Photoreactions of Metarhodopsin III†

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Received April 23, 2004; Revised Manuscript Received June 9, 2004

ABSTRACT: Meta III is an inactive intermediate thermally formed following light activation of the visual pigment rhodopsin. It is produced from the Meta I/Meta II photoproduct equilibrium of rhodopsin by a thermal isomerization of the protonated Schiff base C=N bond of Meta I, and its chromophore configuration is therefore all-trans 15-syn. In contrast to the dark state of rhodopsin, which catalyzes exclusively the cis to trans isomerization of the C11=C12 bond of its 11-cis 15-anti chromophore, Meta III does not acquire this photoreaction specificity. Instead, it allows for light-dependent syn to anti isomerization of the C15=N bond of the protonated Schiff base, yielding Meta II, and for trans to cis isomerizations of C11=C12 and C9=C10 of the retinal polyene, as shown by FTIR spectroscopy. The 11-cis and 9-cis 15-syn isomers produced by the latter two reactions are not stable, decaying on the time scale of few seconds to dark state rhodopsin and isorhodopsin by thermal C15=N isomerization, as indicated by time-resolved FTIR methods. Flash photolysis of Meta III produces therefore Meta II, dark state rhodopsin, and isorhodopsin. Under continuous illumination, the latter two (or its unstable precursors) are converted as well to Meta II by presumably two different mechanisms.

Rhodopsin is the light receptor in vertebrate rod photoreceptor cells and a prototype for class A of the large family of G-protein-coupled receptors (*1*−*3*). It is a membrane protein consisting of seven membrane-spanning α-helices. These helices are arranged in a helix bundle and form thereby the binding pocket for rhodopsin's retinal chromophore that is covalently bound to a lysine residue on helix 7 by a protonated Schiff base. Activation of the receptor is initialized by a light-induced isomerization of this retinal chromophore from an 11-cis to an all-trans isomer and the subsequent structural adaptation of the protein to the changed chromophore geometry in the binding pocket. The finally achieved active state, Meta II, binds and activates the G protein of visual signal transduction, transducin. This Meta II state prevails in a conformational equilibrium with its still inactive precursor, Meta I. In this Meta I/Meta II photoproduct equilibrium Meta I is stabilized only at low temperature or alkaline pH (*4*) such that under physiological conditions Meta II will be formed almost exclusively.

The Meta I/Meta II photochemically induced equilibrium is not stable. Instead it decays via two independent pathways. Under conditions, in which Meta II is the predominant species in the Meta I/Meta II equilibrium, it decays by hydrolysis of the retinal Schiff base and dissociation of the receptor into free all-trans retinal and apoprotein opsin. Under conditions, that favor Meta I, on the other hand, the decay proceeds by a different pathway. Instead of releasing the chromophore, the protein catalyzes a thermal isomerization of the chromophore to allow the receptor to adopt an inactive low-energy conformation, which is, to some extent, similar to that of the dark state (*5*, *6*). During the transition to this thermal decay product, which is termed Meta III (λ_{max} 470 nm), the C15=N double bond of the retinal protonated Schiff base isomerizes thermally from a trans or anti geometry in the Meta I/Meta II equilibrium (all-trans 15-anti) to a cis or syn geometry in Meta III (all-trans 15-syn) (for a full account on the properties of Meta III see, for instance, refs *3* and *5*−*11*).

Already early studies on Meta III had shown that the thermal transition from the Meta I/Meta II equilibrium to Meta III can be photoreverted (*4*), which had been verified as well in more recent studies (*5*, *8*, *12*). Considering the chromophore geometries in Meta I/Meta II and in Meta III, this would imply that the thermal C=N isomerization during the transition to Meta III is reverted by a light-induced C=N back isomerization during the light reaction to Meta I/Meta II. Such light-induced isomerizations of the C=N bond of either protonated or deprotonated Schiff base, however, have never been reported in retinal proteins before. Therefore the question arises whether the light-induced transition from Meta III, characterized by a 15-syn chromophore configuration, to Meta I/Meta II is indeed induced by a simple light-induced C=N isomerization back to 15-anti. In addition, can this photoreversal process be reconciled with other previously published results, which suggested that photolysis of Meta III restores the 11-cis dark state and 9-cis isorhodopsin (*13*, *14*)?

