Partial Agonism in a G Protein-coupled Receptor

ROLE OF THE RETINAL RING STRUCTURE IN RHODOPSIN ACTIVATION

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The visual process in rod cells is initiated by absorption of a photon in the rhodopsin retinal chromophore and consequent retinal cis/trans-isomerization. The ring structure of retinal is thought to be needed to transmit the photonic energy into conformational changes culminating in the active metarhodopsin II (Meta II) intermediate. Here, we demonstrate that cis-acyclic retinals, lacking four carbon atoms of the ring, can activate rhodopsin. Detailed analysis of the activation pathway showed that, although the photoproduct pathway is more complex, Meta II formed with almost normal kinetics. However, lack of the ring structure resulted in a low amount of Meta II and a fast decay of activity. We conclude that the main role of the ring structure is to maintain the active state, thus specifying a mechanism of activation by a partial agonist of the G protein-coupled receptor rhodopsin.

Rhodopsin is the visual pigment of the rod photoreceptor cell and gains its spectral properties from the chromophore 11-cis-retinal, which is covalently linked by a protonated Schiff base to Lys-296 of the apoprotein opsin (see Fig. 1). Rhodopsin is also the only G protein-coupled receptor (GPCR) whose crystal structure is solved (1–3). Insight into its mechanism of activation increases our understanding of how GPCRs work. Rhodopsin activation is triggered by the chromophore capture of a photon, which causes ultra fast cis/trans-isomerization of retinal. The photonic energy is used for conversion of the chromophore from an inverse agonist to a potent agonist and is channeled into protein conformational changes. This enables the cytoplasmic surface of the receptor to catalyze nucleotide exchange in the heterotrimERIC G protein transducin (Gt) (4).

The retinal-binding pocket is flexible enough to harbor a variety of synthetic retinal analogs, making them convenient probes of rhodopsin function (5). Specific protein-chromophore interactions ensure the low activity of rhodopsin in the dark ground state as well as in the rapid activation process. Removal of parts of the retinal structure is thus useful in identifying important elements of the retinal structure utilized in rhodopsin function. In a previous study, we investigated a retinal analog lacking the methyl group at C-9, termed 11-cis-9-demethylretinal (9-dm-retinal) (see Fig. 1C). 9-dm-retinal exerts its effect on the late metarhodopsin (Meta) photoproducts, Meta I and Meta II, by acting as a partial agonist (6, 7). Meta I is inactive and in equilibrium with the G protein-activating Meta II, thus corresponding to low and high affinity states of GPCRs (4). Recent evidence has further specified the nature of the protein-chromophore interaction in active Meta II. It was shown that, in Meta II and not in earlier intermediates, the central part of the polyene chain is firmly locked in the all-trans-configuration and resists re-isomerization to the ground state (8). Absorption of light in this state does not reverse the activation pathway back to the ground state, as observed for the intermediates up to Meta I (9). It rather leads to the Meta III storage form, showing that, in Meta II, the chromophore environment is fundamentally different from its precursors. In agreement with this finding, a 5–6-Å movement of retinal was suggested on the basis of recent high resolution solid-state NMR studies (10).

Here, we have investigated the role of the retinal ring structure by studying the 11-cis-acyclic retinal (ac-retinal) analog (see Fig. 1C), which lacks four carbon atoms of the ring. This part of retinal appears to be important for the activation process of rhodopsin because it is in close proximity to Pro-267 in transmembrane helix (TM) VI. Pro-267 is the assumed pivotal point for the movement of the cytoplasmic part of TM-VI out of the helix bundle (see Fig. 1A), which is a prerequisite for G protein coupling (11–13). Helix motion does not occur prior to the formation of Meta II (14). This suggests that the retinal ring is involved in the activation process via specific interactions, which are maintained up to the final activation stage. Recent studies with 13C-labeled retinal, allowing the C-16/C-17 ring methyl groups (Fig. 1B) to be distinguished, have indeed led to the postulate that the ring is retained with strong interactions within the binding site to the active Meta II state (15, 16). However, the idea that the ring structure is held in place throughout the activation process has questioned previous photo-cross-linking experiments that suggested a flip-over of the ring, parallel to the membrane plane and toward TM-IV, as an immediate consequence of retinal cis/trans-isomerization and before the Meta states are reached (17).

To solve the question of when the retinal ring structure comes into play in rhodopsin activation, we investigated ac-retinal-containing rhodopsin (ac-rhodopsin). By applying UV-visible spectroscopy and Fourier transform infrared (FTIR) difference spectroscopy, intrinsic trypto-
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FIGURE 1. A, seven-helix structure of rhodopsin. The chromophore 11-cis-retinal is linked to Lys-296 in TM-VII via a protonated Schiff base. The counterion Glu-113 in TM-III stabilizes the Schiff base and becomes protonated in the active Meta II conformation. The cytoplasmic part of TM-VI is in close contact with the retinal ring structure. Glu-134 in TM-III is part of the conserved D(E)RY motif of GPCRs. B, chemical structures of the retinylidene chromophore formed by 11-cis-retinal and Lys-296 of opsin. C, retinal analogs ac-retinal and 9-dm-retinal.

phosphine fluorescence measurements, and G, binding and activation assays, we followed the fate of the analog pigment from the early photoproducts through the receptor activation and deactivation phases. We found that the early photoproducts of rhodopsin, bathorhodopsin (Batho) and luminorhodopsin (Lumi), and the equilibrium of Meta I and Meta II intermediates were basically conserved in ac-rhodopsin. The coupling of photonic energy to conformational changes was less efficient, resulting in a Meta I/Meta II equilibrium, which was shifted toward inactive Meta I. This indicates that ac-retinal, like 9-dm-retinal, is a partial agonist. The formation of active Meta II occurred with almost normal kinetics, in sharp contrast to 9-dm-retinal-containing rhodopsin (9-dm-rhodopsin). Intriguingly, the lack of an intact ring structure became most crucial when the barrier to the active state was surpassed and the chromophore was held in the new structural environment. The ac-retinal failed to maintain the active Meta II state, resulting in a rapid decay of activity toward the G protein. These findings reveal a plausible mechanism of partial agonist function in GPCR activation.

