The Activation Pathway of Human Rhodopsin in Comparison to Bovine Rhodopsin

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Background: Rhodopsins are the photoreceptors of the vertebrate eye.

Results: We characterized human rhodopsin by spectroscopic methods and developed a structural model based on the well studied bovine rhodopsin.

Conclusion: The activation pathways of the two receptors are similar. Differences exist between the inactive receptor conformations.

Significance: Our findings will allow a better mechanistic understanding of disease-causing human rhodopsin mutations.

Rhodopsin, the photoreceptor of rod cells, absorbs light to mediate the first step of vision by activating the G protein transducin (Gt). Several human diseases, such as retinitis pigmentosa or congenital night blindness, are linked to rhodopsin malfunctions. Most of the corresponding in vivo studies and structure-function analyses (e.g. based on protein x-ray crystallography or spectroscopy) have been carried out on murine or bovine rhodopsin. Because these rhodopsins differ at several amino acid positions from human rhodopsin, we conducted a comprehensive spectroscopic characterization of human rhodopsin in combination with molecular dynamics simulations. We show by FTIR and UV-visible difference spectroscopy that the light-induced transformations of the early photointermediates are very similar. Significant differences between the pigments appear with formation of the still inactive Meta I state and the transition to active Meta II. However, the conformation of Meta II and its activity toward the G protein are essentially the same, presumably reflecting the evolutionary pressure under which the active state has developed. Altogether, our results show that although the basic activation pathways of human and bovine rhodopsin are similar, structural deviations exist in the inactive conformation and during receptor activation, even between closely related rhodopsins. These differences between the well studied bovine or murine rhodopsins and human rhodopsin have to be taken into account when the influence of point mutations on the activation pathway of human rhodopsin are investigated using the bovine or murine rhodopsin template sequences.

Rhodopsin (Rho), the rod cell photoreceptor that mediates dim light vision in the vertebrate eye, is the archetype of class A G protein-coupled receptors (GPCR) and consists of 348 amino acids. It shares with other GPCRs a seven-transmembrane (TM) α helical structure. Rho is unique among GPCRs as its light-sensitive ligand 11-cis-retinal is covalently bound to the apoprotein via a protonated Schiff base. Light absorption induces 11-cis to all-trans isomerization thus replacing the inverse agonist by a strong agonist in situ. Subsequently the receptor proceeds through a number of intermediates, eventually leading to the late metarhodopsin states, which include the active form Meta II that binds and activates the G protein (1). Rho activation is the first step of a highly optimized catalytic system allowing single photon response due to high amplification on the background of virtually no dark noise.

The majority of biochemical and biophysical investigations on Rho were undertaken with the easily accessible bovine rhodopsin (bRho) (2). Bovine and human rhodopsin (hRho) show a sequence identity of 93.4% (3), with different amino acids at 23 positions (Fig. 1). Remarkably some sequence differences occur in regions that may play a crucial role during receptor activation. This applies to the connection from TM5 to the extracellular loop 2 (EL2, amino acids 175–198), hereafter defined as

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The abbreviations used are: Rho, rhodopsin; hRho, human Rho; bRho, bovine Rho; GPCR, G protein-coupled receptor; TM, transmembrane; EL2, extracellular loop 2; G, G protein transducin; bis-tris-propane, 1,3-bis(tris(hydroxymethyl)methyl)amino)propane; GTPγS, guanosine 5'-O-(thiotriphosphate); Batho, bathorhodopsin; Lumi, lumirhodopsin; TEC, T5E2C region; SB, Schiff base.
the T$_5$E$_2$C region, and amino acids 297–300 in the direct proximity of the Schiff base, which deprotonates during the transition from inactive Meta I to the active Meta II state (Fig. 2).

Because both regions are connected to activating conformational changes, it is possible that these sequence alterations significantly affect the conformations of the dark state and light-activated photointermediates and thereby change the functional properties of the photoreceptor.

For our studies, bRho and hRho were expressed and purified in COS-1 cells, which yields sufficient quantities and purity to conduct spectroscopic investigations. Subsequent incorporation into lipid vesicles allows their investigation in a native-like membrane environment. The main technique of our investigation is FTIR difference spectroscopy, which has high structural sensitivity and allows investigation of Rho samples at physiologically relevant millimolar concentrations. Furthermore, the FTIR peptide binding assay applied here is capable of monitoring the conformational changes induced by binding of the C-terminus of the G$_{\alpha}$ subunit to the active receptor (4–6).

The spectroscopic data are complemented by classical molecular dynamics (MD) simulations of the dark state and active conformations of hRho and bRho, and the G protein activation capability was tested using a catalytic fluorescence assay.

At least 100 mutations at 68 positions (see Fig. 1) in Rho genes are known to cause retinitis pigmentosa, which is linked to a gradual loss of peripheral and night vision (see Ref. 2 for examples). Some of these mutations have an effect on the structural integrity of the protein like impairment of protein folding and transport (7). Other mutations primarily have an impact on the conformational equilibria of the agonist-bound receptor (Meta-equilibria) (8), whereas congenital night blindness is predominantly caused by an increase in constitutive activity (9).

Many of these diseases are caused by single amino acid mutations. The structural and mechanistic differences between bRho and hRho found in this study should thus be considered when the activation pathway of malfunctioning human receptor mutants is investigated.

**Experimental Procedures**

**Sample Preparation**—For expression in COS-1 cells (ATCC, CRL-1650), a human codon-optimized synthetic Rho DNA fragment (corresponding to amino acids 1–348 of the native protein, accession number NP_000530) containing the C-terminal TETSQVAPA sequence (1D4 epitope; Ref. 10) was designed and purchased from MWG (Eurofins MWG Operon, Ebersberg, Germany). The synthetic Rho DNA was inserted between the EcoRI-NotI sites of the expression vector pMT4 (11). Human Rho expressed equally well as wild type bRho. Purification and reconstitution of hRho could be performed in the same way as described for bRho, including the elution procedure and incorporation into egg-phosphatidylcholine vesicles (12). To ensure that the detergent was completely removed after the dialysis procedures, the samples were centrifuged for 1 h at 200,000 g, and the supernatant was checked by UV-visible spectroscopy for protonated retinal Schiff Base.

