Supporting Information

Piekchnick et al. 10.1073/pnas.1117268109

SI Text

FTIR Spectroscopic Characterization of N2C/D282C and the Channel Mutants. For a spectroscopic characterization of the N2C/D282C opsin mutant, we recorded the FTIR difference spectra between the cis-retinal bound dark state and the Ctα peptide-stabilized opsin/all-trans-retinal complex ("Rho minus Ops*\atrans" difference spectrum; Fig. SI A, red spectrum). For regeneration of the rhodopsin dark state, cis-retinal was formed by illuminating the opsin/all-trans-retinal/Ctα peptide mixture with ultraviolet light (395 nm). The Rho minus Ops*\atrans difference spectrum (in the spectral range that predominantly reflects protein activation 1,400–1,800 cm\textsuperscript{-1}), a mirror image of the difference spectrum obtained by illumination of the dark state in the presence of Ctα peptide ("Rho* minus Rho" difference spectrum, Fig. SI A, red spectrum). This mirror image includes the pattern in the carbonyl region (1,768, 1,748, 1,713 cm\textsuperscript{-1}) as well as prominent bands in the amide I (1,661 and 1,644 cm\textsuperscript{-1}) and amide II (1,555 cm\textsuperscript{-1}) regions. A positive band at 1,238 cm\textsuperscript{-1} is proof for the regeneration of a 11-cis-retinal bound state. The lower intensity of the band in Rho minus Ops*\atrans vs. Rho* minus Rho is due to considerable amount of 9-cis-retinal formed by illumination of all-trans-retinal, which leads to the formation of isorhodopsin in which the main retinal band is downshifted to 1,206 cm\textsuperscript{-1}. Note that the 1,661 cm\textsuperscript{-1} band distinguishes the decay and formation of a Ctα peptide-bound form of the active receptor from peptide-free Meta II (1, 2).

We selected eight mutants for FTIR difference spectroscopy (Fig. S1B). In general, all spectra show the signature typical for formation of the Meta II state, as observed in the spectrum of the wild type recorded in egg-PC vesicles (Fig. SI B, black spectrum). Meta II formation is indicated by the carbonyl difference bands at 1,768 cm\textsuperscript{-1}, 1,748 cm\textsuperscript{-1} and 1,713 cm\textsuperscript{-1}, the pattern around the large amide mode at 1,644 cm\textsuperscript{-1} and the typical hydrogen out of plane modes around 969 cm\textsuperscript{-1}. The spectrum of the N2C/D282C background control recorded in DDM (Fig. SI B, red spectrum) is almost identical to the spectrum of the wild type and slight differences might be attributed to different contributions of the Meta II subspecies (1) induced by the detergent.

Further deviations from wild type/ N2C/D282C background control that occur in the spectra of the mutants suggest minor structural impact of the mutants on the activation path. The light-induced transitions of almost all mutants are therefore largely similar to those observed in the wild type/ N2C/D282C background control. Solely the M44F mutant showed a largely similar to wild type not only in M44F but also in the other mutants of opening A (A292F and T299F). Y268E, located in opening B, exhibits a smaller band at 1,644 cm\textsuperscript{-1}, indicating that the structural alterations accompanying light activation are smaller in these mutants. However F208L, also located in opening B, exhibits a larger band at 1,644 cm\textsuperscript{-1} band comparable to wild type. Finally, changes of hydrogen bonding around Asp83 and Glu122, assigned to the bands at 1,768; 1,748; and 1,733/1,727 cm\textsuperscript{-1} (see ref. 3 and citations therein), appeared normal in all tested mutants. We can conclude that the activating changes in the hydrogen bonded network comprising these residues are similar in all mutants investigated.

The FTIR difference spectrum of K296G/11-cis-PrSB (Fig. S1 B, light green spectrum) revealed light induced conformational alterations of the mutant that are also largely similar with the normal activation process of rhodopsin. Specifically, the bands due to changes in hydrogen bonding around the key residues Asp83 and Glu122 are like in the wild type. The absence of the retinal Schiff base in the binding pocket is clearly reflected in the retinal fingerprint around 1,238 cm\textsuperscript{-1}, in the ethylenic region around 1,556 cm\textsuperscript{-1} and in the HOOP region around 950 cm\textsuperscript{-1}.

