Rhodopsin bears 11-cis-retinal covalently bound by a protonated Schiff base linkage. 11-cis/all-trans isomerization, induced by absorption of green light, leads to active metarhodopsin II, in which the Schiff base is intact but deprotonated. The subsequent metabolic retinoid cycle starts with Schiff base hydrolysis and release of photolyzed all-trans-retinal from the active site and ends with the uptake of fresh 11-cis-retinal. To probe chromophore-protein interaction in the active state, we have studied the effects of blue light absorption on metarhodopsin II using infrared and time-resolved UV-visible spectroscopy. A light-induced shortcut of the retinoid cycle, as it occurs in other retinal proteins, is not observed. The predominantly formed illumination product contains all-trans-retinal, although the spectra reflect Schiff base reprotonation and protein deactivation. By its kinetics of formation and decay, its low temperature photointermediates, and its interaction with transducin, this illumination product is identified as metarhodopsin III. This species is known to bind all-trans-retinal via a reprotonated Schiff base and forms normally in parallel to retinal release. We find that its generation by light absorption is only achieved when starting from active metarhodopsin II and is not found with any of its precursors, including metarhodopsin I. Based on the finding of others that metarhodopsin III binds retinal in all-trans-C_{15}-syn configuration, we can now conclude that light-induced formation of metarhodopsin III operates by Schiff base isomerization (“second switch”). Our reaction model assumes steric hindrance of the retinal polyene chain in the active conformation, thus preventing central double bond isomerization.

Living cells react to stimuli, which are realized in physical or chemical signals and are often detected by specialized membrane receptor proteins. G-protein-coupled receptors (GPCRs) transmit their signal to heterotrimeric G-proteins via cytoplasmic domains of their seven-transmembrane α-helical structure. The majority of signals are chemical ligands, such as hormones or pheromones, which reach their GPCR by diffusion and bind to a site near their extracellular surface (1, 2). Photoreceptors contain a chromophore as a fixed prosthetic group and are specialized for the detection of quanta of visible light in the environment (3, 4). The first step in the signal transduction pathway mediated by these receptors is the generation of a short-lived electronically excited state caused by photon absorption to channel the energy into a conformationally and/or chemically altered state of chromophore-protein interaction (5). Although these early events of light absorption are lacking in ligand binding receptors, G-protein-coupled photoreceptors may use similar mechanisms to spread the local activation near the ligand binding site to the cytoplasmic binding sites, where the G-protein has access. In this view, the chromophore can be understood as a fixed ligand that becomes an agonist by light absorption. Because the isomerized chromophore is still light-sensitive, although at a wavelength different from the ground state, chromophore-protein interaction in the active state can be tested by light. Rhodopsin (Rh), the visual pigment in the disc membranes of retinal rods, consists of the apoprotein (termed opsin) and the visual chromophore, 11-cis-retinal, bound by a protonated Schiff base to Lys^{296} in the center of the seventh transmembrane helix (6). X-ray analysis shows the retinal in a specialized pocket with strong hydrophobic interaction near the β-ionone ring and a stabilizing salt bridge between the protonated nitrogen of the Schiff base and its counterion, provided by the negatively charged carboxylic group on Glu^{113} in the third helix (7). In this environment, electronic relaxation on the excited-state potential surface allows fast initial motions of the retinal chromophore and leads the retinal to a region in the configurational space, where the transition to the all-trans-retinal product state can take place (8). In the subsequent pathway, each of the intermediates (blue-shifted intermediate, bathorhodopsin (Bath), lumirhodopsin, metarhodopsin (Meta) I, and Meta II) is characterized by its own UV-visible absorption spectrum. The conversion into the activating conformation is marked by the near-UV absorbing Meta II intermediate, in which the Schiff base is still intact but deprotonated (9).

Meta II is stabilized by either low pH or high temperature and remains in equilibrium with its Meta I predecessor (10). The large shift of the absorption maximum from 480 nm in Meta I to 380 nm in Meta II is due to deprotonation of the Schiff base and coupled to protonation of the counterion at Glu^{113}. The resulting neutralization of Glu^{113} opens the stabilizing salt

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bridge, a necessary condition for forming the signaling state for the G-protein (see Ref. 11). A second necessary step is proton uptake of Glu\textsuperscript{134}, a conserved residue in the (D/E)RY motif found in many GPCRs, which is linked to a breakage of the hydrogen bonded network around this motif. This structure and the NPXXY(\textit{X} = \textit{F}) stretch that links helices VII and VIII and (D/E)RY exert a dual control, essential for reliable transformation of quiescent rhodopsin to active Meta II (12). In turn, chromophore-protein interaction in Meta II is expected to depend on the activation state of these structures, although the mechanism of coupling is not yet clear.

Understanding the active state and how it can form requires structural information on the Meta II intermediate and a comparison with the dark ground state. One way to approach the problem employs analogs of the native retinal. The chromophore pocket recombines strikingly well with analogs of the native 11-cis-retinal, such as 9-cis-retinal, 10-methyl retinal, or 9-demethyl retinal, and even with “locked” retinals, which have a ring-like bridge between C\textsubscript{10} and C\textsubscript{14} of two to four carbons. The main effect of the modifications in these analogs occurs in the active state and has led to the concept of the modified retinal being “partial agonists,” in analogy to the term used for diffusible ligands (4, 13–15). These studies have already suggested that the retinal in the active state may build up new interactions that are absent in the dark ground state. Interactions may now be stronger near the center of the retinal polypeptide, where the modifications are placed to which Meta II is sensitive. The present investigation addresses this problem using a quite different approach. Rather than employing synthetic constraints on the retinal moiety, it uses the native chromophore and studies the constraints exerted by the protein itself. The actinic light is applied in the active state. This enables us to measure the constraints that come into play when the active state is left, rather than when it is formed. These new constraints may then be compared with those seen in the ground state, where the structural limitations are known.

