Deactivation and Proton Transfer in Light-induced Metarhodopsin II/Metarhodopsin III Conversion

A TIME-RESOLVED FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDY

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Eglof Ritter1, Matthias Elgeti1, Klaus Peter Hofmann2, and Franz J. Bartl1,2

From the 1Institut für Medizinische Physik und Biophysik, Charité, Universitätsmedizin Berlin, Charitéplatz 1, D-10098 Berlin and the 2Zentrum für Biophysik und Bioinformatik, Humboldt-Universität zu Berlin, Invalidenstrasse 42, D-10015 Berlin, Germany

Vertebrate rhodopsin shares with other retinal proteins the 11-cis-retinal chromophore and the light-induced 11-cis/trans isomerization triggering its activation pathway. However, only in rhodopsin the retinylidene Schiff base bond to the apoprotein is eventually hydrolyzed, making a complex regeneration pathway necessary. Metabolic regeneration cannot be short-cut, and light absorption in the active metarhodopsin (Meta) II intermediate causes anti/syn isomerization around the retinylidene linkage rather than reversed trans/cis isomerization. A new deactivating pathway is thereby triggered, which ends in the Meta III “retinal storage” product. Using time-resolved Fourier transform infrared spectroscopy, we show that the identified steps of receptor activation, including Schiff base deprotonation, protein structural changes, and proton uptake by the apoprotein, are all reversed. However, Schiff base reprotonation is much faster than the activating deprotonation, whereas the protein structural changes are slower. The final proton release occurs with \( pK \approx 4.5 \), similar to the \( pK \) of a free Glu residue and to the \( pK \) at which the isolated opsin apoprotein becomes active. A forced deprotonation, equivalent to the forced protonation in the activating pathway, which occurs against the unfavorable \( pH \) of the medium, is not observed. This explains properties of the final Meta III product, which displays much higher residual activity and is less stable than rhodopsin arising from regeneration with 11-cis-retinal. We propose that the anti/syn conversion can only induce a fast reorientation and distance change of the Schiff base but fails to build up the full set of dark ground state constraints, presumably involving the Glu134/Arg135 cluster.

The photoreceptor rhodopsin located in the retinal rods of the vertebrate eye contains the chromophore 11-cis-retinal bound by a protonated Schiff base to Lys296 of the apoprotein (1). Light absorption triggers isomerization around the \( C_{11} = C_{12} \) double bond of the polyene chain of the chromophore (2–4), leading to the strained all-trans-form and storage of two thirds of the light energy in the chromophore–protein system (5–8). The receptor subsequently proceeds through a number of intermediates each characterized by its specific absorption spectrum in the UV-visible and mid infrared range. Related conformational changes of the binding pocket and of other, more remote parts of the apoprotein eventually lead to the active G-protein binding state, metarhodopsin II (Meta II).3 It is in equilibrium with its precursor metarhodopsin I (Meta I), depending on temperature and \( pH \) (9, 10) and on other factors such as lipids, protein environment, and pressure (11–15).

The formation of the active species through the photointermediates has been described as a stepwise lowering of the stabilizing effect of the Schiff base counterion, which is a complex structure that comprises highly conserved Glu181 and Glu113. In Meta I, the counterion appears to undergo a shift relative to the Schiff base, whereas the receptor is still inactive (16). The subsequent deprotonation of the Schiff base linkage between Lys296 and the aldehyde group of the retinal is reflected in the strong shift of the absorption maximum from 480 to 380 nm, which is the spectral signature of Meta II and results in a protonation of Glu113 (17). Proton uptake to Meta II must occur in a spectrally silent conversion forming a separate product Meta II1 (18). This step involves the residue Glu134, which is part of a cluster that stabilizes the apoprotein in the dark (19–21). Under physiological conditions the \( pH \) value of the surrounding medium is higher than the intrinsic \( pK \) of the uptake group of the apoprotein (estimated \( pK \sim 4 \) (22)), so that the light energy stored in the chromophore is partly used to enforce this protonation step and to shift the \( pK \) of the proton uptake group into the neutral range (23, 24). This “forced protonation” occurs as long as all-trans-retinal has all structural determinants, including the 9-methyl group and an intact \( \beta \)-ionone ring enabling it to act as a rigid scaffold (25, 26). In rhodopsin regenerated with 9-demethyl-retinal or 11-cis-acetyl retinal (27, 28) light-induced isomerization of the chromophore leads only to an active conformation when a low \( pH \) of the bulk phase supports proton uptake by the apoprotein. This can be interpreted in terms of an incomplete scaffold function of the retinal in these modified pigments.

Active Meta II eventually decays in opsin and all-trans-retinal (29). For completion of the visual cycle, fresh 11-cis-retinal has to be supplied by a complex retinoid cycle to regenerate

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2 To whom correspondence should be addressed: Tel: 49-30-450-524170; Fax: 49-30-450-524952; E-mail: franz.bartl@charite.de.

3 The abbreviations used are: Meta, metarhodopsin; FTIR, Fourier transform infrared; R-/RR-Meta, intermediates of Meta II photolysis; BTP, 1,3-bis-(tris(hydroxymethyl)-methyl-amino)propane; LED, light-emitting diode; Nd:YAG, neodymium yttrium aluminum garnet.
rhodopsin. This is remarkable because other retinal proteins such as archaerhodopsin and cinobufotalin can simply regenerate the ground state when a second photon in the active state is absorbed (for review, see Refs. 30 and 31).

Spectroscopic data show that blue light absorption of the active Meta II state does not lead back to the ground state \( (\lambda_{\text{max}} = 500 \text{ nm}) \) to a significant extent but to a product with an absorption maximum at 475 nm (32). By UV-visible and FTIR difference spectroscopy, this blue light-induced photoproduct of Meta II could be identified as Meta III (33, 34), a species already known as a side product of the thermal Meta II decay (9). Recent work has shown that this thermal product contains the chromophore in the Schiff base all-trans configuration (35) and that the Meta III formed by light absorption is the same by all available criteria. Hence, Meta III is also triggered by light-induced anti/syn isomerization of the C=N double bond of the Schiff base (36).