**FTIR and UV-visible Spectroscopy**—All spectroscopic measurements were performed on a Bruker ifs66v/s IR-spectrometer coupled via fiberoptics to an OLIS RSM UV-visible-spectrometer for simultaneous data acquisition in the infrared and UV/visible range. The samples were illuminated (10 s) during the measurement with LEDs ($\lambda_{\text{max}}$ = 580 nm, Meta I and Meta II conditions; $\lambda_{\text{max}}$ = 470 nm, Batho and Lumi conditions) to achieve full bleaching. The data acquisition was done in the Rapid-Scan mode with a quasi-logarithmic time resolution >171 ms. The recorded datasets were linearly corrected for small temperature-dependent. To obtain sets of difference spectra, the whole dataset was subtracted from the average of all spectra recorded before illumination. A photostationary state is usually not reached due to the overlay of formation and decay processes of the different species, the difference spectra of the intermediates and their kinetics were determined by a singular value decomposition (SVD) method and global fitting procedure (13). The procedure yields the spectral characteristics of the reaction separated by their different kinetics.
To unveil even minute differences between the species under the same conditions, the double difference spectra were calculated by subtracting the spectra of hRho from the bRho counterparts, normalized to the chromophore fingerprint band at 1237 cm$^{-1}$. For complex formation, a G protein-derived high affinity peptide Gt$\alpha$CT (Gt$\alpha$340–350) K341L, NH$_2$-ILENLKDCGLF-COOH) was used in a 1:5 (Rho:peptide) molar ratio. Absorption maxima determination as well as concentration measurements during purification of all pigments were performed on a Varian Cary 50 Bio UV-visible spectrometer.

**Flash Photolysis**—The kinetics of Meta II formation of bRho and hRho as well as of the chimeras were determined by flash photolysis of diluted vesicle suspensions (5 M) at 20 °C, pH 5, where Meta II formation is complete. After illumination with a laser flash (5 ns) at 532 nm (Orion, New Wave Research), the integrated absorbance change between 375 and 385 nm was recorded with an OLIS UV-visible spectrometer using a time resolution of 100 μs.

**G$\alpha$ Activation Assay**—G$\alpha$ was prepared from bovine rod outer segments, separated into G$\alpha$CT and G$\alpha$γ subunits by chromatography as described previously (14), and dialyzed against 20 mm bis-tris-propane, pH 7.1, 130 mm NaCl, 1 mm MgCl$_2$, and 2 mm DTT. Protein concentration was determined with a Bradford reagent.

As a monitor for the G$\alpha$ activation capacity of the Rhos, changes in intrinsic fluorescence intensity of the G$\alpha$ subunit upon the activating exchange of GDP to GTP$\gamma$S were quantified as previously described (15). The measurements were carried out using a SPEX fluorolog II spectrofluorometer equipped with a 450-watt 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. G$\alpha$ activation rates were measured with 5 nm purified Rho, 0.6 μM G$\alpha$, 25 μM GTP$\gamma$S, 20 mm bis-tris-propane, pH 6.0, 130 mm NaCl, 1 mm MgCl$_2$, 2 mm DTT, and 0.01% (w/v) n-dodecyl-$\beta$-D-maltoside in a final volume of 750 μl. The pH value was adjusted by the addition of HCl or NaOH, respectively, directly into the cuvette to avoid differences in n-dodecyl-$\beta$-D-maltoside concentration. All samples were equilibrated at 10 °C for 4 min. G$\alpha$ activation reactions were triggered with orange light (495-nm long pass filter, Schott GG 495). The G$\alpha$ activation rate was obtained by linear regression of the initial rise in fluorescence emission.

**Human Rhodopsin Model Building**—A homology model of hRho was built based on the high resolution crystal structure of dark and active bRho (PDB entries 1U19 (16) and 3PXO (17)). The amino acid sequence of hRho differs in 23 positions compared with bRho. After mutating the respective residues to create hRho structure, the side-chain conformations were chosen based on the 2002 Dunbrack backbone-dependent rotamer library (18). For each of the mutated amino acids the library contained a standard rotamer that fitted without clashes into the template structure. With the exception of H195K and L216M, the highest or the second highest populated backbone-dependent rotamer was a good fit and thereby indicated a very regular structure. For H195K the first rotamer that did not point the lysine to the groove between the N-terminal -sheets and TM5 was chosen. Met-216 faces toward TM6 and is sur-
rounded by two tyrosines, +/− one turn, so the first rotamer 
crashing with neither was chosen. As part of the molecular 
dynamics protocol (see below), this model was inserted into a 
lipid bilayer, solvated in water, then energy-minimized and 
relaxed within the water/lipid environment. Neither the energy 
minimization nor the relaxation significantly changed the rota-
mers of the mutated residues or their neighboring residues.

Preparation of Bovine and Human Rhodopsin Structures—
The starting conformations used for MD simulations were pre-
pared based on x-ray structures from crystals of dark (PDB 
entry 1U19 (16)) and active bRho (PDB entry 3PQR or 3PXO 
(17)). The far C terminus of active bRho (residues 327–348; 
UniProt entry P02699) is not resolved in these complexes and 
was not modeled, as it does not affect receptor function signifi-
cantly, including G, activation (19). For all MD simulations of 
Rho, two palmitoyl chains were attached to the residues Cys-
322 and Cys-323. For the MD simulations of hRho we used the 
modeled structure as described above.

