at 20 °C using fluorescently labeled rhodopsin samples with about 0.1 mol of the respective label incorporated per mol rhodopsin. At this labeling stoichiometry, almost no homoenergy transfer occurs between the fluorescent dyes attached to rhodopsin molecules in the native disc membrane. Using a low labeling stoichiometry is important, because homoenergy transfer was shown to affect the time course of the fluorescence depolarization (8).

Kinetic Light Scattering. A forward near infrared light scattering instrument ($\lambda = 870$ nm) was built up according to ref. 9. This light scattering instrument is very sensitive to the gain in size of the disk membrane upon arrestin binding, resulting in a proportional increase of scattered light. The binding of arrestin to phosphorylated rhodopsin membranes was triggered by a flash of light (478 nm) at 20 °C. The change in the intensity of scattered light (ΔI) upon arrestin binding is expressed as $\Delta I/I$ with I the intensity of scattered light from rhodopsin membranes before light activation. Control measurements without arrestin were performed.

1. Knowles A, Priestley A (1978) The preparation of 11-*cis*-retinal. *Vision Res* 18:115–116.
2. McDowell JH, Kuhn H (1977) Light-induced phosphorylation of rhodopsin in cattle photoreceptor membranes: substrate activation and inactivation. *Biochemistry* 16:4054–4060.
3. Wilden U, Kuhn H (1982) Light-dependent phosphorylation of rhodopsin: number of phosphorylation sites. *Biochemistry* 21:3014–3022.
4. Gibson SK, Parkes JH, Liebman PA (1998) Phosphorylation stabilizes the active conformation of rhodopsin. *Biochemistry* 37:11393–11398.
5. Alexiev U, Rimke I, Pohlmann T (2003) Elucidation of the nature of the conformational changes of the EF-interhelical loop in bacteriorhodopsin and of the helix VIII on the cytoplasmic surface of bovine rhodopsin: a time-resolved fluorescence depolarization study. *J Mol Biol* 328:705–719.
6. Skegro D, et al. (2007) N-terminal and C-terminal domains of arrestin both contribute in binding to rhodopsin. *Photochem Photobiol* 83:385–392.
7. Kim TY, Winkler K, Alexiev U (2007) Picosecond multidimensional fluorescence spectroscopy: a tool to measure real-time protein dynamics during function. *Photochem Photobiol* 83:378–384.
8. Karolin J, Fa M, Wilzczynska M, Ny T, Johannson LB-A (1998) Donor-donor energy migration for determining intramolecular distances in proteins: I. Application of a model to the latent plasminogen activator inhibitor-1 (PAI-1). *Biophys J* 74:11–21.
9. Heck M, Pulvermuller A, Hofmann KP (2000) Light scattering methods to monitor interactions between rhodopsin-containing membranes and soluble proteins. *Methods in Enzymology* 315:329–347.