These findings substantiated the previous concept of the first and second switch (32), which are now identified as the cis/trans isomerization of the polyene chain and the syn/anti isomerization of the Schiff base, respectively. The end product of the new light-induced pathway is the high-energy state Meta III, which can form up to 80% depending on the conditions, and has remarkable properties. Because of its long lifetime (up to hours (34)), it excludes the chromophore very efficiently from the regeneration pathway, which has led to the concept of a retinal storage form (34, 37, 38). Remarkably, the lifetime of Meta III depends on the presence of G-protein (34) and arrestin (39, 40), which was interpreted as an “inverse catalysis” of receptor conversion by G-protein. Given the fact that these proteins undergo light-dependent transport, and that the rate of rhodopsin regeneration is affected by the decay of Meta II and Meta III in contrast to the phosphorylation rate (41), interesting possibilities of regulation are thereby opened.

The putative physiological implications and mechanistic significance of the light-induced deactivation pathway, which might play a role under conditions of bright light illumination, have led us to investigate its kinetics and the intermediates involved. Time-resolved UV-visible spectroscopy and electrical measurements of the activating and the deactivating pathway have already given indications that the light-induced absorption change related to Schiff base reprotonation occurs at a surprisingly high rate, namely even faster than the deprotonation linked to Meta II formation (20, 33, 42). We have therefore attempted to obtain additional information on the kinetics of the conformational conversions by time-resolved infrared difference spectroscopy, which allows us to follow a variety of structural alterations of the protein connected to its activity occurring on a 10-ms time scale. It turns out that anti/syn isomerization causes proper reprotonation of the retinal Schiff base but fails to couple to structural changes that are mandatory to fully deactivate the protein.

**EXPERIMENTAL PROCEDURES**

Rhodopsin Purification and Preparation—Rhodopsin in washed disk membranes was purified from fresh dark-adapted bovine retinae. In a first step, rod outer segments were prepared by a discontinuous sucrose gradient method (43). Subsequently, washed disk membranes were obtained by repetitive washes with a low ionic strength buffer and fatty acid free bovine serum albumin (44, 45). Rhodopsin membrane suspension was stored at −80 °C until use. As buffer, we used 1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP), and pH values were adjusted with diluted (0.1 m) NaOH and HCl. During all preparation steps the sample was kept under dim red light (\( \lambda > 640 \text{ nm} \)).

**FTIR Measurements**—FTIR samples were prepared by centrifugation procedure as described (32). Rhodopsin membrane suspension was centrifuged (30 min, 100,000 × g, 4 °C) to obtain a 2–3 mM pellet, which was exposed to dry air for 60 s to further reduce water content. Subsequently the pellet was transferred into a temperature-controlled cuvette consisting of two BaF2 windows and a 3-μm polytetrafluoroethylene spacer. FTIR spectra were measured in a BrukerIFS66v/s spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector (J15D-series, E&G Judson).

Before measurement, each sample was equilibrated for at least 30 min in the spectrometer. FTIR spectra were recorded before and after illumination, and FTIR difference spectra were calculated by subtracting the spectra of the initial state (A) from the spectra of the final state (B) and termed as “B minus A difference spectra.”

Illumination was performed with the following light sources: For non-time-resolved measurements, we used a ring of six green or blue LEDs placed directly at the cuvette (520 ± 10 nm or 400 ± 10 nm, respectively). With LEDs, the sample was illuminated for 4 s.

For illumination during the time-resolved experiments, we used two different lasers: For activation of rhodopsin a Nd:YAG laser (Spectron Lasers SL 282G), tuned to 532 nm, was directly focused on the sample. For deactivation of Meta II, a second Nd:YAG laser (SL 456-10), emitting at 355 nm, was used to pump a customized dye-laser emitting at 389 nm (Dye: Exalite 389, Radiant Dyes, Germany). The pulse duration of the Nd:YAG was 6 ns. One flash activated or deactivated ~40% of the rhodopsin on a sample spot of 5-mm diameter as estimated by bleaching the samples with LEDs of the appropriate wavelength. A complete conversion to Meta II or Meta III, respectively, could not be achieved by the flash illumination due to the absorption of a second photon by early intermediates of the reaction pathways. This induces back and/or side reactions to rhodopsin and isorhodopsin (activation) (9) or to so far unknown products (deactivation).

To investigate whether a certain intermediate is active toward the G-protein, the “Extra-Meta II” assay was used (46). A rhodopsin pellet was resuspended in 50 μl of a solution of BTP and 10 mM G-protein-derived high affinity peptide (Gto (340–350) VLEDLKSCLGF) (46). This solution was centrifuged for 30 min at 100,000 × g and 4 °C. By this procedure, a pellet containing the peptide in excess was obtained.

Time-resolved FTIR difference spectra were recorded with the rapid scan technique. To enhance time resolution, double-sided forward-backward measured interferograms were split into four single-sided interferograms. Because one scan takes 100.0 ms at a spectral resolution of 4 cm⁻¹ and 64.8 ms at a spectral resolution of 8 cm⁻¹, respectively, we achieved a time
resolution of 25 ms at 4 cm⁻¹ and 16.2 ms at 8 cm⁻¹. Due to the fast reaction, activation was measured with both resolutions, 4 and 8 cm⁻¹. The slower kinetics of deactivation allowed us to measure this reaction with the slower time resolution of 25 ms but with high spectral resolution of 4 cm⁻¹. Sample excitation by the laser flash was synchronized with the forward signal of the mirror movement to minimize the influence of the flash on the signal.

Each experiment was reproduced at least six times. Non-time-resolved measurements were done by averaging 128 scans; for time-resolved spectroscopy four independent measurements were averaged. Time courses of single bands were smoothed by a digital band-block filter and fitted to first order exponentials to evaluate the half times \( t_\frac{1}{2} \). The three-dimensional plots were smoothed by using the Savatzy-Golay algorithm.

