Signaling States of Rhodopsin:
ABSORPTION OF LIGHT IN ACTIVE METARHODOPSIN II GENERATES AN ALL-TRANS-RETINAL BOUND INACTIVE STATE

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SUMMARY

Absorption of light in rhodopsin leads through 11-cis/ all-trans retinal isomerization, proton transfers and structural changes to the active G-protein binding meta II state. When meta II is photolyzed by blue light absorption, the activating pathway is apparently reverted and rhodopsin photoregenerated. However, the product formed (P500) is different from the ground state, based on the following observations: i) the ground state fingerprint of 11-cis-retinal does not appear in the infrared spectra, although the proton transfers and structural changes are reverted; ii) extraction of the retinal from P500 does not yield the expected stoichiometric amount of 11-cis-retinal but predominantly all-trans-retinal; iii) the infrared spectrum of P500 is similar to the classical meta III intermediate, which arises from meta II by thermal decay; iv) both P500 and meta III can be photoconverted to meta II, with the same changes in the infrared spectrum, and without a significant change in the isomerization state of the extracted chromophore. The data indicate the presence of a “second switch” between active and inactive conformations, which operates by photolysis but without isomerization around the C11-C12 double bond. This emphasizes the exclusivity of the ground state, which is only accessible by metabolic regeneration with 11-cis retinal.
In the ground state of rhodopsin, the chromophore 11-cis-retinal is packed between tight hydrophobic interactions of the β-ionone ring and a salt bridge between the protonated retinal Schiff base bond to Lys296 and its counterion, Glu113 (1). A stable configuration is also adopted with 9-cis- and 7-cis-retinals (2), suggesting flexibility of the ground state in adapting to the retinal hydrocarbon chain. After photolysis of the ground state (λ_{max} = 500 nm) by absorption of green light (λ > 500 nm), two-thirds of the photonic energy (238 kJ mol\(^{-1}\)) are taken up in the strained all-trans isomerized configuration of bathorhodopsin (λ_{max} = 543 nm) (3). The strain is thought to relax through lumirhodopsin (498 nm) by a flip of the retinal β-ionone ring (4). This movement may trigger the chain of conformational changes leading to metarhodopsin I (M I; 478 nm) and the signaling state, metarhodopsin II (M II, 380 nm), which binds the G-protein \(G_t\) (5). Crucial steps in these activating conformational changes are the proton translocation from the retinal Schiff base to its counterion at Glu113, with concurrent breakage of the salt bridge, and the subsequent proton uptake that disrupts interactions near Glu134 (6)(5). Current evidence suggests that the all-trans-retinal provides a rigid scaffold for the correct adjustment of the structurally sensitive proton translocations (7). Specific determinants of the active state include the β-ionone ring (8), the 9-CH3-group (7) and a non-substituted 10-H-group (9) of the polyene chain. These three elements are likely to have different interactions in the active vs. the ground state (4,9), suggesting that the chromophore may have different “points of anchor” (10,11) with the protein in the two states.

This study arose from the idea to probe the retinal site by light absorption in the active M II state. When the normal activating pathway is reverted by photolyzing M II with blue light (λ < 420 nm), one measures a shift of the absorption maximum indicating reprotonation of the retinal Schiff base and proton release; a photoproduct with λ_{max} = 500 nm is formed, which was so far identified with the 11-cis- or 9-cis-retinal bound ground state (12-15)(16). However, we will show that this photoproduct does not represent a photoregenerated ground state of rhodopsin or isorhodopsin but rather a product with new properties. Because photoexcitation of the signaling state fails to restore the ground state in vertebrate rhodopsin, the regeneration through the complex cellular metabolism (see (17)) may be the only way to restore 11-cis-retinal bound rhodopsin.
EXPERIMENTAL PROCEDURES

Purification of Rhodopsin - Bovine rod outer segments were prepared from fresh, dark-adapted retinas, by means of a discontinuous sucrose gradient method and stored at - 80°C. Washed rhodopsin membranes were prepared by removing the soluble and membrane-associated proteins from rod outer segment disc membranes by repetitive washes with a low ionic strength buffer and subsequently washed with fatty acid free BSA (18,19). The membrane suspension was stored at – 80 °C until use.