EXPERIMENTAL PROCEDURES

Materials—The materials and proteins used were as described (6, 18).

Preparation of ac-Retinal Analogs—Preparation of 9-cis-acrylic retinal was carried out according to published procedures (19, 20). For detailed description of the synthesis of ac-retinal, see the supplemental “Experimental Procedures.”

Preparation of Rhodopsins—Pigments containing retinal analogs were obtained by reconstitution of opsin membranes with retinal analogs basically as described (21). Briefly, opsin was reconstituted with a 3-fold molar excess of retinal at 8 °C for 10 h. Excess retinal was subsequently removed by repetitive washes with 2% fatty acid-free bovine serum albumin. Recombinant pigments were expressed in COS-1 cells, reconstituted with 30 μM retinal or retinal analogs, and purified as described (6, 18). Immunofluorescence-purified pigments were obtained in 10 mM BisTris propane (pH 6.0) and 0.03% (w/v) β-dodecyl maltoside containing 100 mM C-1–C-18 C-terminal rhodopsin peptide.

UV-visible Spectroscopy—Spectra were taken using a Varian Cary 50 UV-visible spectrometer with a resolution of 2 nm. The buffers used were 50 mM BisTris propane (pH 7.5 or 6.5) or MES (pH 5.5) containing 0.03% (w/v) β-dodecyl maltoside for purified pigments and 20 mM NaOAc (pH 4.5), 130 mM NaCl, 1 mM MgCl₂, and 1 mM dithiothreitol or 20 mM BisTris propane (pH 6.0), 130 mM NaCl and 1 mM MgCl₂ for membrane samples. Samples were illuminated for 15 s using a fiber optics light source equipped with a long-pass filter (λ > 480 nm Schott GG495 for 11-cis pigments or λ > 465 nm Schott GG475 for 11-cis-acrylic and 11-cis-9-demethyl pigments) and a heat protection filter. The concentration of pigments reconstituted with 11-cis-retinal or 9-dm-retinal was determined spectrophotometrically using ε = 24,700 M⁻¹ cm⁻¹. The ε value at the absorption peak of ac-rhodopsin was obtained by comparing the absorption of regenerated recombinant pigments containing 11-cis-retinal or ac-retinal, yielding ε_ac-retinal = 48,000 ± 3300 M⁻¹ cm⁻¹. UV-visible spectra with a time resolution of 1 μs were recorded with an Olis RSM 16 spectrometer as described previously (9). Aliquots of the FTIR sample preparation (1–2 μM rhodopsin) were measured in a cuvette consisting of two BaF₂ windows and a 50-μm polytetrafluoroethylene spacer.

Flash Photolysis—Sandwich samples as used for time-resolved UV-visible measurements were applied for flash photolysis experiments. To monitor the formation of Meta II, the samples were illuminated by a flash lamp equipped with a Schott GG495 (rhodopsin) or GG475 (ac-rhodopsin) long-pass filter. The absorption change was recorded at 380 nm (8).

G Activation Assay—Experiments were performed using a SPEX Fluorolog 2 spectrofluorometer with λ_ex = 300 nm and λ_em = 345 nm and 2-s integration time essentially as described (6, 18). Protein concentrations in the samples were 2 μM pigment and 250 mM G, in 20 mM BisTris propane (pH 7.5 at 20 °C), 130 mM NaCl, 1 mM MgCl₂, and 0.01% (w/v) β-dodecyl maltoside in a final volume of 650 μL. The final concentration of GTPγS was 5 μM.

Fluorescence Spectroscopy—Decay of Meta II was measured essentially as described (22) using a SPEX Fluorolog 2 spectrofluorometer...
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FTIR Spectroscopy—FTIR spectroscopy, low temperature measurements, and calculation of the difference spectra were performed as described (9). ac-rhodopsin and 9-dm-rhodopsin were illuminated using a Schott GG475 long-pass filter. In the case of purified recombinant rhodopsin, 10 μl of concentrated pigment (~100 μM) was dried on the cuvette window in the dark and subsequently rehydrated with 2 μl of 50 mM BisTris propane. For estimation of the maximum amount of Meta II available with ac-retinal, the “Extra Meta II” assay was used (24). A rhodopsin pellet was resuspended in buffer solution containing 10 mM CTaHAI peptide (VLEDKSCGLF; where CTα is G1, α-subunit-derived C-terminal sequence and HA is high affinity) and centrifuged again for 30 min at 4°C. Subsequently, the FTIR difference spectrum was recorded as described (9).

Near-infrared Kinetic Light Scattering—Changes in light scattering intensities were measured as described previously (25). Measurements were performed in 10-mm path length cuvettes at pH 7.5 (20 mM BisTris propane, 130 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol) or pH 4.5 (20 mM NaOAc, 130 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol) and 23 ± 1°C. Rhodopsin (3 μM) in disk membranes constituted with 11-cis-, 9-dm-, or ac-retinal was activated using flashes of green light (500 ± 20 nm); 32% of the rhodopsin was activated by the flash. Flash activation of 9-dm-rhodopsin and ac-retinal was estimated to be 22% by analyzing the signal amplitudes of a set of subsequent flashes.

Cloning, Expression, and Purification of Maltose-binding Protein (MBP)-CTaHAI Fusion Protein—These were performed as described previously (25).