Molecular Dynamics Protocol—System preparation, subse-
quent minimization, equilibration, and simulation were per-
formed with the GROMACS suite (Version 4.5) (20). The 
proteins were inserted into the equilibrated bilayer of 1,2-
dimyrystoyl-sn-glycero-3-phosphatidylcholine using the GRO-
MACS embedded tool (21). Parameters for the 1,2-dimyrystoyl-
sn-glycero-3-phosphatidylcholine lipids were derived from 
Berger et al. (22) and for water from the SPC/E model (23). A 
salt concentration of 0.15 mol/liter was obtained by adding Na+ 
and Cl− ions to the system. The AMBER99SB-ILDN force field 
(24) was used for proteins and ions. Force field parameters for 
the protonated 11-cis-retinal in inactive Rho were adapted from 
Kandt et al. (25) and for the deprotonated all-trans-retinal in 
active Rho from Mertz et al. (26).

To obtain clash-free structures suitable for MD simulations, 
an energy minimization was performed in GROMACS using 
the steepest descent algorithm until the maximum force went 
below 1000.0 kl/mol/nm. In the following equilibration step the 
energy-minimized structure was simulated for 20 ns with all 
protein backbone atoms restrained to their initial positions. 
During this step the protein-membrane, protein-water, and 
the membrane-water interfaces are relaxed so that voids are filled 
and side-chain packing is optimized. For the production MD 
simulations the position restraints were lifted.

Based on the equilibrated systems, the production runs were 
started with different initial velocities obtained from Boltz-
mann distributions at 320 K. For equilibration and the produc-
tion runs, all bonds were constrained using the LINCS algo-


![FIGURE 3. Comparison of the region around residue 298 (spacefill depic-
tion) of inactive bovine (a) and human (b) rhodopsin. Shown are interior 
pockets that fit a probe of radius 0.8 Å (orange). The change of Ser-298 in 
bovine rhodopsin into Ala-298 in human rhodopsin presumably leads to a 
continuous pore accessible to water molecules (small red spheres) near resi-
due 298 and the retinal SB.]

normal). The integration time step used for all simulations was 
0.002 ps.

Results

A Structural Model of Human Rhodopsin from MD Simu-
lation—The 23 amino acids of the bRho sequence, which differ 
from the hRho sequence, were exchanged to obtain models of 
hRho from crystal structures of the dark (PDB entry c1U19 
(16)) and the active (PDB entry 3PXO (17)) conformations (for 
details see “Experimental Procedures”). The resulting structures 
of the hRho dark state (Fig. 2a) prove to be stable during 
classic MD simulations when embedded in a water/lipid envi-
ronment and closely resemble the bovine template. Structures 
of bRho and hRho were subsequently simulated and analyzed 
with regard to alterations in the region around the Schiff base 
and in the extracellular end of TM5 (T5E2C region).

The amino acid triplet Thr-297, Ser-298, and Val-300, which 
is located in the direct vicinity of the retinal Schiff base (Lys-
296) is changed in hRho to Ser-297, Ala-298, and Ile-300 (Fig. 2, 
b and c). Ala-298 is the only of these three residues that is buried 
inside the protein moiety. In bRho Ser-298 together with 
Met-86 and Trp-265 separates a cluster of conserved water 
molecules from the Schiff base (31). The atomic volume of Ala-
298 (59.87 Å3) is smaller than Ser-298 (65.34 Å3), leaving an 
additional space of 5.47 Å3. Assuming a van der Waals radius of 
1.2 Å (5.43 Å3) for water (32), this additional space suggests the 
existence of a narrow continuous water pore formed in hRho 
connecting the two water-filled cavities above the retinal bind-
ing pocket (Fig. 3). Due to the different physicochemical prop-
erties of serine and alanine, the electrostatics of the retinal 
Schiff base region are distinct in hRho and bRho.

Additionally, the residues 194–198 (TM5 to EL2 connection: 
T5E2C region) are different in hRho (Fig. 2, d and e). To inves-
tigate the effect of this exchange on the structure and dynamics 
of the T5E2C region, we performed a series of 200–400-ns clas-
sic all-atom MD simulations of the hRho and bRho dark and 
active states in a membrane/water environment. Analysis of the 
T5E2C region indicates differences in secondary structure 
between hRho and bRho. In bRho the prevalent structural 
elements are turn structures, whereas in hRho mostly bended and 
310-helix structural elements are found (supplemental Fig. 1).

To obtain a more detailed description of the T5E2C region, we
performed a cluster analysis of its structure during simulation. The three largest clusters in each series of MD simulations show the conformational flexibility in the T$_5$E$_2$C region of dark/active state of bovine/human Rho (Fig. 4). A considerable difference is seen in the variability of the T$_5$E$_2$C region relative to TM5. In bRho the T$_5$E$_2$C moves parallel to the TM5 axis (so called flapping motion), whereas in hRho it additionally moves perpendicular (sliding motion), which is most pronounced in the active state of hRho. This difference can be explained by the variation of hydrogen bonding networks stabilizing the T$_5$E$_2$C region in the two pigments. In bRho the T$_5$E$_2$C region is attached to the end of TM5 through a rigid backbone-backbone hydrogen bond between Glu-196 and Thr-198 (Fig. 4e), hampering motions perpendicular to the TM5 axis. In hRho this interaction is displaced in favor of a hydrogen bond between Glu-5 and Lys-195 (Fig. 4f).