FTIR Measurements - FTIR samples were prepared by centrifugation (20). In ca. 40 µl of washed membranes (0.3 mM rhodopsin), pH was adjusted by a few µL of diluted NaOH or HCl. The suspension was centrifuged for 25 min at 100,000 g, yielding 2.2 mM rhodopsin in the pellet (from absorption at 500 nm). The buffer solution was removed and the pellet transferred to a 30 mm-diameter temperature controlled transmission cell with two BaF₂ windows and a 5 µm PTFE-gasket. FTIR measurements were performed with a Bruker ifs 66v spectrometer equipped with an LN₂-cooled HgMnTe-detector (EG&G Judson, J15D-series). Following equilibration for 1 hr, a set of 4 transmission spectra was recorded (see (20) for details). After 20 s illumination with a 150 W fiber-optic light source, filtered through heat (Schott KG2) and 495 nm long-pass filter, a second set of spectra was recorded; sets of spectra were averaged and the M II minus Rh difference spectrum generated (we use the convention that the spectra of the conversion A → B are calculated as B minus A and termed B minus A difference spectrum). Spectra were recorded in the absorbance mode. For photolysis of M II, the sample was illuminated through a band pass filter optics (400 ± 20 nm) resulting from the cut-off characteristics of the fiber-optics and a Schott UG1 filter. After illumination for 30 s, the P (photoreverted M II) minus M II difference spectrum was generated as described above.

To obtain the difference spectra of M III, an M II sample was allowed to decay for 2 hours at room temperature and pH 7. Then spectra were taken from the decay product and the sample was illuminated with green light for 30 s. Subtraction of the decay product spectra (after minus before illumination) yielded the M II´ minus M III difference spectrum. Throughout this study, we will use the term M II´ when an M II-like species is formed from states other than the rhodopsin ground state.

Flash Photolysis - For flash experiments, aliquots of the FTIR preparation were used. M II was photolysed by a 400 nm light flash (bandwidth ca. 50 nm, Schott BG1) (see (15) for details). To follow the formation of P-products, the absorption change at 540 nm was recorded. This wavelength was chosen to minimize reconversion of the P products (λₘₐₓ =
470–500 nm) to M II’. To monitor the formation of M II, the samples were photolysed by a 500 nm light flash and the absorption change was recorded at 380 nm.

**HPLC Analysis** - After recording the infrared spectra, samples (Rh, M II, or P, see Fig. 1) were removed from the BaF$_2$ window of the IR-cuvettes and immediately solved in ice-cold ethanol (21). The solution was stirred (2 min) and the same amount of heptane added. After stirring for another 2 min, the sample was centrifuged (1 min) and the heptane-phase immediately analyzed in a Hewlett Packard HPLC device (Series 1050) equipped with a silicagel column (Si 60, 5µ), with 5% diethyl ether/ heptane at a flow rate of 0.5 ml/ min. Traces are absorption changes at 350 nm and are normalized to total retinal. The procedure to extract retinal oximes was as published (22)(17), but with 10mM hydroxylamine, 10% SDS; mobile phase: 12% diethyl ether/ heptane.

The proportion of each isomer in the samples was determined from the sum of the total peak areas of its syn and anti retinal oximes and calculated according to the following extinction coefficients ($\varepsilon_{360}$, in heptane): syn-all-trans, 54,900; anti-all-trans, 51.600; syn-11-cis, 35,000; anti-11-cis, 29,600; syn-13-cis, 49,000 and anti-13-cis, 52,100 (23).
RESULTS

**Extraction of Retinal from Photoreverted Metarhodopsin II (P-product)** - Concentrated rod disc membrane suspensions were placed in transmission FTIR cuvettes (20) and either used directly for UV/Vis flash photolysis and FTIR difference spectroscopy, or resuspended for retinal extraction and HPLC analysis. To ensure quantitative extraction with retention of configuration, the retinal extraction was performed in the presence of hydroxylamine (17). Both the syn and the anti forms of retinaloxime were fully resolved. Fig. 1 A shows HPLC traces of both the native retinaldehydes (left) and of retinaloximes formed with hydroxylamine (right). Data are from the freshly prepared dark kept samples (Rh), an aliquot after green illumination (20 s, generating M II) and an aliquot after green and subsequent blue illumination (generating the photolysis product of M II, termed P). The relative amounts of retinal extracted from these samples were similar for retinal aldehydes and retinal oximes (Fig. 1A, right).