G Protein Activation by Retinal Channel Mutants. We tested 15 mutants for their ability to catalytically activate G\textsubscript{t} using the intrinsic tryptophan fluorescence change of the G\textsubscript{tα}-subunit upon GTP\textsubscript{S} uptake (4). G\textsubscript{t} activity was assayed with 11-cis-retinal regeneratted pigments after exposure to light (Fig. S5 A) or with purified opsins in the absence of the retinal ligand (Fig. S5 B). With the exception of K296G (see below), the amino acid replacements had little effect on the light-induced activity of the mutant pigments (Fig. S5 A). We can conclude that in these mutants the active cytoplasmic domain responsible for G\textsubscript{t} activation develops properly in spite of the replacements in the channel. The results obtained with mutants T94I, E181Q, and S186A are in agreement with a previous report (5). The activity of mutant Y268F (80%) is higher than reported in an earlier study (20%, ref. 5).

The catalytic activity of the purified opsin mutants in the absence of retinal is shown in Fig. S5 B. Under the conditions of the experiment (0.03% DDM, pH 6.0), the basal activity of the N2C/D282C background control was 1% of the light-induced activity (Fig. S5 C). Several opsin mutants displayed a significant increased basal activity, including K296G (see below), M44F, T289F, A202F, and F276Q. Two mutant opsins (Y43A and T94I) exhibited significant lower activation rates. Interestingly, the reduced basal activity of the night blindness mutant T94I was not observed in a previous study using isolated COS cell membranes (6). Taken together, the effect of the mutations on opsin activity is more pronounced than on the light-activated pigments. This finding may reflect the stabilizing effect of the all-trans-retinal agonist, which is present in Meta II but not in opsin.

G\textsubscript{t} Activity by Mutant K296G. Consistent with a previous report (7), our data (see Fig. 3 in the main text) strongly suggest that illumination of 11-cis-PrSB reconstituted K296G results in an immediate release of the retinal derivative from its binding pocket. Surprisingly, the light-induced activity of K296G (Fig. S5 C, red trace) significantly exceeded that of ligand-free K296G opsin over a period of >60 s (Fig. S5 C, green trace). In the presence of excess 11-cis-PrSB, however, the activity of K296G is rapidly quenched as soon as the activating light is turned off (Fig. S5 C, orange trace). This finding demonstrates that 11-cis-PrSB has immediate access to the binding pocket after light activation of K296G and confirms the fast uptake and release of the retinal chromophore in this mutant. The light-induced activity that apparently persists after release of the retinal ligand from its binding pocket, indicates interaction of all-trans-PrSB with an additional binding site on K296G, which stimulates G\textsubscript{t} activity as previously suggested for native opsin in disk membranes (8–10). Further experiments are necessary to fully investigate this phenomenon.

SI Materials and Methods. Preparation of G\textsubscript{t}. Transducin (G\textsubscript{t}) was prepared from bovine rod outer segments, separated into G\textsubscript{tα} and G\textsubscript{tβγ} subunits by chromatography as described (11) and dialyzed against 20 mM bis-tris-propane, pH 7.1, 130 mM NaCl,
1 mM MgCl₂ and 2 mM DTT. Protein concentration was determined with Bradford reagent.

**Analysis of regeneration kinetics.** Uptake of 11-cis-retinal by opsin was measured by the increase of the 500 nm (or respective λₘₐₓ) absorption of the rhodopsin dark state. To analyze the influence of the mutations on the regeneration rate, we assume that the regeneration process follows an irreversible bimolecular reaction scheme:

opsin + 11-cis-retinal \( \xrightarrow{k} \) rhodopsin.  

\[ S1 \]

Under the non pseudo-first order conditions used in this study (0.5 μM opsin and 1 μM 11-cis-retinal) the regeneration of the mutant opsins followed a bimolecular time course. The rate constant (k) of the reaction was thus obtained by applying a bimolecular reaction fit (Eq. S2); (12) to the data points:

\[ P(t) = \frac{A_0 B_0}{A_0 - B_0} \left( 1 - e^{-k(A_0 - B_0)t} \right) \]

\[ S2 \]

where \( P(t) \) represents the amount of rhodopsin formed, and \( A_0 \) and \( B_0 \) are the initial concentration of opsin and 11-cis-retinal, respectively.