† This work was supported by grants from the DFG (Nos. Si 278/16-3,4 to F.S. and R.V.), Fonds der Chemischen Industrie (to F.S.), and the Israel National Science Foundation (to M.S.). M.S. holds the Katzir-Makineni professorial chair in chemistry.

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10.1021/bi049182q CCC: $27.50
Published on Web 07/14/2004

Unlike UV−visible spectroscopy, FTIR[1] spectroscopy is sensitive to the conformation of the protein and the isomeric state of the retinal chromophore (*15*). In the present study, using rapid-scan FTIR spectroscopy in combination with laser flash photolysis of Meta III, we reveal that the photoreactions of Meta III are characterized by a quite complicated process, involving a branched reaction scheme: Meta III may be photoconverted directly to Meta II by a light-dependent isomerization of the protonated Schiff base of Meta III from 15-syn to 15-anti. Alternatively, Meta III may be photoconverted by a light-induced isomerization of either C9═C10 or C11═C12 of the polyene of the chromophore, followed by a thermal isomerization of the protonated Schiff base C═N bond, yielding isorhodopsin and dark state rhodopsin as stable products. Under continuous illumination with visible light, dark state rhodopsin and isorhodopsin may not accumulate and Meta III is therefore quantitatively converted to Meta II.

MATERIALS AND METHODS

Pigment Preparation. Rhodopsin in washed disk membranes was prepared from cattle retinae according to standard procedures (*16*). For Meta III experiments, the membranes were adjusted to pH 8.0 in 1 mM BTP (Bis-Tris-Propane) and stored at −20 °C. Preparation of rhodopsin with isotopically labeled chromophores was accomplished as described previously (*5*).

Sample Preparation. UV−visible and FTIR spectroscopy were performed with sandwich samples that were prepared as described elsewhere (*15*, *17*) with 0.5−1.0 nmol of pigment and 20 μL of 200 mM BTP buffer. For H/D exchange at the Schiff base, we twice equilibrated the sample film with D_2O and dried it under nitrogen before adding BTP buffer prepared in D_2O.

Meta III samples were prepared by illumination of the dark state at 30 °C and pH 8.0 (20 s, >530 nm orange light, see below for setup) and decay for 20 min at the same temperature. For experiments below 30 °C, these samples were then rapidly cooled (in the cryostat or in the thermostated sample holder) to the desired final temperature. Please note that Meta III samples contain about 30% Meta III, the remainder being mostly opsin and all-trans retinal. The latter does, however, not respond efficiently to illumination in the visible range at alkaline pH (*5*, *12*), because free retinal or its deprotonated Schiff bases with peripheral amino groups absorb at 360 to 380 nm.

FTIR Spectroscopy. FTIR spectroscopy was performed with a Bruker IFS 28 spectrometer with an MCT (mercury cadmium telluride) detector and a thermostated sample holder. In steady-state experiments, IR spectra were recorded in blocks of 512 scans with a spectral resolution of 4 cm^{-1} and an acquisition time of 1 min and corrected for temporal baseline drifts. Unless specified differently, samples were photolyzed for 20 s by a 150 W slide projector equipped with heat filter, through fiber optics and color or cutoff filters as specified. Low-temperature experiments were performed in a liquid nitrogen cooled cryostat. Time-resolved experiments were done at 4 cm^{-1} resolution in rapid-scan mode with on average 65 ms per scan. Spectra used for evaluation comprised at least 48 scans per experiments, subsequently averaged over at least three independent experiments. Laser excitation was performed with the 477 nm output of a dye laser (FL 2000, Lambda Physik, Göttingen, Germany) run with Coumarin 307 dye (Radiant Dyes, Wermelskirchen, Germany) and pumped by the output of an excimer laser. Pulse duration was 20 ns; the delivered pulse energy at the 6 mm diameter sample spot was typically 1.0−1.1 mJ.

To quantify the yield of the different photoproducts, we determined the amplitude of characteristic IR chromophore bands of Rho (at 1237 cm^{-1}), Iso (at 1204 cm^{-1}), and Meta III (at 1349 cm^{-1}) in initial state to Meta II difference spectra obtained by 30 s illumination under similar conditions (30 °C, pH 8.0) and normalized by the characteristic Meta II photoproduct bands above 1700 cm^{-1}. Under these conditions, the same photoproduct (largely Meta II with about 20% of Meta I) will be formed, irrespective of the different initial states. Normalized to the value for the Meta III band, we determined the relative amplitude of the Rho and the Iso bands to be 1.60 and 2.15, respectively. In Meta III to photoproduct difference spectra, these values allow us to quantitatively compare the amount of depleted Meta III with the amount of Rho and Iso being formed in the subsequent thermal reaction.