The basic technique of probing the active site by blue light (λ < 420 nm) illumination of the Meta II state dates back to the 1960s (9, 16). Later, when Rh was recognized as a GPCR, it was used to study the influence of G-protein on the blue light-induced reaction (17). The general observation in these studies is that after illumination, Meta II goes through a shift of the absorption maximum, indicating reprotonation of the retinal Schiff base and proton release, i.e. just the inverse of the proton transfer reactions observed in the forward pathway. Because the absorption change was partially inhibited by G-protein (17) and C-terminal peptides (18), it was assumed that up to 50% of the inactive product (P subspecies with λ\textsubscript{max} = 500 nm (P\textsubscript{500}: P is used henceforth to represent the blue light illumination product of Meta II) was formed that does not interact with the G-protein. It was then generally assumed that blue light absorption can revert the forward pathway (see Refs. 17 and 18), as it does in bacteriorhodopsin or halorhodopsin. However, a more recent analysis based on Fourier transform infrared (FTIR) spectroscopy and retinal extraction has shown that this does not apply to vertebrate rhodopsin. The spectral features in the chromophore “fingerprint” region indicating reversal of the changes in retinal geometry and retinal-protein interaction were lacking in the difference spectra. The data showed that most of the retinal was still in the all-trans-retinal configuration, as it was in the Meta II state, from which the reaction had started. The spectral features that reflect the activating changes in the protein backbone were, however, fully reversible (19). The data allowed the conclusion that (i) the conditions imposed on the retinal in the active state are such that isomerization around a central double bond does not occur to a significant degree and (ii) rhodopsin can be switched between an active and an inactive state without such isomerization.

In this study and the accompanying article (44), we wanted to further explore the nature of the alterations in chromophore-protein interaction behind the “second switch” and the properties of the intermediate formed, respectively. We had previously noted that the product formed by blue light illumination of Meta II is similar to that arising from its thermally activated decay. This is the metarhodopsin III (Meta III) intermediate, a “retinal storage” product that forms in parallel to the pathway through Schiff base hydrolysis and release of the photolyzed retinal from the active site. In Meta III, retinal remains covalently bound, still in the trans configuration, but with the retinylidene linkage reprotontated (19–21). Vogel et al. (22) have recently shown that Meta III harbors the retinal in an all-trans-5-syn-retinal configuration, in which the Schiff base bond and not the central C\textsubscript{11} = C\textsubscript{12} double bond is isomerized. This gives us the chance to identify the Meta III arising from blue light illumination of Meta II and to come closer to an understanding of this product, which is of interest both for its interesting mechanism of formation and for its potentially important role in the regeneration of a light-sensitive photoreceptor.

**EXPERIMENTAL PROCEDURES**

**Preparation of Samples—**Rod outer segments were prepared from fresh, dark-adapted retinas of cattle eyes (23). Rhodopsin disc membranes were subsequently purified by repetitive washes with a low ionic strength buffer (24). Opsin membranes were prepared from rhodopsin membranes by bleaching, extraction of retinals with hydroxylamine, and repetitive washes with fatty acid-free bovine serum albumin. All membrane suspensions were stored at −80 °C. Rhodopsin with isotopically labeled retinals was prepared by reconstitution of opsin with a 3-fold excess of the labeled retinal at 8 °C for 10 h. Excess retinal was removed by repetitive washes with 2% fatty acid-free bovine serum albumin. Isotopically labeled retinals were a generous gift from Mordechai Sheves. BTP was used as buffer. pH was adjusted with diluted NaOH or HCl. Deuterated samples were prepared by repetitive washes of the rhodopsin membranes with BTP buffer prepared in D\textsubscript{2}O.

pD in deuterated samples was adjusted with diluted NaOD or DCl. All steps were performed under dim red light (λ > 640 nm).

**FTIR Measurements—**The sample (200 μM rhodopsin membrane suspension) was centrifuged for 30 min at 100,000 × g and 4 °C to obtain a 2.4 mm rhodopsin pellet (25). The supernatant was removed, and the pellet was transferred to a temperature-controlled (±0.5 °C) transmission cuvette with two BaF\textsubscript{2} windows and a 3-μm polytetrafluorethylene spacer. For infrared spectroscopy, we used a Bruker 166 spectrometer equipped with a liquid nitrogen-cooled HgCdTe detector (J5D-series; EG\&G Judson). Before the measurement, the sample was equilibrated for at least 30 min in the spectrometer. Illumination was performed with a 150-watt fiberoptic light source (Spindler & Hoyer) equipped with a heat reduction filter (Schott KG2).