In the fresh membranes, most of the extracted chromophore is in the 11-cis form, with a fraction of ca. 13 % ± 3 % (maximum deviation in three experiments, relative to the total retinal) of all-trans-retinal. The green illumination led to virtually complete conversion into M II and a correspondingly higher amount (95 %) of extracted all-trans-retinal (Fig. 1A). Subsequent blue illumination for 30 s photolysed > 60 % of the M II formed, as estimated from the characteristic M II bands in the FTIR difference spectra (see below). It generated a corresponding amount of „photoreverted“ P-product with reprotonated retinal Schiff base, as monitored by the red-shifted absorption maximum in the UV/Vis spectra ($\lambda_{\text{max}} = 470-500$ nm (14), data not shown). The amount of trans-retinaloxime extracted from the sample after blue light illumination and formation of P-product was slightly lower than in the M II sample (Fig. 1A, right). However, the decrease relative to the trans isomer formed with M II was 25 % at maximum, and not 60 %, as expected from the amount of P-product. The fraction of cis isomers extracted was 16 % and 9 % for 11- and 13-cis, respectively.

To minimize the effect of variable amounts of all-trans retinal in the Rh preparation (freshly prepared dark kept membranes; Fig. 1 A), the fractions of the retinal isomers were calculated relative to the increase of all-trans-retinal between rhodopsin and M II.

**FTIR Difference Spectra of the Rh → M II and M II → P Conversions** - Fig. 1 B shows the FTIR difference spectra of the Rh → M II and M II → P conversions, measured on the same samples as they were used for HPLC. In the M II minus Rh difference spectrum (green), a first class of spectral features (protein bands) reflects structural alterations in the protein, i.e. changes in the hydrogen bonding and protonation of carboxyl groups (Asp$^{33}$, Glu$^{122}$, and Glu$^{113}$, at 1768, 1748, and 1712 cm$^{-1}$) and in the peptide backbone (amide I and II.
bands at 1700-1620 and 1570-1500 cm\(^{-1}\), respectively (24)). The second class of bands (retinal bands) arises from changes in retinal geometry and retinal-protein interaction, reflected in C-C stretching vibrations (fingerprint region; 1238 cm\(^{-1}\) band and its satellites) and hydrogen out of plane vibrations (HOOP region; 960/970 cm\(^{-1}\)). Retinal related bands also appear in the 1550-1570 cm\(^{-1}\) region (C=C stretching vibration), thus interfering with the amide II bands. The difference spectrum of M II photolysis (blue; normalized to the 1768 cm\(^{-1}\) band) expresses the protein bands with inverse polarity, producing a mirror image of the difference spectrum of M II formation. This does not apply to the retinal C-C stretching vibrations reflecting the geometry of the chromophore (24); the fingerprint and HOOP bands are small and show a new pattern, different from the forward path. Specific for M II photolysis is a new band at 1350 cm\(^{-1}\), which is only seen with photoreversal and not detected in the forward pathway. This band may reflect new interactions of the reprotonated Schiff base with the receptor environment. Band positions at 1302 cm\(^{-1}\) and 1400 cm\(^{-1}\) were recently assigned to the C-H and N-H in-plane bending vibrations, respectively, of the retinal chromophore in bacteriorhodopsin (25).

**Characterization of P\(_{470}\) and P\(_{500}\)** - Blue flash photolysis of the FTIR samples (Fig. 2 A) reproduced the previous finding on suspensions (14) that P is a mixture of two products with reprotonated Schiff base, P\(_{470}\) and P\(_{500}\) (subscripts indicate \(\lambda_{\text{max}}\)). A high affinity C-terminal peptide from the G\(_{i}\) \(\alpha\)-subunit inhibits the conversion of M II to P\(_{500}\), which identifies P\(_{470}\) as a state that does not interact with G\(_{i}\) (15). At pH 4, the only product formed is P\(_{470}\), which enables the separation of both products in the FTIR spectra. Fig. 2 A shows the spectral differences between M II and the P products. The only distinct feature in the P\(_{470}\) minus M II difference spectrum (Fig. 2 A) is the positive band at 1558 cm\(^{-1}\), suggesting that P\(_{470}\) is structurally similar to M II. This assigns the ensemble of protein bands reflecting deactivation to P\(_{500}\) and fits to the observation that P\(_{500}\) but not P\(_{470}\) is affected by the peptide.