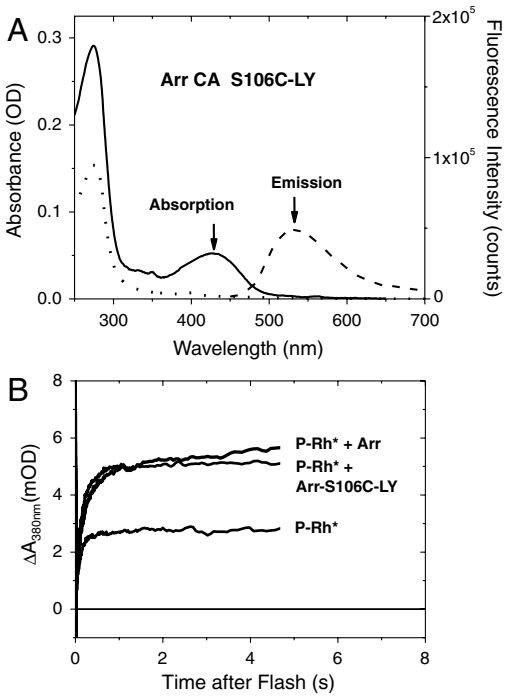

Fig. S1. Characterization of the fluorescently labeled mutant arrestin ArrCA S106C-LY (*A*) Absorption (—) and emission (- - -) spectra of the fluorescent dye LY covalently bound to S106C on the outer surface of the N-domain cupola of arrestin. For comparison the absorption spectrum for unlabeled arrestin (…) is shown. LY displays an absorption maximum of $\lambda_{max} = 428 \pm 1$ nm and an emission spectrum with a maximum of $\lambda_{max} = 533 \pm 1$ nm. The excitation was at 428 nm. (*B*) Time trace of Meta-II formation at 380 nm for phosphorylated rhodopsin disk membranes in the presence and absence of wild-type (WT) arrestin and the fluorescently labeled arrestin mutant CA S106C-LY. Meta-II stabilization (Extra-Meta-II) is indicative of arrestin binding; i.e., arrestin binding shifts the Meta-I/Meta-II equilibrium towards Meta-II under the experimental conditions. Conditions: 3 μM rhodopsin, 1 μM arrestin, 150 mM NaCl, pH 8.0, 3 °C.

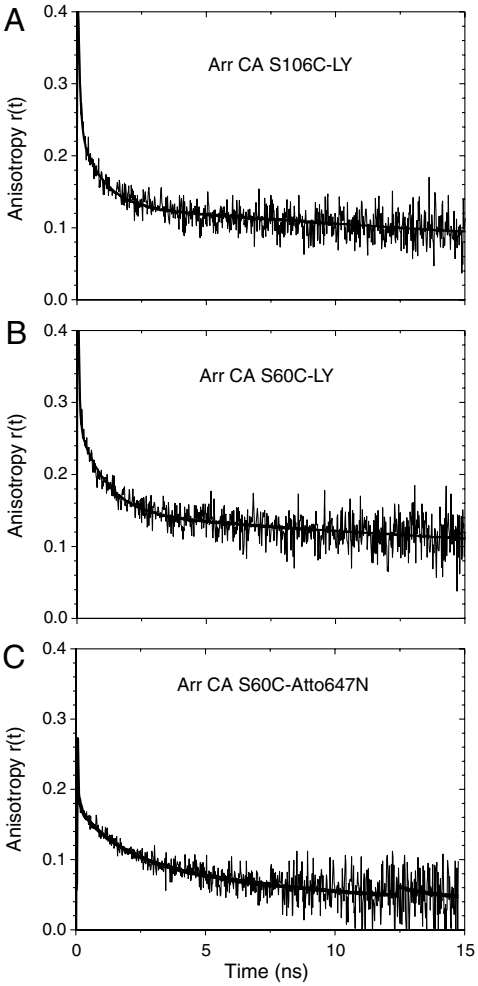

Fig. S2. Time-resolved anisotropy decays of fluorescently labeled arrestin. (*A*) ArrCA S106C-LY, (*B*) ArrCA S60C-LY, (*C*) ArrCA S60C-Atto647N. Absorption and emission spectra of the covalently bound fluorescent dyes are shown in Figs. S1 and S3. The rotational correlation time of arrestin at 20 °C as determined from the anisotropy decays (slowest decay time) is 38 ± 8 ns. The size of the molecule estimated from this correlation time under the assumption of a spherical rotator yields an effective hydrodynamic diameter of $d = 66$ Å, in good agreement with the average diameter of about 52 Å obtained from the crystal structures (Fig. 1*A*).

Conditions: 5 μM arrestin, 150 mM NaCl, 50 mM potassium phosphate buffer pH 7.5, 20 °C. Fluorescence excitation was at 428 nm for LY and 617 nm for Atto647N.