Additionally, the following filters were used: a 495 nm long pass filter (Schott GG495) for illumination of Rh and Meta III at room temperature, a 400 ± 20 nm band pass filter (combination of Schott UG1 and fiber optics) for illumination of Meta II, a 480 ± 2 nm band pass filter for illumination of Meta I, a 470–480 nm band pass filter for illumination of Rh or Meta III at 80 K, a 530 nm long pass filter for illumination of Batho, and a 480 ± 5 nm filter for illumination of Meta III at 173 and 273 K. Illumination times were 4 s for Rh, 40 s for Meta II, and 200 s for Meta III, unless noted otherwise.

Measurements at low temperatures were performed using an Oxford Instruments Optistat DN variable temperature liquid nitrogen cryostat.

Infrared absorbance spectra (256 scans; spectral resolution, 2 cm\textsuperscript{-1}) were recorded before (stage A) and after (stage B) illumination. The difference spectrum was calculated by subtracting the spectrum measured after illumination from the spectrum measured before illumination, unless noted otherwise. Henceforth, it will be termed the “B minus A” difference spectrum for the conversion A→B.

**UV-visible Measurements—**For measurements in the spectral range between 200 and 800 nm, the same sample preparation and centrifugation procedure as described for FTIR were used. Pellets (~2 mm) were transferred to a temperature-controlled transmission cuvette made of two 10-mm-diameter BaF\textsubscript{2} windows with a 50-μm polytetrafluorethylene spacer. An OLIS RSM-16 spectrometer was used for data aquisi-
**RESULTS**

**UV-visible and FTIR Spectroscopy of Meta II and Meta III Illumination Products**

The UV-visible spectra in Fig. 1A were recorded from native disc membranes using the OLIS spectrometer and FTIR sample preparations to make them comparable with the FTIR data (see the text below and “Experimental Procedures”). Dark-adapted Rh exhibits an absorption maximum of 500 nm. Illumination of Rh (pathway a) generates the active Meta II intermediate (380 nm).

When the Meta II sample is subsequently illuminated with blue light (pathway b), the absorption maximum is shifted to the 475 nm region (blue light product P). Even saturating blue light illumination does not recover the 500 nm absorption maximum of Rh. However, when blue light product P is illuminated with green light (>500 nm), a Meta II-like product absorbing at 380 nm is formed (pathway c).

On the basis of the UV-visible data shown in Fig. 1A, the scheme in Fig. 1B was deduced. Pathway a is the normal activating pathway of Rh. Pathway b is the blue light-induced formation of the P product (P-Meta III), which shows the same absorption maximum as the thermal decay product Meta III (T-Meta III). Pathway c illustrates that, as with Meta III (9, 20), illumination of P leads to Meta II (19). The time-resolved difference spectra in Fig. 1C, a (Meta II minus Rh) were taken at pH 5.5 and 10 °C every 20 ms after flash illumination of Rh with green light. In the first trace (20 ms), the pronounced negative band at 520 nm is due to the decrease of the 500 nm absorption of Rh. The 20 nm red shift can be explained by the...
and 293 K. Note that the band at 1348 cm$^{-1}$ already occurs in the low-temperature photoproduct at 80 K. The black lines are the difference spectra of the intermediates of Rh illumination, namely, Batho, lumirhodopsin, Meta I, and Meta II. FTIR samples of Rh, P-Meta III, and T-Meta III were cooled to the respective temperature and illuminated for 60 s, and the difference spectrum was recorded at this temperature.

The difference spectra shown in Fig. 1, reflecting changes in protonation states, hydrogen bonding, the secondary structure of the protein, and also the chromophore fingerprint, including bands at 1348 and 1181 cm$^{-1}$. These fingerprint characteristics are lacking in the Meta II minus Rh difference spectrum of the first illumination and are replaced with features such as the negative band at 1238 cm$^{-1}$ and the spectral pattern around it, reflecting the well-known change in the 11-cis to all-trans geometry of the retinal that triggers activation of Rh from the dark ground state (19). From previous work (16, 17), it is known that the first Schiff base reprotonated products of Meta II illumination include P sub-species with $\lambda_{\text{max}} = 470$ nm (P$\text{}_{470}$) and P$\text{}_{500}$, in which the suffix denotes the absorption maxima of these products in detergent solution. Both in solution and in native membranes, as used in this study, two products are kinetically distinguishable (with formation times of <1 and 20 ms, respectively) and exhibit different sensitivity toward the retinal G-protein (transducin) and its C-terminal peptides (17, 19). However, in native membranes, an apparently uniform product with $\lambda_{\text{max}} = 475$ nm is formed (Fig. 1A), suggesting that the two species are more isochromic under these conditions. We know that formation of the fast product is not linked to a change in activity, despite its UV-visible absorption that indicates Schiff base reprotonation. Consistently, it does not contribute much to the FTIR difference spectrum. We will therefore focus on the product P formed with a change in activity and neglect the fast “hybrid” side product, which remains Meta II-like by its structure but contains a protonated Schiff base. The following experiments were undertaken to explore how the P product, which we will term P-Meta III, relates to the normal Meta III arising from thermal decay of Meta II (T-Meta III; see Fig. 1B).