**FIGURE 4.** Conformational flexibility of T$_5$E$_2$C and adjacent residues analyzed by MD simulations of bovine (a and c) and human (b and d) rhodopsin holoproteins in their dark (a and b) and active (c and d) state. For each system three representative conformations (red, orange, yellow) obtained by cluster analysis are shown. The tube thickness relates to fluctuations of each residue (root mean square fluctuations) within a given cluster. In the simulations of bovine rhodopsin a subset of the EL2/TM5 region shows a flapping motion (solid arrows), whereas in human rhodopsin the whole region shows a sliding (dashed arrows) and, in the active state additionally, a flapping motion (solid arrow) effecting more residues. e and f, hydrogen bonds in the TEC region of bovine and human rhodopsin. e, in bovine rhodopsin a backbone-backbone hydrogen bond is prevalent between Glu-196 and Thr-198, whereas in human rhodopsin a hydrogen bond is observed between Glu-5 and Lys-195 (f).

**Early Intermediates of Human and Bovine Rhodopsin**—In Fig. 5, a and b, we compare the FTIR difference spectra (illuminated state minus dark state) of hRho (green lines) and bRho (gray lines) of the early intermediates bathorhodopsin (Batho) and lumirhodopsin (Lumi), recorded at 80 K and 173 K, respectively. The Batho spectra of both species exhibit the typical difference bands, which mainly reflect changes of the retinal/Schiff base geometry due to cis/trans isomerization (C= N stretching at 1663 cm$^{-1}$; C=C ethylenic modes at 1560/1537 cm$^{-1}$, see Fig. 5).

**FIGURE 5.** FTIR difference and double difference spectra of the early intermediates of human (green lines) and bovine (gray lines) rhodopsin. a, Batho spectra were recorded at pH 7.4, 80 K. The double difference (human minus bovine, blue line) indicates marginal deviations at this early stage of the photoreaction. b, Lumi spectra obtained at pH 7.4, 173K; the double difference (red line) reveals more distinct shifts of characteristics bands in the amide-I region.
Activation Mechanism of Human Rhodopsin

+1537 cm⁻¹; C—C stretching, C—H bending, and N—H bending vibrations at 1237 cm⁻¹, and hydrogen out of plane (HOOOP) vibrations at 920 cm⁻¹ (33, 34). The superscripts + and − indicate the polarity of the bands in the difference spectra. For a better comparison, the bovine minus human Batho double difference spectrum (hereinafter dd spectrum) is given in Fig. 5a (blue line). The weak double difference bands indicate small structural differences between the two dark states and/or the intermediates of both species, presumably within the proximity of the altered amino acids near the retinal pocket. The absorption shift of 4 nm in hRho (494 nm) versus bRho (498 nm) in the dark state (see the inset in Fig. 5a) shows the influence of these altered amino acids already in the dark state.

The FTIR spectra of the bovine and human Lumi intermediates are compared in Fig. 5b (hRho, green line; bRho, gray line). In both spectra, the characteristic Lumi difference bands are seen at +1772/−1767, +1741, −1725, and −1658/+1653 cm⁻¹. The −1658/+1653 cm⁻¹ bilobe is located in the region indicative for changes of the C—N stretching vibration of the protonated Schiff base (35) and most likely corresponds to the slightly different dark state UV-visible absorptions of both pigments as described above. Similar to Batho, the Lumi difference spectrum is dominated by bands caused by the retinal cis/trans isomerization. More pronounced bands as compared with Batho are observed in the dd spectrum (Fig. 5b, red line) at +1556 (ethylenic modes, amide-II range), −1634 (amide-I range), and +1240 cm⁻¹ (chromophore fingerprint region). Especially the negative bands at −1658 and −1239 cm⁻¹ may be attributed to slightly altered interactions of protein and chromophore in the dark state due to an altered Schiff base environment.

The Meta I States of Human and Bovine Rhodopsin—We next recorded difference spectra of hRho and bRho (Fig. 6a, green and gray lines) under conditions that favor formation of the Meta I intermediate in bRho (0 °C, pH 8) (36, 37). To ensure that in hRho a Meta I species is also formed, we conducted the same experiment at lower temperatures (pH 8, −40 °C), conditions under which the transition to Meta II is expected to be completely blocked. We observed the same difference spectrum as seen at 0 °C (data not shown), demonstrating formation of a human Meta I intermediate. In contrast to Batho and Lumi, the spectra now show significant deviations, leading to strongly increased band intensities in the dd spectrum (Fig. 6a, blue line). This bands are caused by deviations in the amide I and amide II regions around 1650 and 1550 cm⁻¹ and around −1768/−1745 cm⁻¹, indicative of hydrogen bond changes of Asp-83 and Glu-122 as well as bands at −1237 and −1190 cm⁻¹, suggesting altered chromophore/protein interactions (34). To clarify whether the difference band at −1745 cm⁻¹ was caused by an altered TM3-TM5 hydrogen-bonded network (nearby Glu-122) or has its origin in a different Schiff base vicinity (nearby Asp-83), we recorded difference spectra under the same conditions but after H₂O/H2O buffer exchange (Fig. 6b), which leads to a specific isotopic shift of all hydrogen-coupled vibrations (38). The spectra show no deuteration-induced shift of the double difference band at −1745 cm⁻¹, indicating altered interactions of amino acids with e.g. lipid, rather than by changes of Glu-122 or Asp-83 (12, 39).

The most significant deviation between the wild type spectra of hRho and bRho refers to the Meta I marker band of bRho at +1661 cm⁻¹ (−1663 cm⁻¹ in the dd spectrum), which is strikingly not observed in the spectrum of hRho (reflected as a negative band in the dd spectrum). The band is replaced by two bands at +1648 and +1638 cm⁻¹, respectively. Remarkably, in a hRho chimera, in which the T_E1C region was replaced by the corresponding bovine sequence (TEC chimera; Fig. 6c, blue line), this Meta I marker band of bRho (+1661 cm⁻¹) arises again, albeit with lower intensity. This finding indicates that structural changes in the T_E1C region are at least partially responsible for the +1661 cm⁻¹ band in Meta I and that the altered sequence in hRho causes the overall smaller intensity of this band. The dark state UV-visible absorption maximum of the TEC chimera was determined to be 492 nm (data not shown).
In a further hRho chimera, the sequence 297SAAI300 in the Schiff base (SB) proximity was replaced by the bRho sequence 297TSAV300 (SB chimera). Although Meta I formation was not significantly influenced in the SB chimera, the absorption maximum of the chimera (497 nm) resembled that of bRho (498 nm), indicating the influence of the region on wavelength tuning. The Meta I difference spectrum of the SB chimera was almost identical to that of hRho (Fig. 6d, green and red line), although the band at -1237 cm^-1 was shifted back to the frequency of bRho at -1238 cm^-1.