**Effect of Extended Illumination** - Extended blue illumination in photoequilibrium (80 % of the initial M II converted into P-products, as defined by the infrared spectrum) produced a steady increase of extracted cis-retinal forms (50 % after 20 min; data not shown). At pH 4 (P\(_{470}\) present), 11-cis and 13-cis predominated, while at pH 6 (both P\(_{470}\) and P\(_{500}\) present), the 7-cis isoform was seen in addition. During the formation of the cis-species in equilibrium, the infrared spectra (of both P\(_{500}\) and P\(_{470}\)) did not change significantly.

**Photoreversibility of the P-products** - Fig. 3 A compares the difference spectra for the conversions M II \(\rightarrow\) P and P \(\rightarrow\) M II\(^{+}\) (top, blue and green). The two difference spectra are a perfect mirror image of each other including the fingerprint region around 1238 cm\(^{-1}\). This
shows that chromophore/protein interactions in these two products are reversible in contrast to the rhodopsin $\rightarrow$ M II conversion (see Fig. 1 B).

**Photolysis of the Decay Product of M II, Metarhodopsin III (M III)** - Thermal decay of M II leads in parallel to the hydrolysis of the retinal Schiff base (yielding opsin and free all-trans-retinal) and to the formation of one or several species with protonated retinal Schiff base, termed metarhodopsin III (M III, broad UV/Vis absorption with $\lambda_{\text{max}} \approx 470$ nm). M III is inactive towards Gt (26); with the formation of M III, the FTIR protein bands assigned to M II have disappeared (data not shown; (27,28)). Photolysis of M III for 30 seconds with green light reverts up to 40% back to an M II-like product, M II’, with a difference spectrum (Fig. 3 A, bottom, green) that shows all essential features of the “reverted reversal” M II’ minus P spectrum, including the protein bands and the fingerprint. Flash illumination (Fig. 3 B) of M III for 20 microseconds generates a fast increase of 380 nm absorption. Although this experiment does not identify the product, it is likely a form of M II, because it is formed with a rate similar to that of M II formation from the rhodopsin ground state. Also the efficiency of formation is similar, when accounting for the reduced spectral overlap between the excitation spectrum of the flash and M III, as compared to the ground state. Moreover M II’ is an active species which is able to bind G-protein (M. Heck, unpublished results). Extraction of retinals from M III (generated by decay of M II for 1.5 hr at 20 °C) and M II’ shows a small shift towards cis species in the relative weight of retinal isomers that accompanies the formation of M II’ (Fig. 3 C).
DISCUSSION

The most significant states of the photoreceptor rhodopsin are the light-sensitive ground state and the active state metarhodopsin II (M II), which binds and activates the G-protein. The switch between these two states is operated by cis/ trans isomerization of the chromophore retinal, followed by thermal relaxation and proton transfer reactions. However, it has long been known that the presence of all-trans-retinal in the chromophore binding site is compatible with both active and inactive states of the receptor, linked to photoproducts M II and M III, respectively (26)(29,30). The salient result of this study is that the conversion between M II- and M III-like products can also be induced by absorption of blue or green light, respectively. Although the trigger mechanism of this reversible “second switch” remains to be elucidated, the results indicate that the underlying chromophore-protein interaction may not involve isomerization around the retinal C_{11}-C_{12} double bond.

**Reaction Scheme** - The data indicate a reaction scheme as shown in Fig. 4 A. The normal pathway is shown as an outer circle; it comprises the formation of M II via the Batho/ Lumi intermediates, its decay into opsin and all-trans-retinal and the regeneration of ground state rhodopsin from opsin and metabolically supplied 11-cis-retinal. The inner circle contains the thermal decay of M II into M III and the photolytic pathways that were newly identified in this study. We neglect here the “hybrid” product P_{470}, which is M II-like by its structure but bears a protonated Schiff base. The products P_{500} and M III are significantly different in $\lambda_{\text{max}}$, indicating alterations in chromophore-protein interaction. We have grouped them into the same class of intermediates, because they have in common the photoconversion by green light to an M II-like product. They are also similar in their infrared spectrum (including the new band at 1350 cm\(^{-1}\)) and they both show the largely red-shifted absorption which indicates a reprotonated retinal Schiff base.