**Identity of Thermally Generated and Photogenerated Meta III**

Light-induced Pathways—P-Meta III and T-Meta III were illuminated at low temperatures, and the respective intermediates overlap with a positive band at 480 nm caused by the formation of Meta I within microseconds after illumination. The rise of the 380 nm absorption in the subsequent traces reflects an increasing amount of Meta II, which appears within milliseconds in the equilibrium with Meta I. The corresponding decrease of 475 nm absorption and the isosbestic point at 417 nm, characteristic for the Meta I/Meta II equilibrium, are clearly seen. After 140 ms, only a minor amount of Meta I is present in the equilibrium; therefore, the spectrum reflects the transition from Rh to Meta II characterized by the negative band at 500 nm and the positive band at 380 nm, respectively. When the sample is subsequently flashed with blue light, the difference of Meta I within microseconds after illumination. The rise of 475 nm absorption and the isosbestic point at 417 nm, characteristic for the Meta I/Meta II equilibrium, are clearly seen. After 140 ms, only a minor amount of Meta I is present in the equilibrium; therefore, the spectrum reflects the transition from Rh to Meta II characterized by the negative band at 500 nm and the positive band at 380 nm, respectively. When the sample is subsequently flashed with blue light, the difference spectrum of the first illumination and are replaced with features such as the negative band at 1238 cm$^{-1}$ and the spectral pattern around it, reflecting the well-known change in the 11-cis to all-trans geometry of the retinal that triggers activation of Rh from the dark ground state (19). From previous work (16, 17), it is known that the first Schiff base reprotonated products of Meta II illumination include P sub-species with $\lambda_{\text{max}} = 470$ nm (P$\text{}_{470}$) and P$\text{}_{500}$, in which the suffix denotes the absorption maxima of these products in detergent solution. Both in solution and in native membranes, as used in this study, two products are kinetically distinguishable (with formation times of <1 and 20 ms, respectively) and exhibit different sensitivity toward the retinal G-protein (transducin) and its C-terminal peptides (17, 19). However, in native membranes, an apparently uniform product with $\lambda_{\text{max}} = 475$ nm is formed (Fig. 1A), suggesting that the two species are more isochromic under these conditions. We know that formation of the fast product is not linked to a change in activity, despite its UV-visible absorption that indicates Schiff base reprotonation.

Consistently, it does not contribute much to the FTIR difference spectrum. We will therefore focus on the product P formed with a change in activity and neglect the fast “hybrid” side product, which remains Meta II-like by its structure but contains a protonated Schiff base. The following experiments were undertaken to explore how the P product, which we will term P-Meta III, relates to the normal Meta III arising from thermal decay of Meta II (T-Meta III; see Fig. 1B).

**Identity of Thermally Generated and Photogenerated Meta III**

Light-induced Pathways—P-Meta III and T-Meta III were illuminated at low temperatures, and the respective intermediates
were stabilized at 80 K, 173 K, 273 K, and 293 K. The amount of P-Meta III formed is ~70%; the amount of T-Meta III formed is only 30%. The spectra were therefore normalized using the band at 1348 cm⁻¹. In the difference spectra of the intermediates (Fig. 2), blue represents the intermediate minus P-Meta III difference spectrum, and red represents the intermediate minus T-Meta III difference spectrum. P-Meta III and T-Meta III form very similar intermediates. Slight differences, especially in the spectra of the intermediates at 173 K and 273 K, can be attributed in part to different content of residual cis isomers, which are able to regenerate with opsin to form Rh and isorhodopsin (Iso), and to deviations in water content. The spectra are surprisingly similar to the spectra of the corresponding intermediates generated by Rh illumination (Fig. 2, black), termed Batho, lumirhodopsin, Meta I, and Meta II, apart from bands in the region between 1348 and 1206 cm⁻¹, which are related to retinal geometry or retinal protein interaction.

The intermediates at 80 K show positive bands for both pathways at 1688, 1670, and 1653 cm⁻¹ and a negative band at 1662 cm⁻¹. This spectral region is dominated by the amide I bands of the protein. The low intensities of these bands show that, similar to the Batho intermediate, structural changes of the protein only occur to a limited extent. There is an intense negative band at 1559 cm⁻¹ and an intense positive band at 1535 cm⁻¹ characteristic for changes in the C=C stretching mode of the retinal and similar to those observed in the Batho minus Rh intermediate. However, the negative band at 1238 cm⁻¹ and the spectral pattern around it, which are characteristic for these spectra. This pattern is replaced with bands at 1348, 1181, 1191, and at 1198 cm⁻¹. This observation was interpreted as a deactivating movement of the chromophore different from the cis/trans isomerization (19) and has recently been assigned by Vogel et al. (22) to an antisynd isomerization of the Schiff base during the thermal decay of Meta II to Meta III (T-Meta III in our nomenclature). The identical band pattern in this spectral region for all four intermediates (80 K, 173 K, 273 K, and 293 K) suggests that the antisynd isomerization of the Schiff base has already occurred in the 80 K intermediate.

The close spectral similarity between T-Meta III and P-Meta III at all temperatures and in the whole 1800 to 1400 cm⁻¹ range argues again for a close similarity between the pathways starting from T-Meta III or P-Meta III, respectively. Small deviations in the Amid I and Amid II region in the spectra of the intermediates at 173 K and 273 K may come from slightly different water content and/or from the initially different proportion of P-Meta III and T-Meta III.