**UV-visible Measurements**—For measurements in the UV-visible spectral range, the same centrifugation procedure as described for FTIR was applied. The rhodopsin pellets were transferred to a temperature-controlled transmission cuvette consisting of two BaF₂ windows and a 50-μm spacer of polytetrafluoroethylene. Measurements were performed with an OLIS RSM-16 spectrometer equipped with a photomultiplier tube as detector. Time resolution was 1 ms, and the total data collection time was 3 s. Flash illumination of the sample was applied using a flash lamp (Rapp OptoElectronic GmbH) with 2-ms flash duration. For illumination of rhodopsin and Meta II, a 480 nm shortwave cut-off (Schott GG 475) and a 400–450 nm band pass filter were used, respectively. During the flash, the detector of the spectrometer was covered by a shutter to avoid any influence of the flash light on the detector signal. For the decay measurement in Fig. 4B we used a Varian Cary 50 spectrophotometer scanning every 12 s over 3.5 min.

**RESULTS**

**Time-resolved UV-visible Spectroscopy of Meta II Deactivation**

In Fig. 1, we show the UV-visible spectra of the light-induced deactivation of Meta II and of the light-induced activation of rhodopsin for comparison, initiated by a blue and a green flash, respectively. The black line in Fig. 1 with an absorption maximum of 498 nm represents the spectrum of the ground state. At pH 6.0 and 10 °C, rhodopsin was activated by a green flash (\( \lambda > 480 \) nm, 2 ms). Spectra were recorded on a millisecond time scale and plotted every 20 ms (thin green lines). The first spectrum measured immediately after the flash shows an absorption maximum of 480 nm, indicative for the Meta I intermediate. Subsequently, this band vanishes while a new band at 380 nm appears, which reflects the formation of Meta II. A single exponential fit to the time course (trace a in the inset of Fig. 1) yields \( t_\frac{1}{2} = 70 \) ms. We achieved a maximum bleaching of \(~40\%\) of total rhodopsin by one flash as described under “Experimental Procedures.” Quantitative conversion to Meta II was subsequently performed by bleaching the sample with green light (\( \lambda > 500 \) nm) for 10 s (Fig. 1, thick green line).

A blue flash (400–450 nm, 2 ms) was then applied to induce deactivation of Meta II. The blue lines in Fig. 1 show the respective UV-visible spectra recorded on a millisecond time scale. Traces were plotted every 20 ms. The blue flash induces an immediate reduction of the intensity of the 380 nm absorption (half time, \( t_\frac{1}{2} < 5 \) ms) and a corresponding rise at \( \sim 475 \) nm. Neither any significant further reduction of the 380 nm band nor any other notable change of the absorption spectra could be detected within a time scale comparable to the activation process. \(~40\%\) of total Meta II were converted by the flash and quantitative conversion to the 475 nm product (dashed blue line) was subsequently achieved by continuous (10 s) blue light illumination of the sample.

The absorption change at 380 nm evoked by the blue light flash is shown in trace b in the inset of Fig. 1 (blue). The fast kinetics cannot be resolved with the rapid scanning UV-visible spectrometer; the same experiment performed with a flash photolysis setup with microsecond time resolution (32) yielded a half time of 4.5 ms at 10 °C for this process.

These experiments indicate a fast, light-induced reprotonation of the Schiff base, which is faster than the deprotonation step in the activating pathway. Because the absorption maxima in the UV-visible spectra solely report on the protonation state of the chromophore, time-resolved FTIR difference spectroscopy was used to compare the kinetics of conformational changes of the apoprotein.

**Time-resolved FTIR Spectroscopy of the Activating and Deactivating Pathway**

**Activation**—Fig. 2A shows the FTIR difference spectra of rhodopsin illuminated by a 6 ns laser flash (532 nm,
Nd:YAG) as a function of time in a three-dimensional plot. To demonstrate that the formation of both species, Meta I and Meta II, can be fully resolved with our experimental setup, the spectra were recorded at 0 °C and pH 6 every 25 ms. Under these conditions, the first spectrum after the flash is a Meta I minus rhodopsin difference spectrum, as indicated by the bands at 951/970 cm⁻¹, the typical fingerprint approximately 1238/1205 cm⁻¹, the doublet at 1536/1549 cm⁻¹, and the characteristic band pattern approximately 1700 cm⁻¹ (Fig. 2, A and B, red lines) (47, 48). Subsequently, bands at 1748/68 cm⁻¹, 1643 cm⁻¹, and 1556 cm⁻¹ reflect an increasing contribution of a Meta II minus rhodopsin difference spectrum. After a few seconds the final state is reached and the spectrum exhibits now the typical Meta II difference bands. The black line in Fig. 2B is the last spectrum recorded 8 s after the activating flash.
Light-induced Deactivation of Metarhodopsin II

The green lines in Fig. 3 show the kinetics of selected characteristic FTIR bands, representing the formation of active Meta II as extracted from a three-dimensional plot, recorded at 10 °C and pH 6 (spectral resolution 8 cm⁻¹, time resolution 16.2 ms). Difference bands reflecting proton transfer processes, changes in hydrogen bonding, and the secondary structure occurring during the formation of Meta II show kinetics similar to those observed in the literature (49) and to our own time-resolved UV-visible spectra shown in Fig. 1. This applies to the band at 1748 cm⁻¹ (A) indicating changes of the hydrogen bonding environment of Asp⁸³ and of Glu¹²², to the bands at 1643 cm⁻¹ (B; amide I-region) and at 1556 cm⁻¹ (C; amide II-region and the C=O stretch of the chromophore) typical for changes in the secondary structure of the protein and to the band at 1713 cm⁻¹ (D) characteristic for the protonation of the Schiff base counterion Glu¹¹³. These bands appear with comparable kinetics as observed for the 380 nm absorption shown in the inset of Fig. 1 reflecting Schiff base deprotonation in Meta II. The band at 1205 cm⁻¹ (E), a marker band of both the protonated Schiff base in Meta I and of the 9-cis-retinal in the isorhodopsin ground state shows a biphasic behavior: a fast increase in absorption (t₁/₂ < 5 ms) due to the formation of isorhodopsin and Meta I, followed by a slower decrease (t₁/₂ ~ 90 ms) representing formation of Meta II from Meta I. The negative band at 1238 cm⁻¹ (F, green line) in the chromophore fingerprint region is typical for the light-induced 11-cis to all-trans isomerization, a process already completed in the early photointermediates. Therefore, this event is too fast to be resolved with the FTIR rapid-scan technique.