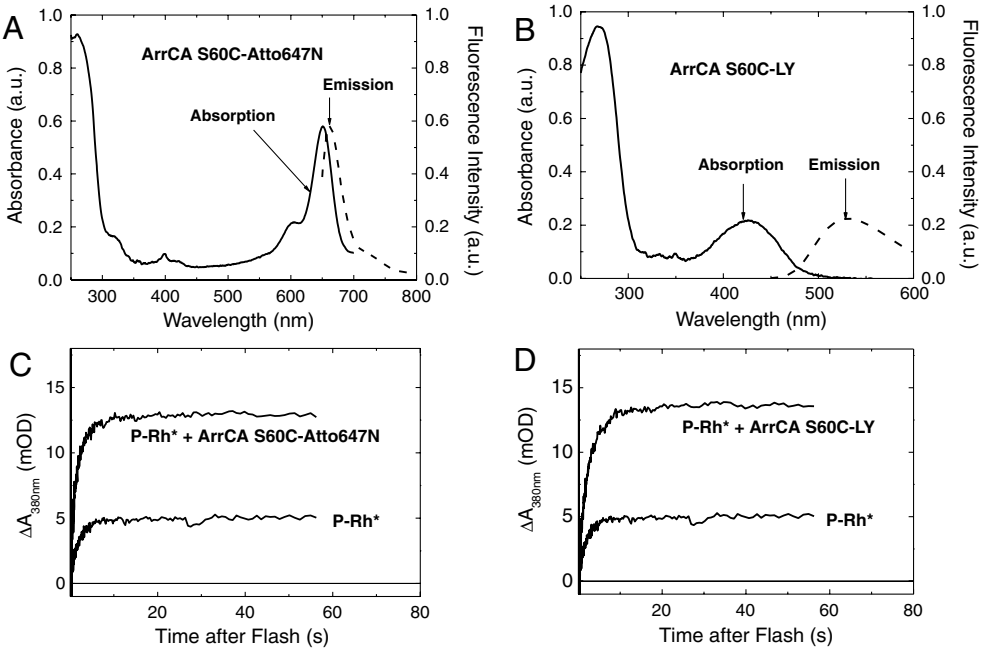

Fig. S3. Characterization of fluorescently labeled arrestin mutants ArrCA S60C-LY and ArrCA S60C-Atto647N. (*A*) Absorption spectra of Atto647N-labeled (—) arrestin ArrCA S60C. The corresponding emission spectrum is shown in (- - - -). Bound Atto647N has its maximum absorption at $\lambda_{max} = 651 \pm 1$ nm and an emission maximum at $\lambda_{max} = 663 \pm 1$ nm. (*B*) Absorption spectra of LY-labeled (—) arrestin ArrCA S60C. The corresponding emission spectrum is shown in (- - - -). LY has its maximum absorption at $\lambda_{max} = 427 \pm 1$ nm and an emission maximum at $\lambda_{max} = 530 \pm 1$ nm. The excitation was at 428 nm for LY and 617 nm for Atto647N. (*C*) Time trace of Meta-II formation at 380 nm for phosphorylated rhodopsin disk membranes in the presence and absence of the fluorescently labeled arrestin mutant ArrCA S60C-Atto647N. (*D*) Time trace of Meta-II formation at 380 nm for phosphorylated rhodopsin disk membranes in the presence and absence of the fluorescently labeled arrestin mutant ArrCA S60C-LY. Meta-II stabilization (Extra-Meta-II) is indicative of arrestin binding; i.e., arrestin binding shifts the Meta-I/Meta-II equilibrium towards Meta-II under the experimental conditions.

Conditions: 3 μM rhodopsin, 1 μM arrestin, 150 mM NaCl, pH 8.0, 3 °C.