**Thermal Decay of P-Meta III and T-Meta III**—Fig. 3A shows the FTIR difference spectra of the P-Meta III—Meta II conversion in the region from 1400 to 1100 cm⁻¹, taken immediately after formation of P-Meta III (green). Characteristic features of this spectrum are the negative bands at 1348 and 1181 cm⁻¹. A P-Meta III sample was now allowed to decay for 480 min, and subsequently, the FTIR difference for the light-induced conversion to Meta II was recorded (Fig. 3A, black). In this spectrum, the band at 1348 cm⁻¹ is absent and replaced with bands at 1238 and 1206 cm⁻¹ assigned to the Rh or Iso ground state. The kinetics of the decay for both products, P-Meta III (green) and T-Meta III (black), are shown in Fig. 3B. P-Meta III and T-Meta III were characterized by the intensity of the 1348 cm⁻¹ band in the FTIR difference spectrum and by the 480 nm absorption in the UV-visible spectrum, respectively. The data were fitted to an exponential function yielding a half-time of 1.8 h for P-Meta III and 2.1 h for T-Meta III, respectively.

To explore whether P-Meta III, like T-Meta III, decays thermally into opsin and all-trans-retinal, a protocol was applied, in which the opsin formed is identified through its regeneration with 9-cis-retinal to Iso. In Fig. 3C, Rh was first illuminated in the ground state with green light for 4 s (a, green) in the presence of a 10-fold excess of 9-cis-retinal. The Meta II minus Rh difference spectrum shows the characteristic negative band at 1238 cm⁻¹. After recording the FTIR spectrum (i.e. 60 s after...
the illumination), the same sample was subsequently illuminated with blue light to generate P-Meta III (Fig. 3, a, blue). After a 3-h incubation in the dark, the sample was then illuminated with green light, and the FTIR difference spectrum was recorded (Fig. 3, b, green). The strong negative band at 1206 cm\(^{-1}\), characteristic for 9-cis to all-trans isomerization appears, demonstrating that regeneration with 9-cis-retinal had taken place, rendering possible a second round of Meta II formation (with 9-cis to all-trans isomerization as the activating trigger). This sample was again illuminated with blue light to form P-Meta III (Fig. 3, C, b, blue). As a final control, the protocol was repeated after 4 more minutes; no further formation of Iso could be detected (Fig. 3, c, green and blue).

G-protein-induced Depletion of P-Meta III and T-Meta III—

The data in Fig. 4 demonstrate that both forms of Meta III can be depleted by interaction with transducin. UV-visible difference spectra were taken from P-Meta III (Fig. 4, blue) and T-Meta III (Fig. 4, red) immediately after their formation (0 min) and after 1–30 min of incubation. In both cases, the 480 nm absorption decreases, and products with absorption in the 360–380-nm range are formed, which may comprise Meta II, free all-trans-retinal, and opsin/all-trans-retinal complex (26). The overall process is virtually completed after 30 min. The data extend the similarity between P-Meta III and T-Meta III, it can be concluded that, similar to T-Meta III by Vogel and coworkers (22), the Meta I formed in the dark ground state. The same illumination protocol was now applied to Meta I (Fig. 6, a green). First, Meta I was generated by illumination of Rh at pH 8.2 and 1 °C with >500 nm light. The Meta I minus Rh difference spectrum is shown in Fig. 6, b (green). Characteristic bands for Meta I are observed at 1737, 1726, 1653, 1550, and 1537 cm\(^{-1}\). The chromophore region in Meta I exhibits typical bands at 1238 and 1196 cm\(^{-1}\) reflecting the 11-cis to all-trans isomerization. The Meta I formed in the sample was now illuminated with 480 nm light. In Fig. 6, b have occurred. In Fig. 5B, the dashed line identifies the unlabeled retinal, and the solid line identifies a \(^{13}\)C label in the C\(_{14}\) and C\(_{15}\) position. In this spectrum, the only band that undergoes a significant shift upon labeling is the band at 1181 cm\(^{-1}\). These observations are in agreement with those made for T-Meta III regenerated with \(^{13}\)C\(_{14}\)=\(^{15}\)C\(_{15}\)-labeled retinal. Meta II minus P-Meta III FTIR difference spectra. A, dashed line, sample recorded in H\(_2\)O; solid line, sample recorded in D\(_2\)O. The intensity of the band at 1348 cm\(^{-1}\) is significantly reduced. The band at 1181 cm\(^{-1}\) has vanished, and new bands at 1247 and 1238 cm\(^{-1}\) have occurred. B, dashed line, sample with unlabeled retinal; solid line, sample with \(^{13}\)C\(_{15}\)-labeled retinal. The band at 1181 cm\(^{-1}\) is significantly influenced by the label.