Deactivation—We return to Fig. 2, to see FTIR difference spectra of light-induced deactivation. In Fig. 2C, at pH 6 and 10 °C, 25 ms after a blue flash, a difference spectrum is observed with a characteristic, intense difference band at 1556 cm⁻¹ (red line in Fig. 2, C and D). However, most other spectral regions display only minor difference bands. Subsequently, difference bands typical for Meta III, including the Meta III marker band at 1348 cm⁻¹, arise within 8 s (Fig. 2D, black line).

The blue lines in Fig. 3 show the kinetics of selected difference bands characteristic for the light-induced deactivation. Interestingly, the kinetics is different from the activating pathway. Bands assigned to deactivating changes of hydrogen bonding or carboxylic acids or to changes of the secondary structure of the receptor occur on a much slower time scale than observed during the activation. Generally, most of the bands representing the deactivation process show a biphasic behavior with an additional fast component (t₁/₂ < 15 ms), which cannot be resolved with the available setup.

For deactivation, the half time of the band at 1748 cm⁻¹ (A), showing changes of the carboxylic acid residues Glu¹²² and Asp⁸³ is t₁/₂ = 1700 ms and of the band at 1643 cm⁻¹ (B), assigned to the amide I vibration and thus sensitive to changes of the secondary structure, is t₁/₂ = 1350 ms. These kinetics are not only significantly slower than the kinetics of the reprotonation process of the Schiff base during the deactivation (see blue line in the inset of Fig. 1B and Fig. 3, D and E) but are even slower than the kinetics of the same bands during the activation (green lines in Fig. 3, A and B).

For comparison, the time constants for the 1748 cm⁻¹ band during activation is 150 ms (1700-ms deactivation) and 80 ms (1350-ms deactivation) for the 1643 cm⁻¹ band (secondary structure).

The intense band at 1556 cm⁻¹ (C), arising within a few milliseconds after the blue flash, indicates the formation of a species, which we term RR-Meta. The difference spectrum of this intermediate is identical to the spectrum of the previously observed species, which forms at acidic pH (32, 50). This band is dominated by the fast strong component (t₁/₂ < 15 ms). The difference band at 1713 cm⁻¹, indicative for the blue light-induced deprotonation of the counterion Glu¹¹³ during the deactivation process, shows fast kinetics and appears within the first 15 ms after the flash (D).

Similar kinetics was also observed at 1205 cm⁻¹ (E), a position assigned to the reprotonation of the Schiff base. The fast kinetics of the bands at 1713 cm⁻¹ and 1205 cm⁻¹ indicate that the reprotonation of the counterion and the reprotonation of the Schiff base occur at once and in a common conversion early in the deactivation process. These data are in agreement with the time-resolved UV-visible spectra, which also have shown that the reprotonation of the Schiff base occurs on a fast time scale.

The kinetics of the band at 1348 cm⁻¹ assigned to syn/anti isomerization of the Schiff base (Fig. 3F, blue line) is interesting. In contrast to the marker band for cis/trans isomerization at 1238 cm⁻¹, this band shows an additional slow component with a half time of ~1900 ms. Half times of selected bands are summarized in Table 1. Standard deviations are given in parentheses.
**FTIR and UV-visible Spectroscopy of Samples at Low pH and in the Presence of Peptide**

Blue light illumination of Meta II at low pH values predominantly leads to an active photoproduct (20) with an absorption maximum of 462 nm, different from Meta III ($\lambda_{max} = 475$ nm) and with structural similarities to Meta II as indicated by FTIR difference spectroscopy. Additionally, the FTIR difference spectrum of this species shows striking similarities to the FTIR difference spectrum of RR-Meta obtained milliseconds after a blue flash was applied to Meta II. Therefore, it was assumed that RR-Meta is active toward the G-protein (32). In Fig. 4A we compare the UV-visible and FTIR difference spectra of blue light photoproducts of Meta II, stabilized at different conditions, with the spectrum of the early blue light photoprodct of Meta II, RR-Meta, obtained by time-resolved spectroscopy. Fig. 4A, trace a, shows the photoproduct minus Meta II FTIR difference spectrum, obtained by 5-s blue illumination of Meta II at pH 4.5, measured 8 s after illumination. The spectrum is dominated by an intense positive band at 1556 cm$^{-1}$. Bands in other spectral regions, i.e. the spectral range characteristic for protonation changes of carboxylic acids (1800–1700 cm$^{-1}$) or the chromophore fingerprint region (1350–1050 cm$^{-1}$), are only observed to a limited extend. This indicates a Meta II-like conformation of the respective groups in this photoprodct. The light gray line in Fig. 4A, trace a, represents the products finally observed after 4-min decay time. The half time of this process is $\sim$ 3 min at 20 °C, similar to the half time of the Meta II decay under comparable conditions. The spectral changes demonstrate that the photoprodct undergoes deactivating conformational changes at this pH value. Presumably opsin and all-trans-retinal are the main decay products instead of Meta III, because no band at 1348 cm$^{-1}$ appears and, apart from a small fraction (10%) of isorhodopsin and rhodopsin, no major light-sensitive components are observed when a second green illumination is applied after this decay time. However, when the pH is adjusted to more alkaline values (pH > 5) immediately after the initial blue illumination of Meta II at pH 4.5, it is converted to Meta III, arguing for an equilibrium between the active photoprodct and Meta III. This was probed by applying green illumination after the change of the pH value (data not shown).