RESULTS

Early Photointermediates of ac-rhodopsin—To investigate whether ac-retinal affects the photolytic pathway of rhodopsin, infrared spectroscopy was performed at low temperatures suited to trap early photointermediates (Fig. 2). By measuring FTIR difference spectra of the intermediates (Fig. 2), two superimposed spectra are highlighted as line

FIGURE 2. FTIR difference spectra of intermediates of native rhodopsin and ac-rhodopsin in disk membranes stabilized at low temperatures. A, simplified reaction scheme of UV-visible photointermediates of native rhodopsin. Approximate lifetimes at room temperature, absorption maxima, and temperatures allowing trapping of Batho and Lumi are indicated in parentheses. B, FTIR difference spectra recorded at 80 K (upper) and at 173 K (lower) of rhodopsin and ac-rhodopsin intermediates. The spectrum of rhodopsin (upper blue line) represents the rhodopsin → Batho intermediate transition for reference. The corresponding spectrum of ac-Batho is shown (upper red line). Negative bands correspond to vibrations present in the ground state, and positive bands correspond to vibrations in the respective intermediates. Note that both spectra are equal except for spectral ranges concerning C=C and C–C stretching modes and the HOOP region. The lower blue line shows the spectrum of the rhodopsin → Lumi intermediate transition, and the lower red line shows the corresponding spectrum of ac-Lumi. Abs., absorption.

cis/trans-isomerization because the band position of this vibration was only slightly shifted in the 80 K intermediate of ac-rhodopsin (ac-Batho; 1240 cm⁻¹).

Clear differences between the spectra of Batho and ac-Batho were observed in the spectral range between 1500 and 1580 cm⁻¹ characterized by the C=C stretching vibration of the retinal. The strong band at 1536 cm⁻¹ in the spectrum of Batho was not observed in the spectrum of ac-Batho. Instead, a band at 1574 cm⁻¹ appeared, indicating that the twist had not relaxed. In the Lumi spectrum recorded at 173 K (Fig. 2B, lower blue line), a positive band at 948 cm⁻¹ was observed, indicating that the distortion of the chromophore persisted. The similar intensities and small shift to 945 cm⁻¹ in the 173 K intermediate of ac-rhodopsin (ac-Lumi) (Fig. 2B, lower red line) showed that the remainder of the ring in ac-retinal was sufficient to sustain chromophore distortion. Apart from small differences in the spectral region between 1560 and 1530 cm⁻¹, the spectra of Lumi and ac-Lumi were similar.

Meta States of ac-rhodopsin—To explore whether ac-rhodopsin in disk membranes devoid of peripheral proteins is capable of forming Meta-like species (denoted ac-Meta I and ac-Meta II), FTIR difference spectra of ac-rhodopsin were taken under various conditions and compared with the Meta I and Meta II reference spectra of native rhodopsin.
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FIGURE 3. Comparison of the FTIR difference spectra of the Meta states of rhodopsin and ac-rhodopsin in disk membranes. Difference spectra were calculated as spectrum of the illuminated sample minus the spectrum of the sample kept in the dark. A, the spectrum of ac-rhodopsin in membranes recorded at pH 7.5 and 0 °C (upper black line) is superimposed with a rhodopsin Meta I reference spectrum recorded at pH 8.0 and 0 °C (upper brown line). The spectrum of ac-rhodopsin recorded at pH 6.5 and 20 °C (middle blue line) is also superimposed with the rhodopsin Meta I reference spectrum (middle brown line). The spectrum of ac-rhodopsin recorded at pH 4.0 and 20 °C (lower green line) is superimposed with a rhodopsin Meta II reference spectrum recorded at pH 5.5 and 20 °C (lower brown line). Note that the spectra of ac-rhodopsin (at pH 6.5, 20 °C) and Meta I reference are similar. Also the spectra of ac-rhodopsin (at pH 4.0, 20 °C) and Meta II reference are similar. A. Abs, absorption. B, FTIR difference spectra were recorded at the pH values indicated. The Meta II content of ac-rhodopsin samples was calculated from the intensity of the band doublet at 1751 and 1768 cm⁻¹ (left panel) and is plotted as a function of pH (right panel). A hyperbolic fit to the data (black line) yielded a pKₐ value of 5.2. The dashed line represents the maximum amount of ac-Meta II, which was estimated by Meta II stabilization with a synthetic peptide (CThA1, VLEDKSCGLF) derived from the G₁-a-subunit C terminus (24). For comparison, the Meta II contents obtained with native rhodopsin (C) and 9-dm-rhodopsin (D) are plotted. Hyperbolic fits to the data yielded pKₐ = 7.5 for native rhodopsin (dotted line) and pKₐ = 5.0 for 9-dm-rhodopsin (gray line). Rel, relative. C, the pH-dependent interaction of the MBP-CThA1 fusion protein with ac-rhodopsin and 9-dm-rhodopsin was investigated by kinetic light scattering (25). Left panel, light-induced binding of MBP-CThA1 to ac-rhodopsin in disk membranes was monitored by an increase in the relative intensity of scattered near-infrared light (ΔI/I) at pH 4.5 and 7.5 at the concentrations indicated. Arrows indicate time of flash activation. Right panel, signal amplitudes evaluated from the binding signals at pH 4.5 (black line) and pH 7.5 (brown line) are plotted against MBP-CThA1 concentrations added and fitted hyperbolically. Closed symbols, ac-retinal; open symbols, 9-dm-retinal. A hyperbolic curve for ac-rhodopsin at pH 7.5 is shown for comparison (dashed brown line).

The Meta I reference spectrum was recorded at pH 8.0 and 0 °C and shows the Meta I-specific bands at 1701, 1691, 1660, and 1538 cm⁻¹ (Fig. 3A, upper brown line). In the spectrum of ac-rhodopsin (Fig. 3A, upper black line) recorded under similar conditions (pH 7.5 and 0 °C), no significant ac-Meta I content could be detected. Instead, bands at 1696 and 1662 cm⁻¹ and a pronounced doublet at 1552 and 1540 cm⁻¹ indicated the formation of a species different from Meta I. A similar spectrum was obtained by Jäger et al. (27) at pH 5.5 and 280 K with a pigment containing acyclic retinal with a 9-cis-configuration. For comparison, we also recorded spectra from rhodopsin containing 9-cis-acyclic retinal (supplemental Fig. 1S).