The “second switch” between the M III/ P_{500} and M II/ M II´products is illustrated in Fig. 4 B. M III and M II are separated by a difference in energy of ca. 35 – 40 kJ mol\(^{-1}\), based on $\Delta H^0$ of M I \(\rightarrow\) MII (42kJ mol\(^{-1}\) (31)) and the $\Delta G^0$ between M I and M III (ca. - 8 kJ mol\(^{-1}\) (26)). Thus the second switch is expected to operate on a shallower energy profile (ca. 15 %) than the ground state/ Batho activation switch.

**Does the M II \(\rightarrow\) P_{500} Deactivation Switch Involve C_{11}-C_{12} double bond Isomerization?** - With the limited time resolution of the available techniques, any conclusions about the mechanism of the deactivation switch rely on a comparison of the starting and final products of thermal decay and photolysis. However, the FTIR spectra have clearly shown that neither the fingerprint nor the hydrogen out of plane bands of the retinal
seen in the M II minus rhodopsin difference spectrum are reverted in the P_{500} minus M II or M II' minus M III spectra. This demonstrates that the chromophore protein interaction in the M II decay or photolysis products (M III and P_{500}, respectively) is not the same as in the ground state. It raises the question which form of the chromophore is actually present in P_{500}, and whether a reversal of the cis/ trans isomerization is the trigger that reverses the structural changes seen in the spectra. Not only the spectrometric data but also the retinal extraction data are in conflict with this obvious explanation, because the amount of C_{11}-C_{12} cis isomer extracted from P_{500} was significantly smaller than stoichiometric. Explanations for this latter finding include:

i) P_{500} contains the C_{11}-C_{12} cis isomer, but cis is partially transformed to trans during the extraction procedure.

ii) The conversions between the M II- and the M III- or P_{500} - like products proceed in a two-photon process, involving successive trans/ cis and cis/ trans isomerizations.

iii) The photochemical trigger of the conversion is different from C_{11}-C_{12} double bond isomerization.

Although an extraction artefact (i) cannot be excluded, it is unlikely by the observations made with M III photolysis. In this case, both the starting and the final product are supposed to bind the retinal in the all-trans form. And indeed, from both M III and its photolysis product, M II', all-trans was the predominantly extracted isomer, with a slight, if any, shift towards 11- and 7- cis-retinal. In principle, a possible solution could arise from a two photon process (ii): for example, in the M III photolyzed in the experiment (Fig. 3), the chromophore could have been transferred from its original binding site to another site, in concert with the formation of protonated retinal Schiff base. Such forms of M III have indeed been observed (29). In the two photon process, a first absorption would isomerize the chromophore. After rapid breakage of the Schiff base bond, the chromophore would return into the original binding site, and a second photon would then photoconvert this newly formed ground state into M II'. In the present study, this possibility was addressed by flash photolysis of M III. The data are consistent with the notion that M II' is formed by flash photolysis from M III with the same relative efficiency as with continuous illumination. This would limit the time interval between the two photoexcitations in the assumed two photon process to 20 µs. Although we cannot exclude that low amounts of M III-like byproducts are formed, most of the M III species present under our experimental conditions and identified in the FTIR spectra, will contain the retinal in its original site or just “put aside”, so that it can readily return into the position it had in M II. A rough measure for the time it takes to assemble the free retinal chromophore with
its binding site is given by the regeneration of rhodopsin from 11-cis-retinal and opsin apoprotein, which takes seconds (18,32).

We therefore come to the conclusion that the third mechanism (iii) must be considered as a real possibility. The data show that not only 11-cis- but also 7- and 13-cis-isomers are readily detected by the extraction procedure applied. This makes it unlikely that other persistently formed isomers would have escaped the analysis.