**TABLE 1**

| Band position | Feature | $t_{1/2}$ (ms) |
|---------------|---------|----------------|
| 380 nm        | Deprotonation Schiff base | 70 (2)* |
| 1748 cm$^{-1}$ | Hydrogen bonding Asp$^{153}$/Glu$^{122}$ | 150 (20) |
| 1713 cm$^{-1}$ | Protonation Glu$^{113}$ | 100 (20) |
| 1643 cm$^{-1}$ | Amide I | 80 (5) |
| 1556 cm$^{-1}$ | Amide II, C=C stretch | 70 (5) |
| 1205 cm$^{-1}$ | Deprotonation Schiff base | 90 (20) |

*Standard deviations are given in parentheses.

**FIGURE 4. Photoprodcts of blue light illumination of Meta II.** A, for identification and characterization of the fast blue light photoprodct RR-Meta in the time-resolved measurements we compared the difference spectrum of this photoprodct obtained by time-resolved spectroscopy with the FTIR photoprodct minus Meta II difference spectra obtained under different conditions. Trace a, black: difference spectrum of the photoprodct (stabilized at pH 4.5), measured within 20 s after the flash; gray: difference spectrum of the decay product (10 min decay time, 20 °C). Trace b, black: photoprodct of Meta II minus Meta II difference spectrum obtained by time-resolved FTIR difference spectroscopy 25 ms after the flash; gray: photoprodct of Meta II minus Meta II difference spectrum obtained 8 s after the blue flash. Trace c, black: photoprodct of Meta II minus Meta II difference spectrum, recorded at pH 6, 10 °C in the presence of a C-terminal-derived peptide of the transducin $\alpha$-subunit, 25 ms after the blue flash. Gray: photoprodct of Meta II minus Meta II difference spectrum, obtained in presence of a C-terminal peptide, 8 s after the blue flash. Inset: black, UV-visible spectrum obtained by blue light illumination of Meta II at pH 4.5; gray, UV-visible spectrum of Meta III, obtained by blue light illumination of Meta II at pH 6. B, decay of RR-Meta in the presence of 10 mM peptide (pH 8.0, 20 °C). A rhodopsin sample was first illuminated with green light and subsequently with blue light to obtain RR-Meta. UV-visible difference spectra (decay product minus RR-Meta) obtained every 12 s after the blue illumination are shown. Inset, time course of the 370 nm and 462 nm absorbance change induced by blue illumination of Meta II in the presence of the peptide (black) and time course of the 1644 cm$^{-1}$ band of the corresponding FTIR difference spectra (gray). A first order exponential fit to the data yields a half time of $t_{1/2}$ $\approx$ 30 s for all datasets.

For comparison, Fig. 4A, trace b, shows an FTIR difference spectrum RR-Meta minus Meta II, measured within 25 ms after the blue light flash was applied to Meta II (black line). The spectrum exhibits the same band positions and intensity distri-
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...as observed in the spectrum measured at low pH shown in Fig. 4A, trace a, in particular the large positive difference band at 1556 cm\(^{-1}\). This demonstrates that the blue light photoproduct formed at low pH is RR-Meta or an intermediate with strong similarity. At higher pH values, RR-Meta evolves within seconds to Meta III as shown in Fig. 4A, trace b (light gray line). This spectrum was obtained 8 s after the blue flash. The inset in Fig. 4A represents the UV-visible spectra of Meta III (gray, \(\lambda_{\text{max}} = 475\) nm) and RR-Meta (black, \(\lambda_{\text{max}} = 462\) nm) obtained by blue light illumination of Meta II at pH 6.0 and 4.5, respectively.

To prove whether RR-Meta is active, illumination of Meta II was performed in the presence of a G-protein-derived peptide. Fig. 4A, trace c, shows the photoproduct minus Meta II FTIR difference spectrum of a sample illuminated under the same conditions as described for Fig. 4A, trace b, but in the presence of high concentration (10 mM) of G-protein-derived high affinity peptide (GtGto-(340–350)) (46, 51, 52), which is known to bind active Meta II. In the presence of the peptide, the primary photoproduct that could be detected after 25 ms was RR-Meta as indicated by its typical difference spectrum similar to the spectra shown in Fig. 4A, traces a and b. The similarity of these spectra implies that there is no detectable effect of the peptide on the formation of this product. Consequently, RR-Meta binds the peptide.

The light gray line shows a difference spectrum recorded 8 s after the first one. No spectral changes can be observed, suggesting that the peptide stabilizes the early RR-Meta intermediate on this time scale and against its decay into Meta III.

Fig. 4B shows the UV-visible difference spectra of the RR-Meta-peptide complex recorded over minutes. Spectra were taken every 12 s. The 462 nm absorbance characteristic for RR-Meta decreased, while the absorbance at 370 nm increased correspondingly. The kinetics of these processes is given in the inset in Fig. 4B.

Additionally the time course of the 1644 cm\(^{-1}\) FTIR difference band, indicative for deactivating structural changes, is shown. Note that these three processes exhibit exactly the same kinetics (\(t_{1/2} = 30\) s) and are even faster than the kinetics of the decay of Meta II under the same conditions (\(t_{1/2} = 400\) s) (37).

**DISCUSSION**

Different Light-induced Pathways of Rhodopsin for Activation and Deactivation—The classic activation pathway of rhodopsin starts with cis/trans isomerization of the retinal. Twists in the retinal polyene chain, reorientation of the \(\beta\)-ionone ring in the early intermediates, and a rearrangement in the complex counterion of the protonated retinylidene Schiff base made up by conserved Glu\(^{183}\) and Glu\(^{113}\) residues (16, 53) enable the net transfer of the Schiff base proton to the counterion.

This proton movement during the transition from Meta I to Meta II, is the first in the coupled metarhodopsin equilibria linked to proton transfer reactions. These equilibria depend on protein–protein interaction, e.g., with the G-protein transducin (46, 51).