To figure out whether and, if so, under which conditions ac-Meta I and ac-Meta II can be formed, infrared spectra were recorded at various temperatures and pH values. A FTIR difference spectrum of ac-rhodopsin was recorded at pH 6.5 and 20 °C (Fig. 3A, middle blue line) and superimposed with the Meta I reference spectrum (middle brown line). Under these experimental conditions (pH 6.5 and 20 °C), the spectrum was similar to the Meta I reference spectrum, indicating formation of ac-Meta I. Slight deviations between the spectra of Meta I and ac-Meta I, especially around 1550 cm⁻¹ (amide II and ethylenic stretching mode) and in the HOOP vibrations between 1000 and 900 cm⁻¹, were due to the different chemical structures of the chromophores. The strong negative band at 1238 cm⁻¹, indicative of 11-cis/all-trans-isomerization, only slightly changed its position in the difference spectrum of ac-rhodopsin, showing that the lack of the ring structure did not influence this vibration significantly. Difference bands between 1720 and 1800 cm⁻¹ in the spectrum of ac-rhodopsin indicated the presence of a small fraction of ac-Meta II.

By further lowering the pH, the fraction of ac-Meta II largely increased at the expense of its precursors, as seen by comparing the spectrum recorded at pH 4.0 (Fig. 3A, lower green line) with a Meta II reference spectrum (lower brown line). Formation of ac-Meta II at lower pH values was indicated by the Meta II-specific bands at 1768, 1745, and 1730 cm⁻¹, resulting from changes in hydrogen bonding of Asp-83 and Glu-122 (26), and by the band at 1713 cm⁻¹, indicative of the protonation of the Schiff base counterion Glu-113 (28). A partial decomposition of ac-Meta II at pH 4.0 and a slight amount of ac-Meta I still present in the equilibrium may have accounted for the reduced intensities of the bands in the range 1800 to 1700 cm⁻¹ (see also Fig. 3B). Similar observations were made for rhodopsin containing the 9-cis-isomer of acyclic retinal (supplemental Fig. 1S).

The strong positive band at 1568 cm⁻¹ in the spectrum of ac-rhodopsin at 20 °C and pH 4.0 was due in part to the different structures of the chromophore as discussed for the earlier intermediates, but mainly to the formation of an ac-Meta II species with a reprotonated Schiff base, influencing the C=O stretching vibrations. The positive band at 1198 cm⁻¹, indicative of a protonated Schiff base, underlined this statement. Accordingly, the band at 1713 cm⁻¹, attributed to the protonated counterion Glu-113 of the Schiff base, was shifted to 1706 cm⁻¹, arguing for stronger hydrogen bonding of Glu-113 due to the presence of the additional charge (29). A reprotonation of the Schiff base at low pH in Meta II (formation of Meta II⁎SB) was also reported previously for 9-dm-rhodopsin (6, 7) and is dealt with for ac-rhodopsin in more detail in supplemental Fig. 2S.
Meta II content was calculated from the infrared spectra recorded at pH values in the range of 3.0–8.0 using the intensities of the Meta II characteristic band doublet at 1768 and 1751 cm⁻¹, which was normalized to the chromophore band at 1240 cm⁻¹. Hyperbolic fits to the data yielded titration curves with an apparent $pK_a$ of 5.2 for ac-rhodopsin, which was shifted by >2 units compared with rhodopsin ($pK_a = 7.5$). For the fit, data points below pH 4 were not evaluated because of the possible denaturation of the receptor under such harsh acidic conditions, as indicated by the deformation of the 1751 cm⁻¹ band in the infrared spectra at low pH. Because quantitative formation of ac-Meta II would require a pH of <3, the maximum amount of ac-Meta II that can be formed in the sample was determined by stabilization of ac-Meta II with a synthetic peptide (VLEDLKSCGLF). This peptide, termed CTαH1, is derived from the Gt $\alpha$-subunit C-terminal sequence (residues 340–350) and is known to stabilize Meta II efficiently (24, 25, 30). By the same method, the amount of 9-dm-Meta II obtained after illumination of 9-dm-rhodopsin was determined and plotted as a function of pH. The calculated $pK_a$ of 9-dm-Meta II formation was 5.0.

Finally, we used a second independent approach to investigate interaction of the CTαH1 peptide and light-activated ac-rhodopsin in disk membranes (Fig. 3C). This was accomplished by the use of a fusion protein consisting of MBP and the CTαH1 sequence. Binding of MBP-CTαH1 fusion protein from solution to rhodopsin following light activation can be monitored by kinetic light scattering (25). MBP-CTαH1 binding led to a gain in mass of the disk membranes, causing an increase in the relative intensity of scattered light (binding signal). The binding signals of MBP-CTαH1 and light-activated ac-rhodopsin measured at 20 °C and pH 7.5 (10 and 26 μM MBP-CTαH1) or pH 4.5 (0.1–12 μM MBP-CTαH1) were superimposed (Fig. 3C, left panel). Plotting of the signal amplitudes against the MBP-CTαH1 concentrations used and hyperbolic fitting of the data points yielded an EC₅₀ value of 0.24 μM for the measurement at pH 4.5, whereas at pH 7.5, binding was ~3 orders of magnitude weaker (EC₅₀ > 100 μM) (Fig. 3C). This indicates that, in disk membranes at 20 °C and pH 7.5, the ac-Meta I ↔ ac-Meta II equilibrium was far on the left side and could be shifted only by high concentrations of CTαH1 (>1 nm), as used in the FTIR experiments. Interestingly, this observed shift in affinity of MBP-CTαH1 for ac-rhodopsin between pH 7.5 and 4.5 of ~3 orders of magnitude is comparable with the difference in the $pK_a$ values of Meta II formation for rhodopsin and ac-rhodopsin ($\Delta pK_a = 7.5 - 5.2 = 2.3$). Similar to ac-rhodopsin, MBP-CTαH1 was capable of interaction with 9-dm-rhodopsin at pH 4.5 with almost the same affinity (EC₅₀ = 0.24 μM) (Fig. 3C), whereas no binding was observed at pH 7.5.