Effect of D\(_2\)O and Reconstitution with \(^{13}\)C\(_{14}\)=\(^{15}\)C\(_{15}\)-labeled Retinals

To identify whether P-Meta III undergoes the same photochemistry on light absorption as T-Meta III, measurements in D\(_2\)O (Fig. 5A) and with \(^{13}\)C isotope-labeled retinals (Fig. 5B) were employed. In the Meta II minus P-Meta III FTIR difference spectra (Fig. 5A), the dashed line represents the spectrum recorded in H\(_2\)O, and the solid line represents the spectrum recorded in D\(_2\)O. The band at 1348 cm\(^{-1}\) has significantly reduced intensity upon deuteration. The band at 1181 cm\(^{-1}\) has vanished, and new bands at 1247 and 1238 cm\(^{-1}\)
**New Pathway from Metarhodopsin II**

**DISCUSSION**

GPCRs bind diffusible ligands or, in the case of photoreceptors, a chromophore to detect chemical or physical signals in the environment. In the active, G-protein binding state, the signal is made available for the cell. Despite the significance of the active state, the interactions of the ligand with the poly peptide chain that maintain activity are not well understood. Even for rhodopsin, for which an x-ray structure of the dark ground state is available (7), the interactions of the retinal chromophore with the protein in the active, G-protein binding Meta II state have not yet been fully elucidated. Known properties of Meta II include that it is formed within milliseconds from its precursor Meta I and that it bears the illuminated all-trans-retinal still bound to a lysine in helix VII, but with the retinylidene (Schiff base) linkage deprotonated. The Schiff base-deprotonated Meta II is distinguished from its precursor in the light-induced pathway by a shift of 100 nm in the absorption maximum to 380 nm. It was recognized early that this opens a simple possibility to probe the native Meta II conformation by illumination with blue light (see “Introduction” for details).

From the present data, it is obvious that the constraints to the chromophore in the active Meta II state must be different from those prevailing in the ground state because most of the illuminated protein does not find a pathway back to the dark ground state. As judged from the spectroscopic information reported, the blue light product is in a deactivated structure compared with Meta II, but despite apparent similarities, it is different from the ground state. What is predominantly formed is in all its measurable properties identical to Meta III, normally generated as a byproduct, when the Meta I/II pool decays through thermal deactivation into opsin and all-trans-retinal. Meta III stores the chromophore in a form in which it does not take part in the normal release/regeneration pathway of the retinoid cycle. In the accompanying article (44), we will show that this product has partial activity and that interaction of the G-protein, transducin, triggers the return of molecules stored in Meta III. Here we are interested in the mechanistic aspects of blue light illumination. Two immediate questions arise, namely, (i) what are the constraints imposed on the chromophore by the surrounding poly peptide structure and (ii) what is the precise identity of the product(s) formed and how do they form?

**In the Active Meta II State, the Central Part of the Polyene Chain Is Locked in the All-trans Configuration**—Relevant properties of chromophore-protein interaction in the dark ground state include that the terminal β-ionone ring of the

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**FIG. 6.** FTIR spectra of the illumination of metarhodopsin intermediates. a, Meta II minus Rh (green) and P-Meta III minus Meta II (blue) FTIR difference spectra (see Fig. 1D for comparison). b, Meta I minus Rh (green) and illumination product of Meta I minus Meta I (blue) FTIR difference spectra. Rh was illuminated with green light at pH 6.1 and 1 °C to form Meta I. Meta I was subsequently illuminated using a 480 ± 5 nm band pass filter. In contrast to the traces in a, the spectra are a mirror image of each other also in the retinal fingerprint region. c, Rh was illuminated at 80 K with blue light (480 ± 20 nm) for 120 s. The product formed (Batho) was then illuminated with orange light (>530 nm) for 120 s. The procedure (blue and subsequent orange illumination) was repeated once more to make sure that light-induced conversions occur only between photostationary states. Now the sample was illuminated with blue light, and the difference spectrum after illumination minus before illumination was recorded (blue). Subsequently, the sample was illuminated with orange light (green).

As can be seen in c of Fig. 6 in the case of rhodopsin, no band at 1348 cm⁻¹, indicating syn/anti isomerization of retinal Schiff base, is observed for this transition. Instead, bands at 1206 and 1238 cm⁻¹ indicating cis/trans isomerization are observed. The band at 1344 cm⁻¹ is assigned to the formation of Iso (27). In Fig. 6, d shows that in the respective difference spectra of P-Meta III, the band at 1348 cm⁻¹ is present in both spectra, indicating syn/anti isomerization and the full reversibility of this process (so that P-Meta III is formed by illumination of its 80 K intermediate).

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**Small deviations, e.g.** the less intense band at 1238 cm⁻¹ and the more intense band at 1206 cm⁻¹, can be explained with the known formation of Iso under the conditions of the experiment. Most important, the band at 1348 cm⁻¹ is lacking in both spectra. The spectra show that in contrast to Meta II, illumination of Meta I regenerates the rhodopsin dark ground state. Starting from Meta I, not only proton transfer reactions and changes in secondary structure but also the geometry of the chromophore are reverted, achieved by all-trans/11-cis or all-trans/9-cis isomerization.

In Fig. 6, c and d show that the same is true for the 80 K intermediates of Rh and P-Meta III, respectively. For both intermediates, the following procedure was applied: Rh or P-Meta III samples were illuminated at 80 K with green light (480 ± 20 nm) for 120 s. Subsequently, the product formed (Batho in the case of Rh) was illuminated with orange light (>530 nm) for 120 s. This procedure (green and subsequent orange illumination) was repeated once more to make sure that light-induced conversions occurred only between photostationary states. Now the sample was illuminated with green light, and the difference spectrum after illumination minus before illumination was recorded (blue). Subsequently, the sample was illuminated with orange light (green).
11-cis-retinal is embedded in a tight hydrophobic pocket and that, on the other end of the polyene chain, a strong salt bridge between the retinal Schiff base and its counterion stabilizes the inactive conformation of the photoreceptor. Despite these specific tight interactions, the ground-state polypeptide structure accepts not only the native 11-cis but also the 9-cis configuration, 10-methyl-retinal (28), and even six locked retinal analogs with a bulky ring in the position between C10 and C13 (14, 15, 29). Photoactivation from the ground state starts with cis/trans isomerization around the central double bond, and the conformational freedom in the central part of the retinal pocket may be an essential requisite for the activation process.