Under cellular conditions and by these interactions the active form of Meta II, Meta II\(_{ap}\), is stabilized. A final proton uptake from solution and formation of Meta II\(_{ap}\), mandatory for the receptor to catalyze nucleotide exchange in the G-protein, occurs, although under physiological conditions the pH of the bulk phase is higher than the intrinsic pK of the opsin apoprotein (22) (forced protonation (24)). Deactivation by light is observed when a light quantum is absorbed in the active Meta II photoproduct (9, 20). Previous work has already shown that Meta II is thereby indeed deactivated, the Schiff base is reprototated, and a proton is released (20). However, although activating reactions are apparently reversed, infrared spectroscopy and retinal extraction experiments have later revealed that only a small percentage of the Meta II that absorbed blue light ends up in 9- or 11-cis re-isomerized rhodopsin (32). Instead, Meta III is formed, in which the chromophore is still in an all-trans configuration. The presence of the all-trans-15-syn configuration is reflected in a number of functional differences between Meta III and the properly regenerated rhodopsin dark state with 11-cis-15-anti-retinal. They include (i) weak interaction of Meta III with the G-protein, (ii) lack of stability of Meta III, and (iii) formation of intermediates other than those observed for the activating pathway from rhodopsin to Meta II. The effect of light is in this case to isomerize the retinylidene Schiff base bond instead of the \(C_{11} = C_{12}\) double bond.

Because Meta II forms only at sufficiently high temperature, temperature-trapped intermediates of the deactivation pathway starting from Meta II cannot be observed with static FTIR spectroscopy. Hence, a model for the light-induced Meta III formation can only be deduced from time-resolved FTIR as presented in this study. The data allow us to identify the new intermediates of this pathway, R-Meta and RR-Meta. A striking result is that significant characteristics of the final product Meta III, namely the protonated Schiff base, already arise immediately after the anti/syn trigger reaction and that the tight coupling between Schiff base deprotonation and structural changes is lost.

Early Events in Light-induced Deactivation—The time-resolved FTIR difference spectra shown in Fig. 2 identify intermediates of the receptor during light-induced activation and deactivation. In the activating pathway, the first difference spectrum taken after 25 ms identifies Meta I (Fig. 2, A and B, red line) by its typical infrared difference bands. Subsequently appearing difference bands are characteristic for formation of active Meta II. These marker bands include the positions at 1713 cm\(^{-1}\) indicative for the protonated Schiff base counterion Glu\(^{113}\), the band doublet at 1748/1768 cm\(^{-1}\), caused by changes of the hydrogen-bonded environment of residues Glu\(^{122}\) and Asp\(^{83}\) and the amide I band at 1643 cm\(^{-1}\) due to changes of secondary structure.

During blue light-induced deactivation, the first FTIR difference spectrum observed within 25 ms after the actinic flash shows an intense band at 1556 cm\(^{-1}\) (Fig. 2, C and D, red line), whereas most other bands related to changes of carboxylic acids or secondary structure appear only to a limited extent. Thus the related photoproduct is still structurally similar to Meta II, although the UV-visible absorption maximum is already red-shifted (Fig. 1 and inset of Fig. 4A), indicating a reprototated Schiff base.

It can be concluded that two steps are necessary for the formation of this photoproduct starting from Meta II. The first...
step is the isomerization of the chromophore leading from an all-trans-15-anti to an all-trans-15-syn configuration. The first product, which so far has not directly been observed, is termed R-Meta (for Reverted Meta II). The second step, the reprotonation of the Schiff base, leads to a product, which we term RR-Meta. Although in RR-Meta the all-trans-15-syn-retinylidene Schiff base is already reprotonated, because it is in the final product Meta III, FTIR difference spectra reveal that its structure is still similar to Meta II. The RR-Meta marker band at 1556 cm\(^{-1}\) in the region of the \(C=\)C stretching vibrations of the retinal undergoes a fast change during deactivation with kinetics comparable to the Schiff base reprotonation observed by UV-visible spectroscopy. The same applies for the bands at 1205 cm\(^{-1}\) and 1713 cm\(^{-1}\) reflecting the reprotonation of the Schiff base and deprotonation of its counterion. From these data we conclude that the salt bridge between the Schiff base and the counterion, most likely Glu\(^{113}\), is restored within 5 ms.

The further fate of RR-Meta can then be followed by time-resolved FTIR difference spectroscopy. The subsequent spectra show the characteristics of Meta III formation, demonstrating that Meta III arises from RR-Meta.

The Deactivation Step Proceeds on a Slow Time Scale—The RR-Meta minus Meta II FTIR difference spectrum recorded within 25 ms after blue light illumination of Meta II shows that this product is in a conformation similar to the active state, although the chromophore is in the all-trans-15-syn configuration as in Meta III, arguing for an activity of RR-Meta toward the G-protein.

To check whether RR-Meta is really an active state, Meta II was illuminated in the presence of a G-protein-derived high affinity peptide (G\(\alpha\)-(340–350)) under conditions favoring the formation of less active Meta III. The FTIR difference spectra of samples with and without peptide were compared.

In the absence of the peptide, RR-Meta is converted to Meta III within seconds. Most of the Meta III difference bands arise with a half time between 700 and 1000 ms. After more than 8 s the transition to Meta III is completed (see Fig. 2C and Fig. 4A, trace b), and the spectra show no further changes. In the presence of the peptide, RR-Meta is still readily formed but remains now stable on this time scale without any decay into Meta III (Fig. 4A, trace c). This shows that in contrast to Meta III RR-Meta binds the peptide with high affinity, in agreement with the structural similarity to Meta II stated above. However, the stabilizing effect of the peptide does not persist and the RR-Meta-peptide complex decays with a half time of \(\sim 30\) s. As the UV-visible and FTIR data in Fig. 4B show, the peptide-induced deactivation of RR-Meta involves hydrolysis of the Schiff base and retinal release and not the formation of Meta III, as observed without the peptide. Interestingly, the decay process through RR-Meta is even faster than the normal Meta II decay.