**Light-induced Changes in Proteins with C11-C12 Double Bond Locked Retinals**

For the related proton pump bacteriorhodopsin, a light-induced conformational change was found when the protein was regenerated with locked retinal, which cannot isomerize around the C11-C12 double bond. It was discussed that polarization changes in the excited state of the chromophore could trigger a conformational change in the protein that persists even after the chromophore has returned to the ground state (33,34). In a recent study on rhodopsin regenerated with locked retinal, it was shown by retinal extraction and HPLC analysis that the chromophore can undergo light-induced isomerization around bonds other than the locked C11-C12 double bond (11). In a parallel FTIR analysis, a retinal fingerprint band at 1206 cm\(^{-1}\) appeared, which likely reflects the isomerization, but indications of structural changes or changes of the protonation state of carboxyl groups could not be obtained to any significant degree. It was concluded that light-induced changes in the chromophore do occur but cannot induce the activating structural changes in the opsin moiety of the receptor, when starting from the protonated Schiff base situation in the ground state (11). A band in the same region appears also in the present reversal or “reverted reversal” spectra (from conversions MII \(\rightarrow\) P or P/M III \(\rightarrow\) M II’, respectively), as part of a chromophore fingerprint, which replaces the normal fingerprint motif around 1238 cm\(^{-1}\) (arising in the forward pathway as a consequence of the steric trigger in Batho/ Lumi and persisting through M I / M II (24)). The presence of the 1206 cm\(^{-1}\) band shows that chromophore alterations other than 11-cis/ trans isomerization do occur in rhodopsin and that they are reflected in the FTIR spectrum.

**Mechanism of the Deactivation Switch**

Because predominantly all-trans-retinal was extracted from the photoreverted species, photoisomerization in the majority of P-product formed can only be transient. However, the protein is persistently deactivated, as seen in the spectral changes and in the blockade of the reversal reactions by bound Gt peptide (Fig. 2). The activating forward pathway of rhodopsin leading to helix motion and the exposure of cytoplasmic interaction sites (5,6,35) involves a proton transfer between the protonated Schiff base and its counterion, the side group of Glu-113. This overcomes the structural constraints imposed by the salt bridge between these two locations. Interactions of the retinal β-ionone ring and of the hydrocarbon chain with the protein environment are
different in the Meta states vs. the ground state configuration (5,8). Anchored in the new interactions, and with the Schiff base bond deprotonated, photochemical conversions may be selected that are fundamentally different from those that determine the forward pathway.

It will take more extended analyses to find out how this new path proceeds and how similar P_{500} is to Lumi and/or M III. On the one hand, the new band at 1350 cm^{-1} is a distinct property of the photoreversible pathway opened by photolysis of M II (Figs. 1 and 3), and not seen when starting from the ground state of normal or locked retinal (11). On the other hand, the band at 1206 cm^{-1}, which appeared in the spectra of the rhodopsin with locked retinal (see above), may be a part of the fingerprint motif in the reversal or “reverted reversal” spectra (Figs. 1 and 3).

What we can state is that, although a different type of chromophore-protein interaction occurs in M III or P_{500}, as compared to the ground state, the full set of structural changes appears in the spectra. We propose that, in forming or leaving the active state, the different crucial elements are coupled to each other (like the spokes of an umbrella), so that the entity of transformations can be triggered by different mechanisms. Although in vitro techniques are not sensitive enough to determine the activity of P_{500} or M III, it is likely that it is higher than the 11-cis-retinal bound ground state. This would mean that only the 11-cis isomer can impose the stringent constraints that make the ground state so inactive.

**Exclusivity of the Metabolically Formed Inactive State** - The presence of a second rhodopsin-like state is in line with findings that photoregeneration from early intermediates restores the visual absorption of the ground state but not all of its physiological functions (36). Under conditions of substantial bleaching, and dependent on the irradiation conditions, the P-products are expected to accumulate, which may influence bleaching adaptation phenomena. The current observations may also be relevant for the potential physiological role of M III as a storage form (29,30).

The new data underline the exclusivity of the normal photolytic pathway in vertebrate rhodopsin. They show that the ground state, which is relevant for normal green light single quantum detection in the rod cell, cannot be reached by photoreversal. It must thus arise from a chemical reaction between 11-cis-retinal and the apoprotein. This may elucidate why it takes a complex metabolic process to regenerate vertebrate rhodopsin. It may be the only possible way to achieve a sufficiently stable chromophore protein configuration, to ensure the exceedingly low noise of the transduction process in rods.
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REFERENCES


The abbreviations used are:

- Rh, rhodopsin in the ground state
- Batho, bathorhodopsin
- FTIR, Fourier Transform Infrared Spectroscopy
- Lumi, lumirhodopsin
- M II, metarhodopsin II
- M III, metarhodopsin III
- P, photoreverted M II
- P$_{470}$, P subspecies with $A_{\text{max}} = 470$ nm
- P$_{500}$, P subspecies with $A_{\text{max}} = 500$ nm
- M II', M II-like species, photoreverted from M III or P
- G$_t$, heterotrimeric G-protein of the rod cell, transducin
FIGURE LEGENDS

Fig. 1 Analysis of the photolysis products of metarhodopsin II by retinal extraction and FTIR difference spectroscopy.