**Table One**

| Meta II formation, Schiff base hydrolysis, Gt activation, and Gt interaction data | Rhodopsin | 9-dm-rhodopsin | ac-rhodopsin | 9-cis-ac-rhodopsin |
|---|---|---|---|---|
| pKₐ<sup>a</sup> | 7.5 | 5.0 | 5.2 | 5.3 |
| tₐo (ms) | <2 | ~140 | <2, ~8<sup>b</sup> | ND |
| Schiff base hydrolysis, tₐo (min)<sup>c</sup> | 12.3 | 17.3 | 12.0 | 11.9 |
| Gt activation (%)<sup>d</sup> | 100 | 21 | 31 | 27 |
| Gt activation (%), E134Q mutants<sup>e</sup> | 97 | 94 | 71 | 64 |
| R<sup>f</sup>/MBP-CTαH1 binding, EC₅₀<sup>f</sup> | ND at pH 4.5, 0.68 μM at pH 7.5<sup>g</sup> | 0.24 μM at pH 4.5<sup>g</sup> | >100 μM at pH 7.5 | 0.24 μM at pH 4.5<sup>g</sup> | >100 μM at pH 7.5 | ND |

<sup>a</sup> Rod outer segment membranes were depleted of peripheral proteins.

<sup>b</sup> This refers to the decay component of transient 380 nm absorption.

<sup>c</sup> Purified recombinant pigments were activated at t = 0 with orange light.

<sup>d</sup> The rhodopsin control sample was obtained by regeneration of opsin with 11-cis-retinal.

<sup>e</sup> MBP-CTαH1 interacts with Meta II and Meta II<sub>IR</sub> forms.

<sup>f</sup> In rod outer segment membranes, Meta II<sub>IR</sub> forms with pKₐ = 2.5 (29).
dopsin and ac-rhodopsin ($t_{1/2} < 2$ ms). In the case of ac-rhodopsin (and in contrast to rhodopsin), the fast absorption increase was followed by a slower decline ($\sim 8$ ms), which indicated the decay of Meta II and formation of a Meta II photoproduct (Meta II$_{Psb}$) (see also supplemental Fig. 2S). For 9-dm-rhodopsin, the formation of Meta II was 2 orders of magnitude slower ($\sim 140$ ms) compared with rhodopsin and ac-rhodopsin (Fig. 4, A and B), in accordance with previous reports (7). Time-resolved UV-visible spectra were recorded every millisecond, and difference spectra (illuminated-minus-dark state) were calculated (Fig. 4C). The difference spectra were plotted at 20-ms intervals for ac-rhodopsin and native rhodopsin and at 50-ms intervals for 9-dm-rhodopsin. In contrast to ac-rhodopsin and native rhodopsin, the formation of Meta II of 9-dm-rhodopsin was slow enough to be time-resolved with the experimental setup as indicated by the gradual appearance of a species with an absorption maximum at 380 nm.

**ac-Meta II Formation and $G_t$ Activation by the E134Q Mutant** — The E134Q mutation in the conserved D(E)RY motif of GPCRs facilitates light-induced Meta II formation in pigments containing 9-dm-retinal (6). To test the effect of this mutation on pigments containing ac-retinal, we expressed opsin in COS-1 cells and reconstituted it with 11-cis-, 9-dm-, or ac-retinal to obtain recombinant rhodopsin, 9-dm-rhodopsin, and ac-rhodopsin, respectively. UV-visible spectra of the purified rhodopsin pigments before (Fig. 5A, unshaded) and after (shaded) illumination were recorded at pH 7.5. Spectra in the dark displayed absorption maxima at 500 nm (rhodopsin), 464 nm (9-dm-rhodopsin), and 460 nm (ac-rhodopsin), which shifted to 380, 466, and 449 nm, respectively, upon illumination. All three pigments showed an increase in the absorption at 380 nm indicative of formation of Meta II to different degrees. The absorption in the region at 450–470 nm was due to a protonated Schiff base, as present in Meta I, for example. In agreement with our previous study (6), a large light-induced absorption at 380 nm was observed for E134Q opsin reconstituted with 11-cis-retinal (E134Q) or 9-dm-retinal (9-dm-E134Q) (Fig. 5A). A similar effect was observed with the ac-retinal-reconstituted E134Q mutant (ac-E134Q) (Fig. 5A, shaded), suggesting that the diminished Meta II formation due to lack of the retinal ring structure could be rescued by the E134Q mutation.

To obtain more detailed information on the effect of ac-retinal on the Meta I $\rightarrow$ Meta II transition, we determined the conformation of the Meta photoproducts by infrared spectroscopy. FTIR difference spectra of purified recombinant ac-rhodopsin (Fig. 5B, black line) and ac-E134Q (gray line) were recorded at pH 8.0 and $-10^\circ$C, representing a mixture of Meta I and Meta II. Conditions were controlled carefully because the Meta I/Meta II equilibrium is sensitive to pH, temperature, and pigment environment. Meta II is favored by temperature increase, pH decrease, and detergent (rather than lipidic) environment. The bands between 1800 and 1700 cm$^{-1}$, indicative of formation of Meta II, were less pronounced in the spectrum of ac-rhodopsin, corresponding
to a fraction of ~30% ac-Meta II. The presence of a large amount of ac-Meta I could be concluded from the positive band at 1541 cm\(^{-1}\) and the strong negative band at 1555 cm\(^{-1}\). In the difference spectrum of ac-E134Q, however, the bands between 1800 and 1700 cm\(^{-1}\) and the band at 1644 cm\(^{-1}\), characteristic for the formation of active Meta II, had a significantly higher intensity. Additionally, the band at 1541 cm\(^{-1}\) was shifted to 1532 cm\(^{-1}\), indicating that mainly ac-Meta II was present. According to UV-visible and FTIR spectroscopic criteria, the E134Q mutation therefore facilitated ac-Meta II formation and rescued the impairment due to ac-retinal.