UV−Visible Spectroscopy. For UV−visible spectroscopy sandwich samples identical to the infrared samples were used in a Perkin-Elmer Lambda 17 double-beam spectrophotometer equipped with a temperature-controlled sample holder in either the scanning mode or the time drive mode at a single wavelength with 100 ms time resolution. Illumination was as in the IR experiments. Spectra were analyzed using Bruker's Opus software for OS/2 (Bruker, Ettlingen, Germany) and Statistica (StatSoft, Hamburg, Germany).

RESULTS

Meta II Minus Dark State and Meta II Minus Meta III Difference Spectra. Illumination of the dark state of rhodopsin (Rho, λ_{max} 500 nm) at 30 °C and pH 8.0 leads to formation of the equilibrium between the photochemically induced intermediates Meta I and Meta II (480 and 380 nm, respectively), which consists under the specific chosen conditions of about 20% Meta I and 80% Meta II (Figure 1, gray spectrum). For simplicity, this equilibrium, which is largely on the Meta II side under these conditions, will be referred to as Meta II in the following. With a time constant of about 4 min, this photoproduct equilibrium decays thermally, yielding opsin and all-trans retinal (380 nm), as well as about 30% Meta III (470 nm). Following complete decay, Meta III can be selectively reverted to Meta II by a second illumination with a >475 nm long-pass filter (Figure 1, black spectrum), allowing thus the exclusive study of Meta III, despite the fact that this species can only be obtained in mixtures with other species (*5*, *8*).

Some of the chromophore-related bands in the Meta II minus dark state and Meta II minus Meta III difference spectra in Figure 1 can be assigned using Raman and IR data on the dark state of rhodopsin (*18*, *19*) and previously published quantum chemical density functional theory (DFT) calculations and IR data for Meta III (*5*). In the case of the dark state (negative bands in the gray spectrum in Figure

[1] Abbreviations: FTIR, Fourier transform infrared; Rho, dark state rhodopsin; Iso, isorhodopsin.

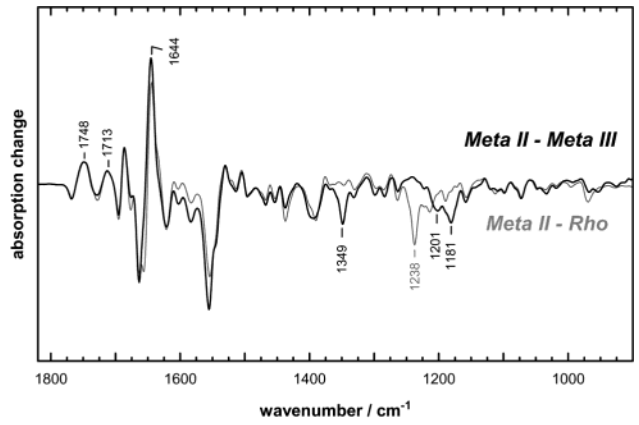


FIGURE 1: Photoreactions of dark state rhodopsin and Meta III. Dark state rhodopsin (Rho) has been illuminated (>530 nm) for 30 s to form the Meta I/Meta II photoproduct equilibrium with about 80% Meta II (gray spectrum, pH 8.0, 30 °C). After full decay of these photoproducts, the Meta III fraction of the decay product has been photolyzed again (30 s, >475 nm) to yield again the Meta I/Meta II photoproduct equilibrium (black spectrum). In this "photoproduct minus initial state" representation, positive bands in the difference spectra belong to the photoproduct state, while negative bands represent the respective initial states with their distinct marker bands. Because only about 30% of the initial photoproduct decays to Meta III, the Meta II − Rho difference spectrum was scaled by a factor of 0.30 for easier comparison.