The Meta II States of Human and Bovine Rhodopsin—Metarhodopsin II, formed at higher temperatures, is characterized by a deprotonated retinal Schiff base leading to a shift of the absorption maximum to \( \lambda_{\text{max}} = 380 \) nm. However, Meta II has been shown to consist of at least three conformationally distinct states, namely Meta IIa, Meta IIb, and Meta IIbH\(^+\), which can be stabilized at different pH values and temperatures (40). Because the pK\(_a\) values of the Meta I/Meta II equilibrium did not show significant deviations between hRho and bRho (compare Fig. 8b), we assumed that the subspecies of hRho are stabilized at similar conditions as their bovine counterparts. In Fig. 7a we show the difference spectra of bRho and hRho (gray and green line) recorded at pH 8 and 30 °C, where a mixture of Meta IIa/IIb prevails, with some residual Meta I. In Fig. 7b the respective Meta IIbH\(^+\) spectra (recorded at pH 5 and 30 °C) are presented. The absorption maxima of the UV-visible spectra at 380 nm (insets in Fig. 7, a and b) show in both cases the proper formation of the deprotonated Schiff base. The spectra taken at alkaline pH, pH 8, exhibit the typical Meta IIa/IIb marker bands at 1659, 1237 cm^-1 and the small shift of the positive amide-I band (+1644 cm^-1 in bovine) are most likely attributed to slightly different amounts of residual Meta I or to differences between the two dark states as already suggested by the analysis of the intermediates Batho, LumI, and Meta I. The similarity between the dd spectra of Meta IIa/IIb and Meta IIbH\(^+\) formation (Fig. 7, a, purple line, and b, blue line) show that, in contrast to Meta I, no significant structural deviations occur between human and bovine Meta II intermediates.

Peptide-bound Meta II State of Human and Bovine Rhodopsin—To investigate the G protein interacting subforms of Meta II, we recorded the Meta II-peptide-minus-dark states difference spectra of hRho and bRho (pH 5, 30 °C, Fig. 7c, green and gray lines) formed in the presence of an 11-mer peptide derived from the C terminus of the G protein \( \alpha \)-subunit (G\(_\alpha\)CT) (41). Compared with the Meta IIbH\(^+\), the difference spectra of both human and bovine Meta II-peptide exhibit bands at +1659 and -1558 cm^-1, which are specific for the Meta IIbH\(^+\)/peptide interaction. These two bands and the positive band at +1536 cm^-1 are much less pronounced in bRho, giving rise to bands in the dd spectra shown in Fig. 7c, red line.

The dd spectra between Meta II-peptide and Meta IIbH\(^+\) (peptide binding spectra pbs, representing structural changes in receptor and peptide due to peptide binding) show, however, the typical bands at +1555 and +1660 cm^-1 (Fig. 7d). They are even more similar, as the pbs are not influenced by structural differences between the dark states of hRho and bRho, which are superimposed in the Meta II-peptide-minus-dark state differences. These results indicate that G\(_\alpha\)CT binds and stabilizes very similar receptor conformations in bRho and hRho and that the same interactions are involved in formation of the stable receptor-peptide complex. The similarity between the double differences of bRho and hRho and the identical peptide binding spectra (pbs, difference spectrum of Meta II-peptide-minus-difference spectrum of Meta IIbH\(^+\) formation) of both pigments are closely similar. Note that, due to difference formation, the influence of the dark state on these spectra is negligible.

**FIGURE 7. Coupled FTIR and UV-visible (insets) spectroscopy of the late human (green lines) and bovine (gray lines) intermediates.** a, conditions favoring a mixture of Meta states (pH 8, 30 °C). In the double difference spectrum (purple line) the bands are less intense as compared with the Meta I conditions. b, spectra recorded at pH 5, 30 °C indicate a transition from the dark state toward the fully active state Meta IIbH\(^+\). The weak bands in the double difference (blue line) demonstrate that the active states of human and bovine rhodopsin are largely similar. c, spectra of the complex formation of active Meta IIbH\(^+\) and high affinity G\(_\alpha\)CT peptide (Meta II-peptide). The double difference (red line) exhibits more pronounced bands than in the case of Meta IIbH\(^+\). d, in contrast, the peptide binding spectra (pbs, difference spectrum of Meta II-peptide-minus-difference spectrum of Meta IIbH\(^+\) formation) of both pigments are closely similar. Note that, due to difference formation, the influence of the dark state on these spectra is negligible.
The influence on G protein binding is, if at all, only marginal. We recorded a series of infrared difference spectra at different pH values. This was expected as even ancestral archosaur pigments activate the retinal G protein with a comparable rate.

**Discussion**

The aim of this study was to compare the activation pathways of bRho and hRho with respect to structure, function, and kinetics of photointermediate formation. For this purpose we first analyzed the sequences of both pigments and modeled and simulated the structures of dark and active state of hRho on the basis of the available crystal structures of bRho. The combination of these modeling techniques with FTIR difference spectroscopy of the photointermediates provides us with a detailed picture of conformational changes involved in activation. Thus we can describe the similarities and differences between these two photoreceptors.