The catalytic activity of purified recombinant rhodopsin and the E134Q mutant containing 11-cis-, 9-dm-, or ac-retinal was determined by monitoring intrinsic tryptophan fluorescence changes in the G protein resulting from GTP\(\gamma\)S uptake at pH 7.5 and 20 °C. The activities were 100% for rhodopsin, 97% for E134Q, 21% for 9-dm-rhodopsin, 94% for 9-dm-E134Q, 31% for ac-rhodopsin, and 71% for ac-E134Q (Fig. 5C). In our previous work (6), we found that 9-dm-retinal acts as partial agonist, explaining the lower activity of 9-dm-rhodopsin as due to a decreased amount of 9-dm-Meta II. Accordingly, the rescue of activity by the E134Q mutation was in agreement with the increased formation of 9-dm-Meta II. Basically, the same rescue effect of the E134Q mutation on G\(\alpha\) activity and Meta II formation was observed when ac-retinal was used as chromophore of the pigment.

**Failure of ac-retinal to Maintain the Active Conformation**—The rhodopsin-catalyzed GTP\(\gamma\)S uptake of G\(\alpha\) measured as intrinsic tryptophan fluorescence change and evaluated to calculate the G\(\alpha\) activation rates of purified pigments containing ac-retinal, showed different amplitudes of GTP\(\gamma\)S uptake traces (Fig. 6A). The reduced amplitude indicated that a pigment could not activate the complete pool of G\(\alpha\), which is most likely explained by a fast decay of the active receptor species during the course of the experiment. In detergent solution, light-activated rhodopsin decomposed within minutes into opsin and all-trans-retinal, along with Schiff base hydrolysis (22). To explore whether the active species of ac-rhodopsin decayed much faster than activated rhodopsin, the recombinant pigment was illuminated and added immediately or after 30, 60, or 120 s of preincubation to the assay mixture to start the GTP\(\gamma\)S uptake reaction of G\(\alpha\) (Fig. 6B). A 30-s preincubation of illuminated ac-rhodopsin led to a 5-fold reduced initial slope of the fluorescence trace, suggesting a decay of the active species of this pigment on the order of tens of seconds in contrast to the 11-cis-retinal-containing pigment, which takes minutes (supplemental Fig. 3S) (22, 31). To test whether this decay of activity parallels Schiff base hydrolysis and formation of inactive opsin, ac-rhodopsin was illuminated, and the protein moeity was denatured by addition of hydrochloric acid at defined times after illumination (Fig. 6C). The absorption peak at 422 nm reflected retinal remaining in a Schiff base linkage. Evaluation of this absorption maximum over time yielded a half-time of 12.0 min for ac-rhodopsin, comparable with the half-time determined for rhodopsin (12.3 min). The half-time for 9-dm-rhodopsin was 17.3 min (supplemental Fig. 3S).

The Schiff base hydrolysis reaction in rhodopsin due to light activation of the pigment is reflected in an increase in opsin tryptophan fluorescence (Fig. 6D, red line) (22). Interestingly, we found a much faster increase in tryptophan fluorescence for solubilized ac-rhodopsin with kinetics on the order of tens of seconds (Fig. 6D, blue trace), similar to
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the fast decay of the active species. In contrast with rhodopsin, illumination of ac-rhodopsin with orange light (indicated by \textit{h}v) activated only a fraction of the pigment because a second and third illumination caused a further fluorescence increase. This was most likely due to restoration of the ac-rhodopsin ground state from Meta I \(9\) during illumination. At the end of the experiment, hydroxylamine (\textit{HA}) was added to remove all chromophore from the pigments as retinal oximes. The fast decay of the active species of ac-rhodopsin may explain why \textit{Jäger et al.} \((27)\) failed to observe any activity for the purified pigment containing the 9-cis-isomer of acyclic retinal.

DISCUSSION

As a photoreceptor and GPCR, rhodopsin shares properties with both of these receptor protein families \((32)\). The complex activation process of the photoreceptor, with its multistep photon capture-triggered mechanism of activation, appears to have little in common with the mechanism by which other GPCRs are activated through the binding of diffusible ligands. However, similarities become obvious under more detailed analysis. The formation of the activating Meta II state is then understood as the stepwise adjustment of the protein structure to the light-generated agonist all-trans-retinal, which arises from the inverse agonist 11-cis-retinal much faster than the protein can react. Three phases can be distinguished in the activation process: (i) \textit{cis}/trans-photoisomerization of retinal, (ii) thermal relaxation of the retinal-protein complex and transmission of the photonic energy into the protein, and (iii) the late metarhodopsin equilibrium states in which the interactions with the G protein are initiated \((4)\). Our results show that the lack of an intact retinal ring affects these three phases, leading to defined differences, all of which we can infer from the experimental data. It appears that the most critical role of the retinal ring is to maintain the active conformation.

Transmission of the Light Signal into the Protein—Previous work has shown that 11-cis-retinal analogs lacking carbon atoms C-2 and C-3 of the ring (compare Fig. 1B) are able to form partially active pigments with opsin \((33)\). By further truncation of the ring moiety, it was found that the retinal ring C-18 methyl group and/or one of the C-16/C-17 methyl groups is required to form stable pigments. However, to prevent Schiff base cleavage by hydroxylamine, two methyl groups mimicking C-18 and one of C-16/C-17 are necessary \((20)\). It was concluded that crucial interactions between these methyl groups and the binding cavity must exist. The present data now show that the light-induced isomerization process is not significantly influenced by the ring modification of ac-retinal, which preserves C-17 and C-18. This conclusion can be drawn because the complex combination of C-C stretching, C-H bending, and N-H bending vibrations seen in the FTIR spectra (band at 1238 cm\(^{-1}\)) \((26)\) display only a minor shift.