In contrast, interactions in the active state are such that allowed configurational changes of the retinal are in the Schiff base bond and that changes in the central part of the polyene chain are inhibited (Fig. 7 A). There is a certain analogy to the locked retinal analogs, in which central isomerization is prohibited by the synthetic hydrocarbon ring structures between C10 and C12. Photoisomerization of these retinals, even if reconstituted with opsin, can still occur, albeit not around the C11=C12 double bond. Two of the four isomers allowed formation of the active state (14, 15). The analogy to the present study is that different chromophore movements than the cis/trans isomerization around the C11=C12 double bond allow the...

Fig. 7. Reaction schemes for light-induced and thermal conversions between rhodopsin intermediates. A, this figure illustrates the first and second switch. The first switch is the normal cis/trans isomerization forming Meta II, which is in a pH- and temperature-dependent equilibrium with its precursor, Meta I. The second switch leads via light absorption of Meta II to Meta III by syn/anti isomerization of the retinal Schiff base. B, pathway 1a describes the normal activating pathway by illumination of Rh with 500 nm light. Light absorption causes cis/trans isomerization of the retinal, changing its configuration from 11-cis 15-anti to all-trans 15-anti. A product with a still protonated Schiff base is formed (Meta I). Subsequent deprotonation of the Schiff base leads to the active species (Meta II). This configuration of the retinal can also be reached thermally by deprotonation of the Schiff base in the 11-cis 15-anti form, leading to rhodopsin with deprotonated Schiff base Rhb, and subsequent isomerization around the C11=C12 double bond (pathway 1b). However, this is an extremely rare event, as shown by the time constant of 10^{11} s. Blue light absorption in the deprotonated Meta II state fails to induce trans/cis isomerization and restore the ground state. Instead, antisy/n isomerization forms reverted Meta. Subsequent reprotonation of the Schiff base leads to the Meta III intermediate, a product with reduced activity (pathway 2a). Illumination of Meta III leads via syn/anti isomerization and deprotonation of the Schiff base back to Meta II (pathway 2b). This pathway is reversible via thermal Schiff base reprotonation, forming Meta I and subsequent thermal antisy/n isomerization (22). In contrast, illumination of Meta I restores Rh and Iso ground state by trans/cis isomerization of the retinal.
protein to change its state of activation (19). In the case of Meta II, however, it is the protein itself that exerts the constraints preventing central double bond isomerization, thus opening a specific pathway that leads into a storage form.

Remarkably, these constraints build up only in Meta II and are not found when the predecessor of Meta II, Meta I, is illuminated. Meta I is still inactive toward the G-protein and keeps the salt bridge between the retinal’s positively charged Schiff base linkage to the apoprotein and the counterion intact. It may therefore be concluded that it is the breakage of the salt bridge that imposes new constraints to the chromophore, which lock it in the all-trans configuration. More support for this notion comes from the finding that not only Meta I but also all other intermediates with an intact salt bridge undergo photoreversion to the ground state, triggered exclusively by cis→trans isomerization, called the “first switch” (Fig. 6, c). The second switch (syn→anti isomerization) seems to be less specific because it is operated from either a deprotonated (Meta II→Meta III conversion) or a protonated Schiff base (Meta III→Meta II or 80 K intermediate→Meta III). The facts that Batho is photoreverted to Rh and Iso and the 80 K intermediate of Meta III is photoconverted to Meta III and not to Rh show that these intermediates are different (Fig. 6, d).

This is in agreement with the notion that the chromophore, in turn, controls even the last steps of rhodopsin activation, so that a rhodopsin harboring the 9-demethyl analog of retinal showed a specific defect in forming the Meta II intermediate (13, 30, 31). It was concluded that only the native chromophore provides a specific tight fit and rigid scaffold to maintain the active conformation and adjust the proton donor and acceptor groups for the proton transfer reaction linked to Meta II formation (13). In contrast, structural changes that were reported to occur as early as in Meta I (32), especially the proposed Schiff base counterion switch (33), do not seem to affect central double bond isomerization.

Light Converts Meta II into the Meta III Storage Form—The identity of the Meta III arising from thermally activated decay of the Meta I/Meta II pool (T-Meta III) with the illumination product (P-Meta III) now allows us to extend the molecular interpretation of T-Meta III to P-Meta III and thus to Meta III in general. Meta III binds the chromophore via a protonated Schiff base (21) in its original binding site and yields all-trans retinal on extraction (19). Vogel et al. (20) have recently shown (22) that the all-trans-retinal is bound to Lys296 via a Schiff base linkage in the 15-syn configuration, whereas in dark-adapted Rh and in Meta II, the configuration of the retinal is 15-anti. Such a syn→anti conversion is not uncommon for retinal proteins and also occurs in the proton pump bacteriorhodopsin during extended periods of darkness (34). Thus, when Meta III forms, the retinylidene bond and not the central C11=C12 double bond is isomerized. Pathway 1a illustrates the constraints in the dark ground and active states, respectively.