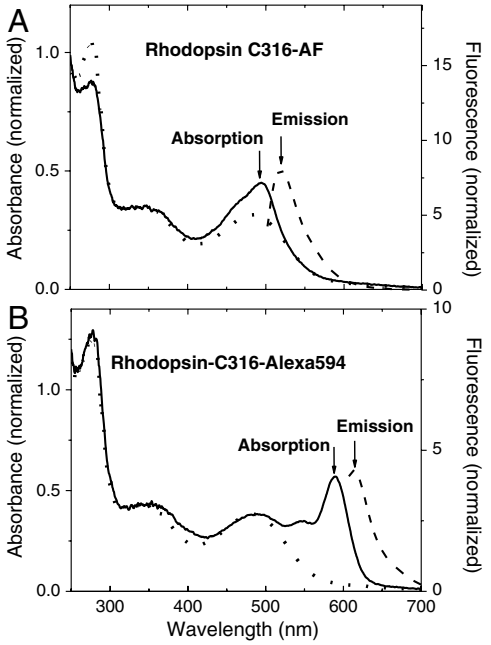

Fig. S4. Characterization of fluorescently labeled rhodopsin. Absorption and emission spectra of the fluorescent dyes iodoacetamidofluorescein (IAF or SF) and Alexa594-maleimide (Alexa594 or LF) covalently bound to C316 on the surface of bovine rhodopsin, yielding Rhodopsin C316-AF, also named Rh H8-SF, and Rhodopsin C316-Alexa594, also named Rh H8-LF. (*A*) Absorption spectra of unlabeled (…) and SF-labeled (—) rhodopsin membranes. The corresponding emission spectrum is shown in (- - - -). SF has its maximum absorption at $\lambda_{max} = 496 \pm 1$ nm and an emission maximum at $\lambda_{max} = 520 \pm 1$ nm. (*B*) Absorption spectra of unlabeled (…) and LF-labeled (—) rhodopsin membranes. The corresponding emission spectrum is shown in (- - - -). LF has its maximum absorption at $\lambda_{max} = 598 \pm 1$ nm and an emission maximum at $\lambda_{max} = 614 \pm 1$ nm. The fluorescence excitation was at 470 nm for SF and 600 nm for LF.