After cis/trans-isomerization, a considerable twist in the polyene chain is present in the early rhodopsin intermediates, as indicated by the strong positive HOOP bands in the Batho and Lumi FTIR difference spectra. A small shift toward higher wave numbers of the positive HOOP band in the ac-Batho spectrum compared with Batho indicates that the ring modification slightly changes the coupling between retinal and the protein environment. However, the intensities of the HOOP bands, as a measure of the twist of the polyene chain, are comparable for both retinals, indicating that dissipation of the light energy into the protein is achieved in a comparable way.

We conclude that, in both the ground state and the ac-Batho intermediate, the two methyl groups corresponding to C-17 and C-18 are sufficient to fix the retinal by defined hydrophobic interactions. Moreover, the amide I spectral features of the Batho and Lumi intermediates show a high similarity for rhodopsin and ac-rhodopsin in these states and indicate that the C-17 and C-18 methyl groups are sufficient to trigger a normal chain of structural changes as a consequence of photonic energy transmission into the protein.

Formation of the Active State—A criterion of how much of the light energy is finally transmitted into the protein active state by a given retinal analog \((7, 34)\) is the effective pH at which the active receptor conformation is formed. This is explained by the fact that proton uptake is required to generate the active conformation of rhodopsin and other GPCRs \((35–38)\) and that energy is needed to protonate a residue at a pH higher than its intrinsic \(pK_a\) (forced protonation). By this criterion, the efficiency of ac-retinal and 9-dm-retinal compared with native retinal can be derived from the difference of the \(pK_a\) values for native rhodopsin \((pK_a = 7.5)\) \((39)\), ac-rhodopsin \((pK_a = 5.2)\) \((3)\), 9-dm-rhodopsin \((pK_a = 5.0)\) \((3)\), and the apoprotein \((pK_a = 4.1 \text{ at } 30 ^\circ C)\) \((40)\). This is a large difference in terms of the biological function of the receptor because ac-rhodopsin cannot form a sufficiently high amount of Meta II at neutral pH \((~5%\text{ and even less for } 9\text{-dm-retinal})\) \((3)\). Amplification of the light signal in terms of \(G\) protein activation is thus strongly reduced. However, it is not much in terms of energy uptake because the \(pK_a\) difference for rhodopsin and ac-rhodopsin of \(-2\) pH units corresponds to only a \(\Delta G = 3 \text{ kcal} / \text{mol} \text{ lower energy input for ac-retinal compared with native } 11\text{-cis-retinal}\. Most likely this behavior results from an increased entropy of the precursor of ac-Meta II, as was discussed for 9-dm-Meta I \((6, 7)\). In line with the shift in \(pK_a\) are our results on Meta II stabilization with MBP-CTdHA1 fusion proteins, as seen in the differences of the \(EC_{50}\) values \((39)\).

Previous work has shown that retinal controls the last steps of the activation process \((6, 41)\) and that the function of the chromophore agonist in the late photointermediates is to enable these last conformational adjustments to reach Meta II. Proton transfer and proton uptake reactions \((\text{involving conserved residues Glu-113 and Glu-134, respectively})\) are behind the protonation-dependent protein conformational changes, and it was proposed that retinal in the all-trans-configuration provides the scaffold for the relevant proton transfer reactions \((6, 28, 42)\). Our results now show a behavior for ac-retinal similar to that for 9-dm-retinal, classifying it as another partial agonist of rhodopsin. This is in line with the ability of the E134Q mutant, in which the protonation changes in the D(E)RY region are anticipated, to overcome the partial agonism of ac-retinal and 9-dm-retinal. Interestingly, the rate of Meta II formation is much slower for 9-dm-rhodopsin compared with rhodopsin and ac-rhodopsin \((4)\) \((7)\). This might be explained by differences in fixing the retinal analogs in the binding cavity of the rhodopsin ground state and emphasizes the importance of the retinal methyl group at C-9 in this regard.

Role of the Retinal Ring Moiety in Maintaining the Active State—We have recently shown that, in active Meta II, the central part of the polyene chain is locked in the all-trans-configuration and resists re-isomerization to the 11-cis-ground state \((8)\). In the present study, we have measured, for the ring-deficient ac-retinal, a fast decay of activity of ac-Meta II once formed. We therefore conclude that the ring portion is also fixed in Meta II. This fast inactivating protein conformational change in ac-Meta II, which is also observable as a change in tryptophan fluorescence, is made possible by an increased degree of freedom for the remainder of the ring portion present in ac-retinal. It would be interesting to know whether introduction of an additional methyl group and double bond (corresponding to C-17 and the C-5=C-6 bond of retinal) into ac-retinal is sufficient to prevent the observed fast decay of the active receptor species. Because the rate of retinal Schiff base hydrolysis is unaltered in light-activated ac-rhodopsin, it can be concluded that the
non-ring part of retinal is sufficiently fixed in the retinal-binding pocket to prevent accelerated retinal release. The view that the ring moiety of retinal actively maintains the Meta II conformation of rhodopsin is supported by solid-state NMR studies showing that the C-16/C-17 methyl groups are more strongly fixed by the protein moiety in Meta II (16).

Conclusion—In this study, we delineated the molecular mechanism of activation by a partial agonist of the GPCR rhodopsin. Although interesting and instructive differences exist in activation of rhodopsin and ac-rhodopsin, the initial energization of the protein structure by retinal isomerization and the transmission of the photonic energy into the protein are basically the same for both pigments. Because the rate of retinal isomerization and the transmission of the photonic energy into the active state is similarly high. The lack of an intact ring structure is decisive as soon as this barrier is overcome and protonation of the cytoplasmic part of TM-VI out of the helix bundle. Such a mechanism is now supported because any impairment in the interaction between the ring ending of the chromophore and TM-VI must lead to a decrease in the energy of the active state. It is conceivable that other GPCRs are activated in a similar manner by partial agonists.