The question of how Meta III can form by thermal activation or illumination would then be answered by the simple assumption that Meta III forms either slowly by thermal (spontaneous) Schiff base reprotonation followed by a thermal isomerization step (the T-Meta III pathway) or rapidly by light-induced Schiff base isomerization and subsequent reprotonation (the P-Meta III pathway). Pathways identified in the present study and in the literature are shown in Fig. 7B and will now be discussed.

Pathway 1a is the normal signal transduction path, starting with cis→trans isomerization around the C11=C12 double bond and leading into the Meta I/Meta II equilibrium linked to Schiff base deprotonation (Fig. 7B, SRH+/SR; time constant, $10^{-3}$ s). The hypothetical pathway 1b goes through thermal (spontaneous) Schiff base deprotonation and subsequent isomerization around the central double bond. This is the path proposed by Birge and Barlow (35) to explain the spontaneous activation of Rh, which is an exceedingly rare event (time constant, $10^{11}$ s). The thermally activated formation of the deprotonated Schiff base would probably be rate-limiting. The reversal of pathways 1a and 1b is, according to the present data, inhibited. It is replaced with pathway 2a, which starts with light absorption and leads through the reverted Meta intermediate (17) to Meta III. Reverted Meta can now be assigned to a retinal syn conversion, with the Schiff base still deprotonated and the receptor still in its active conformation (insofar comparable with the Batho intermediate of the activating Rh→Meta II pathway). According to the much lower barrier provided by the second switch (syn→anti isomerization), Meta III is much less stable than Rh. It undergoes slow decay into opsin and retinal, which is even enhanced by interaction with transducin or its C-terminal peptides (44).

As shown by the present data, illumination of Meta III leads via pathway 2b to Meta II. Light causes syn→anti isomerization and subsequent Schiff base deprotonation. Dependent on the illumination conditions, trans→cis and subsequent syn→anti isomerization of the Schiff base was also observed (36). Pathway 2b can be reverted: Meta II reacts thermally to Meta III, via Meta I (37). In this pathway, reprotonation of the Schiff base precedes anti→syn isomerization (22). The scheme implies that thermal isomerization to Meta III uses (and may need to use) the Schiff base-reprotonated retinal, whereas light-induced deactivation requires the deprotonated form (Fig. 7). Only in the deprotonated situation (Meta II) does light-induced anti→syn isomerization occur, leading to Meta III. This is in remarkable contrast to central double bond isomerization in the dark state, which starts from the protonated Schiff base. Only in the protonated form and only in Meta I is the energy barrier of the syn→anti isomerization low enough to be overcome by thermal activation, whereas the much higher energy barrier necessary for cis→trans isomerization needs to be overcome by photoexcitation. In the deprotonated form, photoactivated syn→anti isomerization is the predominant path.

Related Systems and Perspectives—In view of the present results, it is interesting to compare related retinal proteins that subserve different biological functions. Close relatives of vertebrate rhodopsin are the invertebrate rhodopsins. In these, the active G-protein binding metarhodopsin species is, unlike vertebrate rhodopsin, thermally stable. Rhodopsin regeneration is brought about by the absorption of a second photon (38, 39). Archaebacteria carry the light-driven proton pump bacteriorhodopsin and the sensory rhodopsins (sensory rhodopsins I and II) in their plasma membrane. Bacteriorhodopsin exists in its dark ground state (the so-called dark-adapted state) in the 13-cis-syn configuration, which is converted by light to the all-trans-15-anti light-adapted ground state. Excitation of the light-adapted state ($\lambda_{max} = 568$ nm) starts the photocycle, in which proton pumping or formation of the signaling state is initiated by all-trans-15-anti/13-cis-15-anti isomerization and subsequent Schiff base deprotonation. Late in the photocycle, the Schiff base is reprotonated, and the retinal can return thermally into the all-trans-15-anti configuration, thus closing the photocycle. The cycle is left, and the dark-adapted storage state is restored by back-formation of the 13-cis-15-syn configuration, which requires both thermal cis→trans isomerization around the C11=C12 double bond and anti→syn isomerization around the retinylidene Schiff base. Illumination of the Schiff base-deprotonated M intermediate state can lead in a short cut of the normal photocycle directly back to the (light-adapted) ground state (40). Vertebrate rhodopsin is thus the only retinal protein that does not allow restoration of the ground state by
illumination of the Schiff base-deprotonated Meta state. There is a general analogy to bacteriorhodopsin with respect to a storage form that involves a syn/anti conversion, although the details are different.

Ligand-activated GPCRs and rhodopsin display similarities between the Meta I and Meta II states and the low and high affinity binding conformations (41). High affinity to the ligand and to the G-protein characterizes the ternary ligand-receptor-G-protein complex. The tight interaction between the retinal and the apoprotein in the active Meta II state, as reflected in the constraints that come into play on illumination of Meta II, fits into this general scheme.

The Meta III storage form is of potential significance, not only because of its interesting mechanism of formation but also in terms of visual function and physiology. The enhanced storage of retinal in the light-induced P-Meta III should reduce the amount of free retinal, thus reducing its poisonous effects, such as the formation of a bis-retinoid pyridinium compound called A2E (42). This might be a factor that reduces blue light-induced apoptosis (43).

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New Pathway from Metarhodopsin II

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