The final deactivating structural changes during the Meta II/Meta III transition are much slower than Schiff base reprotonation and even slower than corresponding structural changes in the activating pathway where all FTIR difference bands appear on a time scale comparable to Schiff base deprotonation (see Table 1). When Meta II is illuminated at low pH (Fig. 4A, trace a), the RR-Meta formed is stable for minutes, as indicated by the difference band at 1556 cm\(^{-1}\), and almost no Meta III is formed. However, Meta III forms when the pH is adjusted to neutral or alkaline values, indicating a pH-dependent equilibrium between RR-Meta and Meta III, comparable to that between the species Meta II\(_a\) and Meta II\(_b\), of the activating pathway. This suggests that a proton release is a further prerequisite for the formation of the Meta III state and that a given receptor protein is active as long as the proton release group or network is protonated. The RR-Meta intermediate of the deactivating pathway is insofar comparable to the Meta II\(_b\)-state of the activating pathway, which can only form the active state Meta II\(_a\) when a proton uptake by the protein occurs. Meta II\(_a\) and Meta II\(_b\) are much harder to separate and best seen in solubilized preparations of rhodopsin (18). The observation that the pH- and/or peptide-dependent stabilized receptor can proceed up to the product RR-Meta argues for a start of the light-induced pathway investigated here from active, peptide binding Meta II\(_b\) (Fig. 4A, trace c). Otherwise the deactivation process would require a complex pathway involving a transiently deprotonated intermediate after syn/anti isomerization of the retinal.

No Forced Proton Release in the Deactivating Pathway—Although light-induced deactivation is similar to activation in the sequence of proton transfer reactions, there are fundamental mechanistic differences. After all-trans-15-anti to all-trans-15-syn isomerization of the Schiff base and formation of the R-Meta intermediate, reprotonation of the Schiff base occurs very rapidly. The arising intermediate RR-Meta is active and structurally similar to Meta II, so that one is led to conclude that Schiff base reprotonation is not efficiently linked to and/or does not require major structural changes of the receptor. Proton transfer to the Schiff base occurs on a virtually constant structural background, and the C=N double bond isomerization is the main prerequisite to cause proton transfer. This may explain why Schiff base reprotonation is so fast compared with Schiff base deprotonation during activation, in which structural changes of the apoprotein are necessarily linked to the proton transfer reaction. Only proton release from the apoprotein to the bulk phase enables further major conformational changes eventually leading to Meta III.

Properties of this final product reflect the lack of coupling to structural changes. In contrast to activation, where an active species is formed even when the pH of the bulk phase is much higher than the intrinsic pK of the opsin apoprotein (pK 7.5 versus 4.1, respectively (10, 22, 49)), the pK at which RR-Meta remains in a pH-dependent equilibrium with Meta III is nearly the same as the pK for the opsin apoprotein. Consistently, Meta III has, at neutral pH, an activity similar to a relaxed opsin apoprotein that is not subject to the restrictions from a bound chromophore. The activity of Meta III toward the G-protein is, at neutral pH, orders of magnitude lower compared with Meta II but much higher than dark rhodopsin. So the only effect of anti/syn isomerization is to remove the constraint that was imposed by the extended trans configuration. Deactivation to the exceedingly low activity of the rhodopsin dark ground state would require a (virtual) pK \(\ll 1\) for the relevant protonable group or cluster. Only the 11-cis configuration of the retinal is able to impose the necessary constraints. The individual steps of light-induced activating and deactivating pathways are illus-
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trated in Scheme 1 and opposed to each other in Table 2 for better comparison.

Physiological Role of the Photointermediates—This work was undertaken to identify the photointermediates involved in blue light-induced deactivation and to gain information about its kinetics. One important finding is that under special conditions (low pH) the RR-Meta intermediate arising from Meta II after light absorption can exist for minutes before further decay. Although the low pH under which this happened in vitro, does likely not occur in the functioning photoreceptor cell, such a product opens new possibilities for regulation, as will be discussed below.

First, it can be anticipated that the residual activity of Meta III should lead to an increased noise level of the visual system (34). Under conditions of extended illumination and depending on the spectral composition of the incident light, a certain amount of Meta III accumulates (up to 80% under the artificial conditions of continuous blue illumination). To avoid a high noise level, this species needs to be removed when illumination conditions have changed from bright to dim light.

However, Meta III is comparatively stable, and its decay would take several hours. For fast depletion of Meta III, interaction with transducin is required, which first would need to be translocated to the outer segment (54).

In contrast to Meta III, RR-Meta decays on a faster time scale with kinetics comparable to the Meta II decay, due to the structural similarity of both species as shown in the results section. In other words, the equilibrium between Meta III and RR-Meta provides an alternative possibility for Meta III depletion, because Meta III can also disappear quickly through RR-Meta, enabling regeneration and fast recovery of the low noise dark state even in the absence of transducin.

Furthermore, a decay pathway via RR-Meta would be especially effective in the presence of transducin, as it is the case in the dark, because the high affinity of RR-Meta to the peptide shifts the equilibrium between the RR-Meta and Meta III toward faster decaying RR-Meta. Binding of transducin even accelerates the decay of RR-Meta compared with the normal Meta II decay. This suggests a role of RR-Meta in noise reduction and dark adaptation.

The isolated Meta II/Meta III system keeps the retinal inside the apoprotein and prevents its transfer into the regeneration cycle, constituting a “retinal storage” under conditions of bright light. However, arrestin can release the retinal by converting Meta III (formed either by thermal decay or blue light irradiation) to a Meta II-like species (39, 55). Also the signal transmitter, transducin itself (34), can regulate the amount of Meta III and its precursor RR-Meta. Not only does the effect of transducin depend on the cytoplasmic levels of GDP and GTP, but both arrestin and transducin undergo light-dependent transport to and from the rod outer segment (54, 56, 57). These metabolic processes may serve to regulate the system to achieve an optimal balance between a low noise level and the storage function.