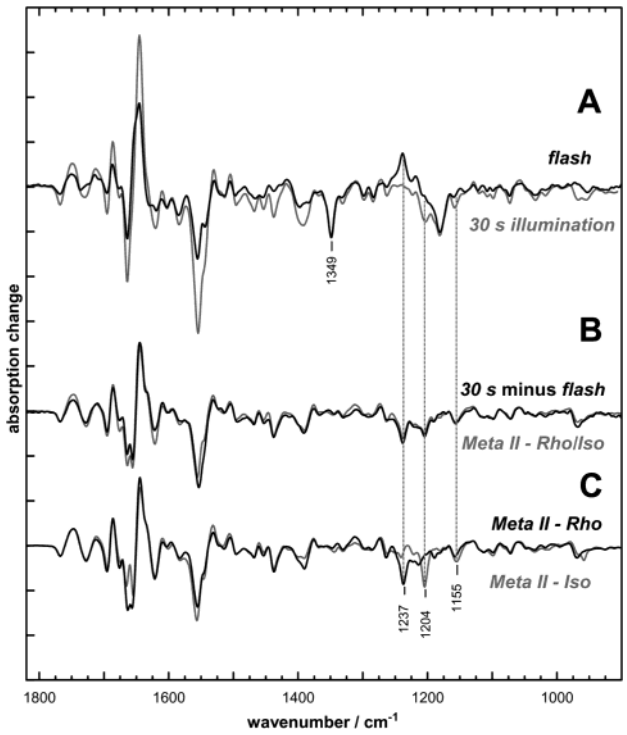


FIGURE 2: Meta III photoproducts obtained after continuous illumination vs flash illumination. In part A, photoproduct minus initial state (Meta III) difference spectra were obtained after a 30 s illumination (460−495 nm) or after a 30 ns laser flash (477 nm) of Meta III samples at 30 °C, pH 8.0. The spectra were obtained in steady-state experiments with a time resolution due to data acquisition of 60 s and normalized to the negative 1349 cm^{-1} band of photolyzed Meta III. Part B presents difference between the two spectra in part A (black) and in comparison a calculated spectrum consisting of 64% Meta II − dark state rhodopsin and 36% Meta II − isorhodopsin. Part C presents difference spectra of the photoreaction Meta II − dark state rhodopsin (11-cis) and Meta II − isorhodopsin (9-cis) used for comparison in part B with their typical marker bands in the range of the chromophore fingerprint vibrations obtained under standard Meta II conditions (20 °C, pH 5.0). Tickmarks correspond to 0.1 mOD.

1), the fingerprint bands were found to comprise C12−C13 and C14−C15 stretching vibrations of the chromophore for the 1238 cm^{-1} band and C8−C9 stretching mode for the 1216 cm^{-1} band. The 1200 and the 1180 cm^{-1} vibrations of Meta III (negative bands in the black spectrum of Figure 1) were assigned to linear combinations of C8−C9, C10−C11, C12−C13, and C14−C15 stretching vibrations, the 1180 cm^{-1} vibration being the one with the strongest C14−C15 contribution, while the band at 1349 cm^{-1} had been assigned to the protonated Schiff base NH in-plane bending mode.

The Light-Induced Transition from Meta III to Meta II. The difference spectrum in Figure 1 represents the transition from Meta III to Meta II induced by a 30 s continuous illumination. We have observed distinct changes in corresponding difference spectra upon reduction of the illumination time to 5 s or shorter or following photolysis of Meta III by a 20 ns laser pulse at 477 nm (Figure 2A). Obviously, the photoproducts obtained after flash photolysis and after prolonged continuous illumination are not identical. Instead, we have observed a much lower amplitude of typical Meta II bands in the flash-induced spectrum compared to the spectrum obtained after continuous illumination. This is particularly evident for the Meta II band at 1644 cm^{-1} or in the frequency range above 1700 cm^{-1} of the C═O stretching mode of carboxylic acids. Concomitantly, positive fingerprint bands appear in the range around 1200 cm^{-1} following flash photolysis that do not correspond to Meta II (*20*) and that are absent after continuous illumination. A closer examination of the Meta II marker bands amplitude reveals that only about 40% of the photolyzed Meta III is converted to Meta II by a light-induced C═N bond isomerization in the laser flash experiment. The remainder is converted by the short laser pulse to one or more thermally stable products different from Meta II, which account for the observed pronounced fingerprint bands. Obviously, these species do not accumulate under continuous illumination and are converted to Meta II instead by further photoreactions.