**Structural Differences between Bovine and Human Rhodopsin**—In the majority of the 23-amino acid alterations, the chemical and physical side-chain properties are preserved (i.e. F88L, T5E2C, V173A, V218I, L266V, L321I), and no major influence on receptor function is expected. However, significant differences occur in two distinct regions with potential influence on receptor structure (Fig. 1) and may thus have functional consequences.

The first region comprises the residues 297–300 in the direct vicinity of the retinal Schiff base with the sequence 297SAAI300 in hRho and 297TSAV300 in bRho (Fig. 2, b and c). The serine to alanine exchange may contribute to two distinct effects on the retinal Schiff base environment. First, the electrostatic properties of the retinal Schiff base environment will be altered when the hydroxyl group is removed. Second, our simulations reveal additional space in hRho (Fig. 3), potentially occupied by an additional water molecule, which influences the vicinity of the Schiff base (47) and thus the UV-visible absorption maximum (48) and the typical infrared absorptions of the dark state. In agreement, the absorption maximum of the SB chimera, in which the bovine 297TSAV300 sequence was introduced, was shifted back toward that typical of bRho, whereas the kinetics of Meta II formation remain the same as seen by hRho, suggesting only a local influence of the TSAV region on the retinal Schiff base.

The second sequence deviation comprises the T5E2C region (residues 194–198) at the connection from TM5 to EL2 (Fig. 2, d and e). The human T5E2C sequence is conserved in many mammalian rhodopsins, except in Rho of sea mammals or nocturnal animals including bRho (see the Weblogo plot in Fig. 2, c and e). The differences in the T5E2C region alter its hydrogen
bond network with the end of TM5 and leads, according to our MD simulations, to a considerable difference in conformational flexibility of the T₅₋₃₋₂₋₁ region. In bRho this region exhibits a flapping motion during the MD simulations that still allows coupling of EL2 motion to TM5 movement, whereas in hRho the whole region shows an additional sliding motion that loosens the tight EL2/TM5 coupling (see Fig. 4). Consequently, in bRho this region functions as a rigid lever (49), whereas in hRho it is rather a flexible linker with limited ability to trigger TM5 movement connected with Meta I formation (50). Together with the faster formation of the Schiff base deprotonated species, this implies that necessary conformational changes must already have taken place before the formation of Meta I or do not have to take place in hRho. Our simulations suggest that the loosening of the EL2/TM5 coupling present in the active conformation of bRho is anticipated in the dark state of hRho. The dynamic properties of the EL2 have previously been connected with the constitutive activity of other GPCRs (51, 52). Note that sequence differences in the 297–300 region around the Schiff base (297SAAI300 in hRho and 297TSAV300 in bRho), which are partially restored the bovine Meta I difference spectrum of both pigments. Especially, the positive band at 1661 cm⁻¹ assigned to conformational changes occurring with Meta I formation, is not observed in human Meta I. In bovine Meta I the primary counterion of the Schiff base is known to switch from Glu-113, which serves as counterion in the dark state, to Glu-181 positioned in EL2 (55). This decisive reorganization of the Schiff base hydrogen-bond network leads to several conformational changes that unlock the photoreceptor irreversibly and facilitate its activation by thermal energy and proton uptake (56). This is achieved by a collective motion of EL2 and TM5, realized by a relatively rigid connection of both regions in bRho (57). The stability of this rigid connection is, however, strongly affected in the simulations of hRho. Our simulations further revealed an increased lateral mobility of EL2 in the dark state of hRho, which enables Glu-181 to move closer to the retinal Schiff base and thereby at least partially anticipates the counterion shift (Fig. 4). This notion is also corroborated by a TEC chimera between hRho and bRho (Fig. 6c). In this hRho based chimera, the “flexible” sequence 194PHE196 of hRho is replaced with the more rigid bovine sequence 194PHE196, thus restoring the putative tight coupling of EL2 movement to TM5 motion. In this mutant the band intensity at +1661 cm⁻¹ is almost restored to the level found in the Meta I difference spectra of bRho.

Formation of the species with deprotonated Schiff base proceeds much faster in hRho as observed in the present time-resolved flash photolysis measurements and is in agreement with results of detergent-solubilized hRho (43) and in vivo experiments (58). This finding supports the view that some rate-limiting structural changes going along with Meta I in bRho already have formed in hRho. The SB chimera exhibits no influence on the Meta II formation kinetics. It is formed with a comparable rate as hRho (Fig. 8a, Table 1), although the absorption maximum of the bRho dark state is restored. More intriguing is the behavior of the TEC chimera. The exchange of EL2/TM5 connection influences not only both rate constants of Meta II formation but also restores the structure of bovine Meta I at least partially and shows a hypochromic shift of the dark state absorption maximum. The pKₐ of the Meta I ↔ Meta II equilibrium is a very sensitive monitor of the conformational equilibrium. The two pKₐ values are virtually identical, which means that both pigments share the same conformational equilibria of Meta II states and that the mechanisms for TM6 outward movement and proton uptake at Glu-134 are conserved.

Finally, binding of the Gt oct-CT peptide surrogate and catalytic activation of the transducin reflect that the functional properties of the activated receptor conformations are similar.

It is tempting to correlate our results with the existence of two different Meta I species that form on the ms and μs timescale, respectively, as it has been described for bRho (59). The two Meta I states are formed in parallel reaction pathways accessed in a temperature-dependent manner. However, because the Meta equilibria are not significantly shifted in hRho, we suggest that the dark state conformation is such that the mutations in the Schiff base and T₅₋₃₋₂₋₁ region facilitate faster activation by allowing conformational changes characteristic for bovine Meta I (e.g. Schiff base counterion shift).