**FTIR difference spectroscopy.** The purified pigments in 10 mM bis-tris-propane (pH 6.0) and 0.03% DDM were concentrated using a microapore 30 kD filter (Millipore). 5 μL of this solution was then slowly dried on a BaF₂ window of the infrared cuvette. After rehydration with 0.2 μL 10 mM bis-tris-propane (pH 6.0) the cuvette was sealed by a second BaF₂ window and a 3 μm polytetrafluoroethylene spacer. FTIR spectra were recorded at 20 °C using a Bruker ifs66v/s spectrometer equipped with a LN₂-cooled MCT-detector (Kolmar Technologies Inc.) and a 1.950 cm⁻¹ optical cut-off filter. Activating illumination was performed by a 3 s illumination with three green LEDs (520 nm, 7,000 mcd; led1.de). FTIR spectra were recorded before, during, and after illumination in the rapid-scan mode with a time-resolution varying from 0.2 s to 15 s. FTIR differences were calculated by subtracting the spectra of the illuminated state from the spectra before illumination. These time-dependent FTIR difference spectra were then analyzed by Singular Value Decomposition and Global analysis (13) to correct the data for decay products of Meta II.

**G₄ activation assay.** As a monitor for G₄ activation, changes in intrinsic fluorescence intensity of the G₄α-subunit upon exchange of GDP to GTPγS were quantified as previously described (14). The measurements were carried out using a SPEX fluorolog II spectrophotofluorometer equipped with a 450 W xenon arc lamp. For all activation measurements, settings were \( \lambda_{ex} = 300 \) nm, and \( \lambda_{em} = 340 \) nm with an integration time of 1 s. G₄ activation rates were measured with 5 nM purified rhodopsin or opsin, 0.6 μM Gt, 25 μM GTPγS, 20 mM bis-tris-propane, pH 6.0, 130 mM NaCl, 1 mM MgCl₂, 2 mM DTT and 0.01% (wt/vol) DDM in a final volume of 750 μL. All samples were equilibrated at 20 °C for 4 min. Reactions were triggered with orange light (495 nm long pass filter, Schott GG 495) or by addition of GTPγS. The initial G₄ activation rate was obtained by linear regression of the initial rise in fluorescence emission.

References:

Fig. S1. FTIR spectroscopic characterization of wild type, N2C/D282C background control and selected retinal channel mutants. (A) FTIR difference spectrum of the cis-retinal regenerated dark state (Rho) minus the Cterminal peptide-stabilized opsin/all-trans-retinal complex (Ops*/atr) (blue spectrum) and the difference spectrum of light-activated rhodopsin (Rho*) minus the cis-retinal regenerated dark state (Rho) (red spectrum). (B) FTIR difference spectrum (photoproduct minus dark state) obtained under conditions favoring the formation of Meta II is nearly identical for wild type rhodopsin (reconstituted in liposomes, pH 6.0, 30 °C, black trace) and the N2C/D282C background control (in DDM, pH 6.0, 20 °C, red trace). Corresponding spectra of the mutants M44F (blue), A292F (magenta), T289F (cyan), K296G (light green), Y268F (dark green), Y268E (turquoise), A272I (red), and F208L (orange) are shown below.
Fig. S2. Spectroscopic properties of the retinal channel mutants. Absorption maxima ($\lambda_{\text{max}}$) of the rhodopsin dark state (A) and spectral ratio of $A_{280}/A(\lambda_{\text{max}})$ of the purified mutant pigments (B).
Fig. S3. UV/visible absorption spectra of wild type, N2C/D282C control, and retinal channel mutants in opening A. Photobleaching properties are shown for each pigment as follows: purified dark state (spectrum 1), immediately following 15-s illumination (spectrum 2), after complete decay of the Meta II state (spectrum 3), and immediately following addition of 25 mM hydroxylamine combined with a second illumination (spectrum 4). For selected mutants the spectrum of a sample immediately following 15-s illumination in the presence of 50 μM CTh peptide is shown (spectrum 5, red). Measuring conditions: 0.5 μM purified rhodopsin mutant in bis-tris-propane (pH 6.0) and 0.03% DDM.
Fig. S4. UV/visible absorption spectra of retinal channel mutants in the binding pocket and opening B. Photobleaching properties are shown for each pigment as follows: purified dark state (spectrum 1), immediately following 15-s illumination (spectrum 2), after complete decay of the Meta II state (spectrum 3), and immediately following addition of 25 mM hydroxylamine combined with a second illumination (spectrum 4). For selected mutants the spectrum of a sample immediately following 15-s illumination in the presence of 50 μM CTPα peptide is shown (spectrum 5, red). Measuring conditions: 0.5 μM purified rhodopsin mutant in bis-tris-propane (pH 6.0) and 0.03% DDM.
**Fig. S5.** $G_{i}$ activation by selected retinal channel mutants. Comparison of the initial rates of $G_{i}$ activation by 11-cis-retinal-regenerated mutants after exposure to light (A) and by mutant opsins in the absence of retinal (B). Activities are plotted as percentages relative to the N2C/D282C control, which was taken to be 100% (dashed lines). Error bars display SD of ten (control) or three (all other mutants) independent measurements. (C) $G_{i}$ activation by N2C/D282C control and K296G mutant. Black trace: $G_{i}$ activation by N2C/D282C rhodopsin (control) induced by a 10-s illumination (arrow). Gray trace: $G_{i}$ activation by N2C/D282C opsin initiated by addition of GTP/$\gamma$S. The arrow marks the point when additional light-activated N2C/D282C rhodopsin (R*) was added to activate the whole $G_{i}$ pool. Red trace: $G_{i}$ activation of K296G regenerated with 11-cis-PrSB induced by a 10-s illumination. Green trace: $G_{i}$ activation by K296G opsin initiated by addition of GTP/$\gamma$S. Blue trace: $G_{i}$ activation by K296G in the presence of 0.5 $\mu$M 11-cis-PrSB induced by continuous illumination. Orange trace: $G_{i}$ activation by K296G in the presence of 0.5 $\mu$M 11-cis-PrSB induced by a 10-s illumination (first arrow), followed by a second 10-s illumination (second arrow) and continuous illumination (third arrow).
Fig. S6. Activation/deactivation cycle of rhodopsin. In Meta II the all-trans-retinal ligand is covalently attached to Lys296 of the opsin apoprotein by a deprotonated Schiff Base linkage. Decay of Meta II (top) comprises hydrolysis of the Schiff base via a carbinolamine intermediate which leads to formation of a noncovalent Ops* all-trans-retinal adduct and eventual release of retinal from its binding pocket. Regeneration of rhodopsin (bottom) starts with uptake of 11-cis-retinal into Ops*, either by binding to the preexisting fraction of Ops* (conformational selection) or by interaction with Ops (induced fit). Receptor-ligand interaction within the noncovalent Ops* 11-cis-retinal adduct then triggers the formation of the deprotonated 11-cis-retinal Schiff base linkage of the rhodopsin dark state. Schiff Base formation proceeds via a carbinolamine intermediate and release of water. Both pathways depend on switches between active (red) and inactive (blue) receptor conformations. Light absorption (hν) closes the cycle by forming Meta II from rhodopsin.

Fig. S7. Influence of CTα peptide on the regeneration of N2C/D282C background control opsin with 11-cis-retinal monitored by the absorbance change at 500 nm. Regeneration in the absence (open circles) and in the presence (closed circles) of 50 μM CTα peptide [G_{340–350}-high affinity analog peptide, VLEDLKSCGLF]. Measuring conditions: 0.5 μM N2C/D282C opsin, 1 μM 11-cis-retinal; 10 mM bis-tri-propane, pH 6.0 and 0.03% DDM, 20 °C.