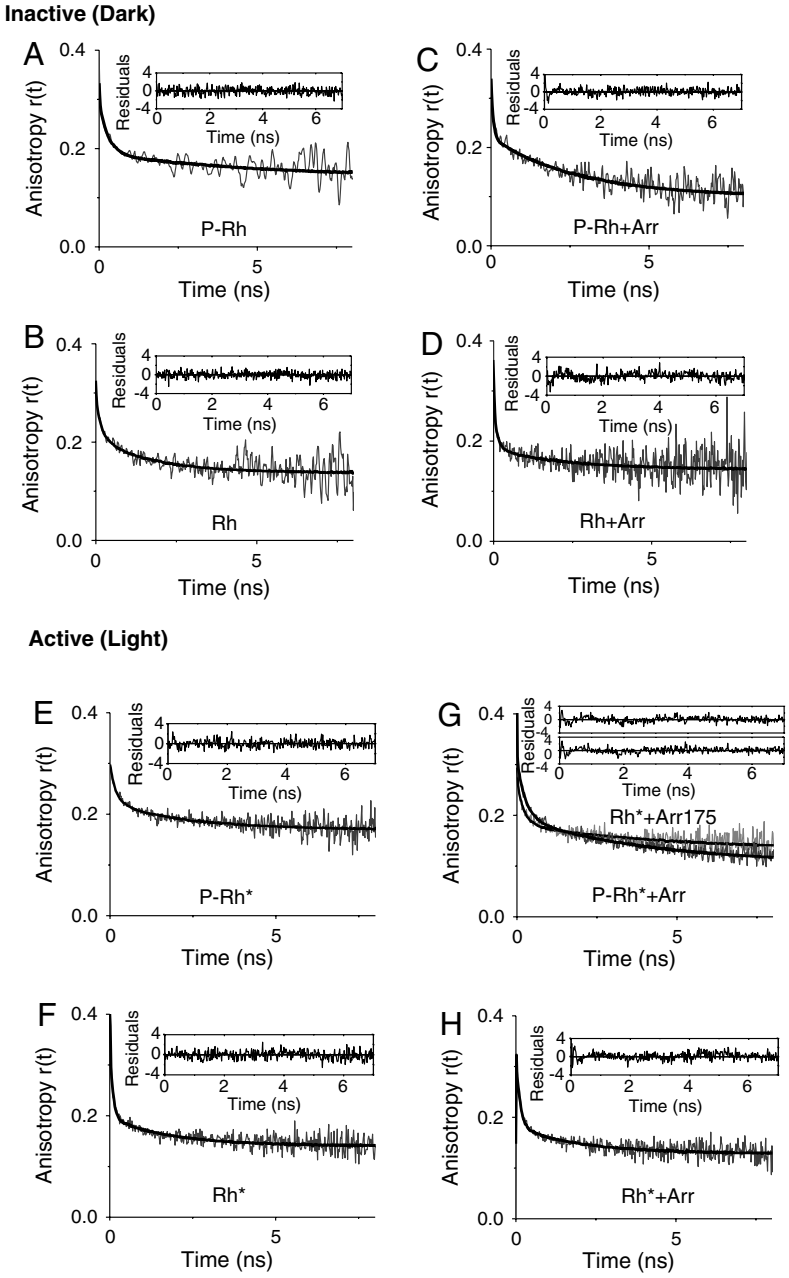

Fig. S5. Time-resolved anisotropy decay curves of SF covalently bound to rhodopsin in position Cys316 for dark (*A*–*D*) and light-activated rhodopsin (*E*–*H*): (*A*),(*E*)—phosphorylated rhodopsin membranes, (*B*), (*F*) unphosphorylated rhodopsin membranes, (*C*), (*G*)—phosphorylated rhodopsin membranes in the presence of arrestin, (*G*)—phosphorylated rhodopsin membranes in the presence of arrestin, and unphosphorylated rhodopsin membranes in the presence of the arrestin mutant R175E, (*D*), (*H*)—unphosphorylated rhodopsin membranes in the presence of arrestin.

Experimental data and fit curves are presented for the time range 0–8 ns. Fit parameter are summarized in Table S1.

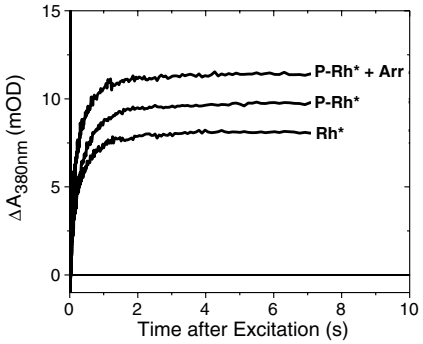

Fig. S6. Binding affinity of arrestin to rhodopsin membranes. Time trace of Meta-II formation at 380 nm, indicative for the deprotonation of the Schiff base linkage of the covalently bound ligand retinal, for phosphorylated rhodopsin disk membranes in the presence and absence of WT arrestin. Meta-II stabilization (Extra-Meta-II) is indicative of arrestin binding; i.e., arrestin binding shifts the Meta-I/Meta-II equilibrium towards Meta-II under the experimental conditions. For comparison, the time trace of unphosphorylated rhodopsin disk membranes is shown.

Conditions: 3 μM rhodopsin, 1 μM arrestin, 150 mM NaCl, pH 8.0, 3 °C.

Table S1. Fluorescence anisotropy decay parameters of fluorescein bound to cysteine 316 in H8 of rhodopsin (H8-SF)

	Amplitudes			Correlation times		χ_R^2
Sample	β_1	β_2	r_∞	ϕ_1 (ns)	ϕ_2 (ns)	
dark						
Rh	0.09	0.07	0.15	0.11 ± 0.01	1.9 ± 0.1	1.02
P-Rh	0.11	0.06	0.17	0.18 ± 0.06	3.4 ± 0.2	1.09
P-Rh + wt Arr	0.11	0.12	0.11	0.07 ± 0.01	2.8 ± 0.2	1.05
light						
Rh*	0.09	0.05	0.16	0.08 ± 0.02	2.0 ± 0.1	1.12
Rh* + wt Arr	0.09	0.05	0.16	0.12 ± 0.01	2.1 ± 0.1	1.06
P-Rh*	0.10	0.05	0.18	0.16 ± 0.01	2.4 ± 0.1	1.07
P-Rh* + wt Arr	0.10	0.09	0.11	0.24 ± 0.01	4.2 ± 0.1	1.07
Rh* + ArrR175E	0.09	0.05	0.14	0.11 ± 0.01	2.1 ± 0.1	1.13

The reduced χ^2 (χ_R^2) is given. Experimental data, fit curves and residuals of the fit are presented in Fig. S4.

Conditions: pH 7.5, 150 mM NaCl, 20 °C. The excitation wavelength was 470 nm and the fluorescence emission was detected after passing through a cut-off color glass filter OG 515.