Conclusion—Despite their close evolutionary relationship, hRho and bRho display differences in their primary structure
Activation Mechanism of Human Rhodopsin

that we connect to specific impacts on structural elements during receptor function. On the one hand the electrostatic environment of the retinal Schiff base linkage is altered. On the other hand we have identified an increase of conformational flexibility in the linker region between extracellular loop 2 and transmembrane helix 5 of hRho, which is a feature of the active conformation and has been reported to increase the constitutive activity in other GPCRs. Flexibility in this region seems to be anticipated in hRho leading to a shifted absorption maximum in the dark state and faster formation of active Meta II. Although the structural properties of the inactive receptor seem to have adapted to the specific environment of the host species (e.g. diurnality or nocturnality), the active conformation itself is surprisingly similar. Apparently, the architecture of the receptor is such that host specific activation pathways evolve; however, the active conformation appears highly optimized for G protein catalysis and is thus conserved.

Our findings highlight that although hRho provides a valuable template for hRho, the differences between the inactive conformations have to be considered carefully, especially when the activation mechanism of disease-associated mutations of hRho are to be examined using the bovine template.

Author Contributions—F. J. B. coordinated the study and wrote the paper. R. K. prepared the samples, performed FTIR UV-visible and photolysis measurements, analyzed the spectroscopic data, wrote the paper, and prepared Figs. 1, 5, 6, 7, and 8. M. S. and P. S. prepared photolysis measurements, analyzed the spectroscopic data, wrote the manuscript, Anja Koch, Jana Engelmann, and Brian Bauer for fruitful discussions. The computer time necessary was provided by the technical assistance and Rho purification, and Martin Heck for fruitfulness of the manuscript, Anja Koch, Jana Engelmann, and Brian Bauer for fruitful discussions. The computer time necessary was provided by the "Norddeutscher Verbund für Hoch-und Höchstleistungsrechner" (HLRN) project bec00085.

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References

1. Hofmann, K. P., Scheerer, P., Hildebrand, P. W., Choe, H.-W., Park, J. H., Heck, M., and Ernst, O. P. (2009) A G protein-coupled receptor at work: the rhodopsin model. Trends Biochem. Sci. 34, 540–552
2. Rakocevic, E. P., Kiel, C., McKeone, R., Stricher, F., and Serrano, L. (2011) Analysis of disease-linked rhodopsin mutations based on structure, function, and protein stability calculations. J. Mol. Biol. 405, 584–606
3. Nathans, J., and Hogness, D. S. (1984) Isolation and nucleotide sequence of the gene encoding human rhodopsin. Proc. Natl. Acad. Sci. U.S.A. 81, 4851–4855
4. Bartl, F., Ritter, E., and Hofmann, K. P. (2000) FTIR spectroscopy of complexes formed between metarhodopsin II and C-terminal peptides from the G-protein α- and γ-subunits. FEBs Lett. 473, 259–264
5. Nishimura, S., Kandori, H., and Maeda, A. (1998) Interaction between photoactivated rhodopsin and the C-terminal peptide of transducin α-subunit studied by FTIR spectroscopy. Biochemistry 37, 15816–15824
6. Szczepak, M., Beyrière, F., Hofmann, K. P., Elgeti, M., Kazmin, R., Rose, A., Bartl, F. J., von Stetten, D., Heck, M., Sommer, M. E., Hildebrand, P. W., and Scheerer, P. (2014) Crystal structure of a common GPCR-binding interface for G protein and arrestin. Nat. Commun. 5, 4801
7. Singhal, A., Ostermaier, M. K., Vishnevskiy, S. A., Panneels, V., Homan, K. T., Tesmer, J. I., Veprintsev, D., Deupi, X., Gurevich, V. V., Schertler, G. F., and Standfuss, J. (2013) Insights into congenital stationary night blindness based on the structure of G90D rhodopsin. EMBO Rep. 14, 520–526
8. Bosch, L., Ramon, E., Del Valle, L. I., and Garriga, P. (2003) Structural and functional role of helices I and II in rhodopsin. A novel interplay evidenced by mutations at Gly-51 and Gly-89 in the transmembrane domain. J. Biol. Chem. 278, 20203–20209
9. Rao, V. R., Cohen, G. B., and Oprian, D. D. (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. Nature 367, 639–642
10. Molday, R. S., and MacKenzie, D. (1983) Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry 22, 653–660
11. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) A single amino acid substitution in rhodopsin (lysine 248 to leucine) prevents activation of transducin. J. Biol. Chem. 263, 2119–2122
12. Elgeti, M., Kazmin, R., Heck, M., Morizumi, T., Ritter, E., Scheerer, P., Ernst, O. P., Siebert, F., Hofmann, K. P., and Bartl, F. J. (2011) Conserved Tyr-223(5.58) plays different roles in the activation and G-protein interaction of rhodopsin. J. Am. Chem. Soc. 133, 7159–7165
13. Elgeti, M., Ritter, E., and Bartl, F. J. (2008) New Insights into Light-induced deactivation of active rhodopsin by SVD and global analysis of time-resolved UV/Vis- and FTIR data. Zeitschrift für Phys. Chemie 222, 1117–1129
14. Heck, M., and Hofmann, K. P. (2001) Maximal rate and nucleotide dependence of rhodopsin-catalyzed transducin activation: initial rate analysis based on a double displacement mechanism. J. Biol. Chem. 276, 10000–10009
15. Ernst, O. P., Gramse, V., Kolbe, M., Hofmann, K. P., and Heck, M. (2007) Monomeric G protein-coupled receptor rhodopsin in solution activates its G protein transducin at the diffusion limit. Proc. Natl. Acad. Sci. U.S.A. 104, 10859–10864
16. Okada, T., Sugihara, M., Bondar, A.-N., Elstner, M., Entel, P., and Buss, V. (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure. J. Mol. Biol. 342, 571–583
17. Choe, H.-W., Kim, Y. I., Park, J. H., Morizumi, T., Pai, E. F., Krauss, N., Hofmann, K. P., Scheerer, P., and Ernst, O. P. (2011) Crystal structure of metarhodopsin II. Nature 471, 651–655
18. Dunbrack, R. L., Jr., and Cohen, F. E. (1997) Bayesian statistical analysis of protein side-chain rotamer preferences. Protein Sci. 6, 1661–1681
19. Chen, J., Makino, C. L., Peachy, N. S., Baylor, D. A., and Simon, M. I. (1995) Mechanisms of rhodopsin inactivation in vivo as revealed by a COOH-terminal truncation mutant. Science 267, 374–377
20. Hess, B., Kutzner, C., Van Der Spoel, D., and Lindahl, E. (2008) GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. J. Chem. Theory Comput. 4, 435–447
21. Wolf, M. G., Hoefling, M., Aponte-Santamaría, C., Grubmüller, H., and Groenhof, G. (2010) G-membred: Efficient insertion of a membrane protein into an equilibrated lipid bilayer with minimal perturbation. J. Comput. Chem. 31, 2169–2179
22. Berger, O., Edholm, O., and Jühling, F. (1997) Molecular dynamics simulations of a fluid bilayer of dipalmitoylphosphatidylcholine at full hydration, constant pressure, and constant temperature. Biophys. J. 72, 2002–2013
23. Berendsen, H. J. C., Grigera, J. R., and Straatsma, T. P. (1987) The missing term in effective pair potentials. J. Phys. Chem. 91, 6269–6271
24. Lindorff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J. L., Dror, R. O., and Shaw, D. E. (2010) Improved side-chain torsion potentials for the Amber ff99SB protein force field. Proteins 78, 1950–1958
25. Kandt, C., Schlitter, J., and Gerwert, K. (2004) Dynamics of water molecules in the bacteriorhodopsin trimer in explicit lipid/water environment. Biophys. J. 86, 705–717
26. Mertz, B., Lu, M., Brown, M. F., and Feller, S. E. (2011) Steric and electronic influences on the torsional energy landscape of retinal. Biophys. J. 20126 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 290 • NUMBER 33 • AUGUST 14, 2015
Activation Mechanism of Human Rhodopsin