TABLE 2
Comparison of light-induced activation and deactivation

| Initial trigger          | Retinal cis/trans isomerization      | Activation |
|-------------------------|--------------------------------------|------------|
| Chain of crucial events | Schiff base syn/anti isomerization   |            |
| Structural changes of apoprotein leading to Schiff base proton transfer | Only light-induced isomerization |            |
| Activity of initial state | Isomerization, Schiff base proton transfer, proton transfer between |            |
| Activity of final state  | apoprotein and bulk phase            |            |
| Role of the retinal in initial state | Relatively high |            |
| Role of the retinal in final state | Extremely low |            |
| Trigger induces shift of pK compared to opsin apoprotein | Extremely high |            |
| Scaffold function of the retinal | Strong inverse agonist |            |
| Stability of the inactive form | Strong agonist |            |
| Kinetics of Schiff base proton transfer (10 °C) | Partial agonist |            |
| Majority of conformational changes of the protein (10 °C) | No (marginal) |            |

| Deactivation              | Only light-induced or thermal     |            |
|---------------------------|----------------------------------|------------|
| In initial and final state | Relatively low                   |            |
| Stable                    | Extremely high                   |            |
| t_{1/2} = 70 ms           | Low                              |            |
| t_{1/2} = 70–100 ms       | Strong agonist                   |            |
| t_{1/2} = 1300–1900 ms    | Partial agonist                  |            |

SCHEME 1. Kinetic skeleton of light-induced activation and deactivation. Activation from the dark state (top) starts with cis/trans isomerization leading to Meta I, which is formed within microseconds. From Meta I, proton transfer from the Schiff base to its counterion Glu113 and Glu181 (yellow) leads to still inactive Meta II, and a further proton uptake to active Meta IIb, mediated by an H3/H6 network, including Glu134 (black). These two steps are directly linked to changes of protein structure, with the largest conformational changes in Meta IIb as shown by intense bands in the FTIR difference spectra reflecting vibrations of amide and carboxylic acids (see text for details). The deactivation pathway observed here starts from Meta IIb. The constraints for the chromophore are such that syn/anti isomerization of the retinal Schiff base C=\( N \) double bond is forced to occur, leading to the first hypothetical intermediate R-Meta, which probably still absorbs around 380 nm. RR-Meta is then formed by reprotonation of the retinal Schiff base, without major structural changes and without a change of activity toward the G-protein so that this process can occur on a millisecond time scale. Only with the formation of Meta III, most structural changes and proton transfer processes have been reverted in a way that an inactivated species is formed.
Further insight into the physiological implications of the reaction pathways will depend on a further development of the technique that includes the effect of signaling or regulatory proteins, i.e. arrestin and transducin, in depth. We have seen above that a peptide from the G-protein transducin not only can be used to identify and stabilize the active, G-protein binding state RR-Meta, but it can also influence the decay of RR-Meta. Naturally, all these investigations will involve intense illumination, because, only under this condition, do Meta II and its tautomeric “reverted” forms (R-Meta and RR-Meta) accumulate to a substantial amount. Under conditions of steady bright illumination, the actual level of Meta III will be adjusted according to the photoequilibria between the species involved and to the spectral overlap between these species and incident light.

Concluding Remarks and Perspectives—Under the structural constraints of the active Meta II state, light absorption does not trigger trans/cis re-isomerization but rather anti/syn Schiff base isomerization around the retinylidene linkage to the apoprotein (33, 35). This type of light-induced conversion of the chromophore deactivates the protein but only partially. The salient result of the study is that the energy of the absorbed light deflates in early Schiff base reprotonation, without an efficient link to structural changes, and is not used to deactivate the protein.

In mechanistic terms, two immediate questions arise, namely (i) what factors are responsible for the channeling of the chromophore into the anti/syn pathway, and (ii) why does anti/syn conversion not revert the full set of deactivating switches? Although the two questions are presumably related, we can, at this point, only narrow down the problem. What we can say is that the Glu\(^{113}\)/Glu\(^{181}\) readjustment alone cannot be the factor that determines the mode of photochemical conversion. It is complete in Meta I, which however does go back to rhodopsin on illumination (33). We can also exclude that the deprotoinated Schiff base as such is responsible, because early photointermediates of Meta III can be isolated at low temperature, which do contain a protonated Schiff base but still perform syn/anti and only a minor cis/trans isomerization. Other determinants of the Meta II state are therefore likely involved, including known differences between Meta II and the ground state, such as the proton uptake into the Glu\(^{34}\)/Glu\(^{54}\) cluster and/or the exposure of a binding site near the rectangular kink between Helix seven and eight (58). This would be consistent with the available evidence that it takes Meta II\(_1\), i.e. the species of Meta II, in which a proton has been taken up from solution, to go over into the anti/syn conversion. If these activating switches were the elements that decide between trans/cis and anti/syn conversion, it would mean that at least one of these (presumably distant) regions that are involved in Meta II\(_1\) but not in Meta II\(_2\) retroacts on the chromophore binding site.

Finally, we come back to the physiological significance of our findings. We can generally state that, as long as the concentration of blue light-sensitive Meta II is high, the prevailing trend will be to form more Meta III. In our current understanding, this is the condition in which the rod cell does not function as a photoreceptor and just waits for dim light conditions to return. Meta III is then assigned the role of a “storage” form (37).

However, Meta III is light-sensitive itself and will be reconverted to Meta II upon light absorption (9, 32). The resulting photoconvertible Meta II/Meta III-system bypasses the 11-cis-retinal bound dark state. This bistable system may function as a retinal storage in bright light, extending the storage concept for Meta III. In addition, the equilibrium between Meta III and the fast decaying RR-Meta provides a new possibility for recovering the low noise dark state.

Finally, one may envisage a rudimentary photoreceptor function of Meta III in the Meta II/Meta III system. It switches between the fully active Meta II and the less active Meta III forms of the receptor, insofar comparable to the conversion between Meta II and the rhodopsin dark state. The essential difference is that light-induced Meta II/Meta III conversions shortcut the complex metabolic retinoid cycle. It remains to be elucidated whether such a mechanism contributes to vision under bright light conditions. Here it is important to note that the perception of “red” and “green” by dichromats, with only two types of cone photoreceptors, may involve a contribution of rod signaling\(^4\) (see also Refs. 59 and 60).

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