(A) HPLC elution profiles of retinal (left) or retinal oxime (right) extracted from Rh in ground state (top), metarhodopsin II (M II, middle) and the P-photoproduct (bottom). (B) FTIR difference spectra of the transitions (green and blue coded) between the products shown in (A). (Green) difference spectrum of M II minus rhodopsin; negative bands correspond with vibrations present in rhodopsin, positive bands with vibrations in M II; (blue) difference spectrum of P minus M II. Negative bands correspond with M II and positive bands with P. See text for details. FTIR spectra were normalized to the band at 1768 cm⁻¹.

Fig. 2. FTIR and flash photolysis of P₅₀₀ and P₄₇₀.

(A) P (P₄₇₀ plus P₅₀₀) minus M II difference spectrum, from spectra recorded at pH 6.0, and P₄₇₀ minus M II difference spectrum recorded at pH 4.0. (B) Flash photolysis of M II; shown are flash-induced absorption changes at 540 nm after a 400 nm flash with (lower) and without (upper) GᵣαₐC-terminal high affinity peptide. Dotted line is the approximate level of the fast absorption change (P₄₇₀). The slow absorption change is due to the formation of P₅₀₀. Absorbance maxima were determined by UV/Vis spectroscopy (data not shown; see (14)). Flash duration was 20 µs; see the Procedures and (14)(15) for experimental details.

Fig. 3 Photoregeneration of metarhodopsin II-like (M II´) species from its photolysis product (P) or decay product (M III).

(A) (Blue) difference spectrum (P minus M II) of the M II → P conversion (same as in Fig. 1 B); (top, green) difference spectrum (M II´ minus P) of the P → M II´ conversion induced by green illumination of P; (bottom, green) difference spectrum (M II´ minus M III) of the M III → M II´ conversion induced by green illumination of the decay product M III. The notation M II´ is used when an M II-like species is formed from states other than the rhodopsin ground state. (B) Flash-induced absorption change recorded at 380 nm, indicating the formation of M II from rhodopsin (black trace) and of M II´ from M III (red); flash duration and spectral profile as in Fig. 2 B. (C) HPLC elution profiles of retinal extracted from M III (top) and M II´ (bottom). See text for details.

Fig. 4 Reaction scheme and energy profile of rhodopsin activation and second switch.

(A) The scheme shows the normal pathway as an outer circle; it comprises the formation of M II via the Batho / Lumi intermediates, its decay into opsin and all-trans-retinal and the
regeneration of ground state rhodopsin from opsin and metabolically supplied 11-cis-retinal. The inner circle contains the thermal decay of M II into M III and the photolytic pathways that were newly identified in this study. Green and blue arrows indicate the conversions induced by green (500 nm) and blue (400 nm) light, respectively.  

(B) Reaction coordinate and qualitative energy scheme of rhodopsin activation and M II → M III conversion. By light absorption and retinal cis/ trans isomerization, the receptor passes irreversibly across the initial activation barrier between rhodopsin ground state and the early Batho/ Lumi intermediates. Illumination in the late Meta intermediates operates a photoreversible “second switch” between M II and M III or the P$_{500}$ photoproduct.
Figure 1

(A) Rhodopsin (Rh)
- illum. 500nm (MII)
- illum. 400nm (P)

(B) Rhodopsin (Rh)
- illum. 500nm (MII)
- illum. 400nm (P)

wavenumber [1/cm]
Figure 2
Figure 3
Batho/Lumi Meta II Meta III/ P

Regeneration with 11- cis-retinal

Opsin + all- trans-retinal

Retinal cis/trans Isomerisation

Energy

Rh Batho/Lumi Meta II Meta III/ P

"first switch" "second switch"

Reaction Coordinate

Figure 4
Signaling states of rhodopsin: Absorption of light in active metarhodopsin II generates an all-trans-retinal bound inactive state
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