Photolysis intermediates of human rhodopsin. *Biochemistry* **30**, 11372–11376

44. Hoersch, D., Otto, H., Wallat, I., and Heyn, M. P. (2008) Monitoring the conformational changes of photoactivated rhodopsin from microseconds to seconds by transient fluorescence spectroscopy. *Biochemistry* **47**, 11518–11527

45. Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., and Ernst, O. P. (2003) Role of the conserved NpxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2290–2295

46. Chang, B. S., Jönsson, K., Kazmi, M. A., Donoghue, M. J., and Sakmar, T. P. (2002) Recreating a functional ancestral archosaur visual pigment. *Mol. Biol. Evol.* **19**, 1483–1489

47. Sekharan, S., Mooney, V. L., Rivalta, I., Kazmi, M. A., Neitz, M., Neitz, J., Sakmar, T. P., Yan, E. C., and Batista, V. S. (2013) Spectral tuning of ultraviolet cone pigments: an interhelical lock mechanism. *J. Am. Chem. Soc.* **135**, 19064–19067

48. Sullivan, J. M., and Shukla, P. (1999) Time-resolved rhodopsin activation currents in a unicellular expression system. *Biophys. J.* **77**, 1333–13357

49. Ahuja, S., and Smith, S. O. (2009) Multiple switches in G protein-coupled receptor activation. *Trends Pharmacol. Sci.* **30**, 494–502

50. Ye, S., Zaitseva, E., Caltabiano, G., Schertler, G. F., Sakmar, T. P., Deupi, X., and Vogel, R. (2010) Tracking G-protein-coupled receptor activation using genetically encoded infrared probes. *Nature* **464**, 1386–1389

51. Wheatley, M., Wootten, D., Conner, M. T., Simms, J., Kendrick, R., Logan, R. T., Poyner, D. R., and Barwell, J. (2012) Lifting the lid on GPCRs: the role of extracellular loops. *Br. J. Pharmacol.* **165**, 1688–1703

52. Willing, D., Bernhardt, G., Dove, S., and Buschauer, A. (2015) The extracellular loop 2 (ECL2) of the human histamine H4 receptor substantially contributes to ligand binding and constitutive activity. *PLoS ONE* **10**, e0117185

53. Nakamichi, H., and Okada, T. (2006) Crystallographic analysis of primary visual photochemistry. *Anat. Histol. Embryol.* **35**, 397–399

54. Nakamichi, H., and Okada, T. (2006) Local peptide movement in the photointermediate of rhodopsin E181Q mutant. *Biochemistry* **45**, 19064–19067

55. Sandberg, M. N., Greco, J. A., Wagner, N. L., Amora, T. L., Ramos, L. A., Chen, M. H., Knox, B. E., and Birge, R. R. (2014) Low temperature trapping of photointermediates of the rhodopsin E181Q mutant. *SOJ Biochem.* **1**, 12

56. Mahalingam, M., Martínez-Mayorga, K., Brown, M. F., and Vogel, R. (2008) Two protonation switches control rhodopsin activation in membranes. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 17795–17800

57. Ye, S., Huber, T., Vogel, R., and Sakmar, T. P. (2009) FTIR analysis of GPCR activation using azido probes. *Nat. Chem. Biol.* **5**, 397–399

58. Pugh, E. N. (1975) Rhodopsin flash photolysis in man. *J. Physiol.* **248**, 393–412

59. Thorgeirsson, T. E., Lewis, J. W., Wallace-Williams, S. E., and Kliger, D. S. (1993) Effects of temperature on rhodopsin photointermediates from lumirhodopsin to metarhodopsin-II. *Biochemistry* **32**, 13861–13872