REFERENCES
1. Palczewski, K., Kumazaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
2. Li, J., Edwards, P. C., Burghammer, M., Villa, C., and Schertler, G. F. (2004) J. Mol. Biol. 343, 1409–1438
3. Okada, T., Sugihara, M., Bondar, A. N., Elstner, M., Entel, P., and Buss, V. (2004) J. Mol. Biol. 342, 571–583
4. Okada, T., Ernst, O. P., Palczewski, K., and Hofmann, K. P. (2001) Trends Biochem. Sci. 26, 318–324
5. Lou, J., Tan, Q., Karnaukhova, E., Berova, N., Nakanishi, K., and Crouch, R. K. (2000) Methods Enzymol. 315, 219–237
6. Meyer, C. K., Böhme, M., Ockenfels, A., Gärtner, W., Hofmann, K. P., and Ernst, O. P. (2000) J. Biol. Chem. 275, 19713–19718
7. Vogel, R., Fan, G. B., Sheves, M., and Siebert, F. (2000) Biochemistry 39, 8895–8908
8. Bartl, F. J., Ritter, E., and Hofmann, K. P. (2001) J. Biol. Chem. 276, 30161–30166
9. Ritter, E., Zimmermann, K., Heck, M., Hofmann, K. P., and Bartl, F. J. (2004) J. Biol. Chem. 279, 48102–48111
10. Patel, A. B., Crocker, E., Eilers, M., Hirshfeld, A., Sheves, M., and Smith, S. O. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10048–10053
11. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
12. Sheikh, S. P., Zvyaga, T. A., Litcharge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350
13. Hubbell, W. L., Altenbach, C., Hubbell, C. M., and Khorana, H. G. (2003) Adv. Protein Chem. 65, 243–290
14. Farahbakhsh, Z. T., Hiekle, K., and Hubbell, W. L. (1993) Science 262, 1416–1419
15. Spooner, P. J., Sharples, J. M., Goodall, S. C., Seedorf, H., Verhoeven, M. A., Lugtenburg, J., Bovee-Geurts, P. H., DeGrip, W. J., and Watts, A. (2003) Biochemistry 42, 13371–13378
16. Spooner, P. J., Sharples, J. M., Goodall, S. C., Bovee-Geurts, P. H., Verhoeven, M. A., Lugtenburg, J., Pistorius, A. M., Degrip, W. J., and Watts, A. (2004) J. Mol. Biol. 343, 719–730
17. Borhan, B., Souto, M. L., Imai, H., Shichida, Y., and Nakashima, K. (2000) Science 288, 2209–2212
18. Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., and Ernst, O. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2290–2295
19. Crouch, R. K. (1982) J. Am. Chem. Soc. 104, 4946–4948
20. Crouch, R. K., and Or, Y. S. (1983) FEBS Lett. 158, 139–142
21. Kuksa, V., Bartl, F., Maeda, T., Jang, G. F., Ritter, E., Heck, M., Van Hooser, J. P., Liang, Y., Filipek, S., Gelb, M. H., Hofmann, K. P., and Palczewski, K. (2002) J. Biol. Chem. 277, 42315–42324
22. Farrens, D. L., and Khorana, H. G. (1995) J. Biol. Chem. 270, 5073–5076
23. Heck, M., Schädel, S. A., Maretski, D., and Hofmann, K. P. (2003) Vision Res. 43, 3003–3010
24. Bartl, F., Ritter, E., and Hofmann, K. P. (2000) FEBS Lett. 473, 259–264
25. Herrmann, R., Heck, M., Henklein, P., Kleuss, C., Hofmann, K. P., and Ernst, O. P. (2004) J. Biol. Chem. 279, 24283–24290
26. Siebert, F. (1995) Int. J. Chem. 35, 309–323
27. Jäger, F., Jäger, S., Krüügel, O., Friedman, N., Sheves, M., Hofmann, K. P., and Siebert, F. (1994) Biochemistry 33, 7389–7397
28. Jäger, F., Fahmy, K., Sakmar, T. P., and Siebert, F. (1994) Biochemistry 33, 10878–10882
29. Vogel, R., Fan, G. B., Siebert, F., and Sheves, M. (2001) Biochemistry 40, 13342–13352
30. Martin, E. L., Rensi-Domiano, S., Schatz, P. J., and Hamm, H. E. (1996) J. Biol. Chem. 271, 361–366
31. Kleiber, I., Mitchell, D. C., Beach, J. M., and Litman, B. J. (1991) Biochemistry 30, 6761–6768
32. Filipek, S., Stenkamp, R. E., Teller, D. C., and Palczewski, K. (2003) Annu. Rev. Physiol. 65, 851–879
33. Karnaukhova, E., Hu, S. H., Boonyasai, R., Tan, Q., and Nakanishi, K. (1999) Bioorg. Chem. 27, 372–382
34. Hofmann, K. P. (2000) in Handbook of Biological Physics (Hoff, A. J., ed) Vol. 3, pp. 91–142, Elsevier Science Publishers B. V., Amsterdam
35. Cohen, G. B., Oprian, D. D., and Robinson, P. R. (1992) Biochemistry 31, 12592–12601
36. Arnits, S., and Hofmann, K. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7849–7853
37. Buczeklo, J., Szaí, J. C., Crouch, R. K., and Palczewski, K. (1996) J. Biol. Chem. 271, 20621–20630
38. Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G., and Coteccchia, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 808–813
39. Parkes, J. H., and Liebman, P. A. (1984) Biochemistry 23, 5054–5061
40. Vogel, R., and Siebert, F. (2001) J. Biol. Chem. 276, 38487–38493
41. DeGrip, W. J., and Rothschild, K. J. (2000) in Handbook of Biological Physics (Hoff, A. J., ed) Vol. 3, pp. 1–54, Elsevier Science Publishers B. V., Amsterdam
42. Arnits, S., Fahmy, K., Hofmann, K. P., and Sakmar, T. P. (1994) J. Biol. Chem. 269, 23879–23881