Flash Photolysis of Meta III Leads to Formation of Rho and Iso. Subtraction of the flash-induced difference spectrum from the difference spectrum obtained by continuous illumination should therefore reflect the photolysis of the stable intermediates mixtures to the final Meta II product (Figure 2B, black spectrum). In the resulting spectrum, the contributions of the stable intermediates are negative with pronounced bands in the fingerprint range at 1237, 1204, and 1155 cm^{-1}. These bands match precisely the positions of the strongest fingerprint bands of dark state rhodopsin (Rho, characterized by 11-cis 15-anti chromophore geometry and a fingerprint marker band at 1237 cm^{-1}) and isorhodopsin (Iso, 9-cis 15-anti chromophore geometry and a fingerprint marker band at 1204 cm^{-1}), as evident from the respective Meta II minus Rho and Meta II minus Iso spectra in Figure 2C. Are these intermediates identical with Rho and Iso? The gray spectrum in Figure 2B is a linear combination of the two spectra in Figure 2C with 64% contributions from Rho to Meta II spectrum and 36% contributions from Iso to Meta II spectrum. The very good agreement of this linear combination with the measured double difference spectrum (black)

in Figure 2B confirms that flash photolysis of Meta III leads, besides a direct formation of Meta II, to formation of Rho and Iso.

What are the molecular isomerization events that underlie the observed results? Direct formation of Meta II from Meta III in the flash photolysis experiment can be achieved only by a light-induced isomerization of the C═N double bond. The significance of this pathway will be discussed further below. The process leading to formation of Iso and Rho is necessarily more complicated, because it should involve two isomerizations associated with the polyene C11═C12 or C9═C10 double bond and of the protonated Schiff base C═N double bond. In the following, we will focus on the sequence of events leading to formation of these two products.

Study of Rho and Iso Formation from Meta III by Time-Resolved Spectroscopy. As mentioned above, we have observed that similar photoproducts as after laser flash photolysis were obtained by reducing the duration of the continuous illumination to 5 s or less (data not shown). This suggested that Rho and Iso are formed on a comparable time scale. This time scale is accessible by time-resolved FTIR spectroscopy in the rapid-scan mode, and therefore, this method was employed to examine the formation of Rho and Iso following laser excitation of Meta III. Figure 3A presents photoproduct minus Meta III spectra obtained in the time interval from 0 to 3 s and from 25 to 50 s after laser pulse at 30 °C. As in Figure 2A, Meta II-like bands are observed indicating direct light-induced formation of Meta II from Meta III by a light-dependent C═N isomerization, as already mentioned above. Besides this reaction, it is evident that Rho and Iso are not formed as initial products of Meta III photolysis but evolve in the dark on the time scale of seconds during the thermal decay of not yet specified initial products. This thermal reaction leads to formation of the final decay products, characterized by bands at 1237, 1204, 1188, and 1155 cm^{-1} in the decay spectrum (Figure 3A, lower spectrum). Exponential fits to the two most pronounced decay bands yield time constants of approximately 9 s for the 1237 cm^{-1} band (Rho marker band) and of about 3 s for the 1204 cm^{-1} band (Iso marker band, all at 30 °C).

The rate of this thermal dark reaction can be slowed considerably by decreasing the temperature. At 10 °C, the time constants are approximately 40 and 20 s for the 1237 and the 1204 cm^{-1} band, respectively (not shown), and 120 and 80 s, respectively, at 0 °C (Figure 3 B). This may suggest that the decay to Iso, giving rise to the 1204 cm^{-1} band, is too fast at 30 °C to be fully resolved by the employed time frame such that at this temperature predominantly the slower decay to Rho of one of the initial products is observed. At 0 °C, on the other hand, both thermal processes can be fully resolved.

Assignment of the Thermal Reaction to the C═N Isomerization Step. The transition from Meta III to Rho and Iso consists therefore of a light-induced reaction, followed by a thermal reaction to yield the final products. Can these processes be correlated with chromophore isomerizations? The chromophore geometry of Meta III is all-trans 15-syn, while those of Rho and Iso are 11-cis 15-anti and 9-cis *15*-anti, respectively (Scheme 1). The necessary two double bond isomerizations are achieved by a light-induced reaction and a subsequent thermal reaction in the dark. From an energetic point of view, it is reasonable to assume that the thermal

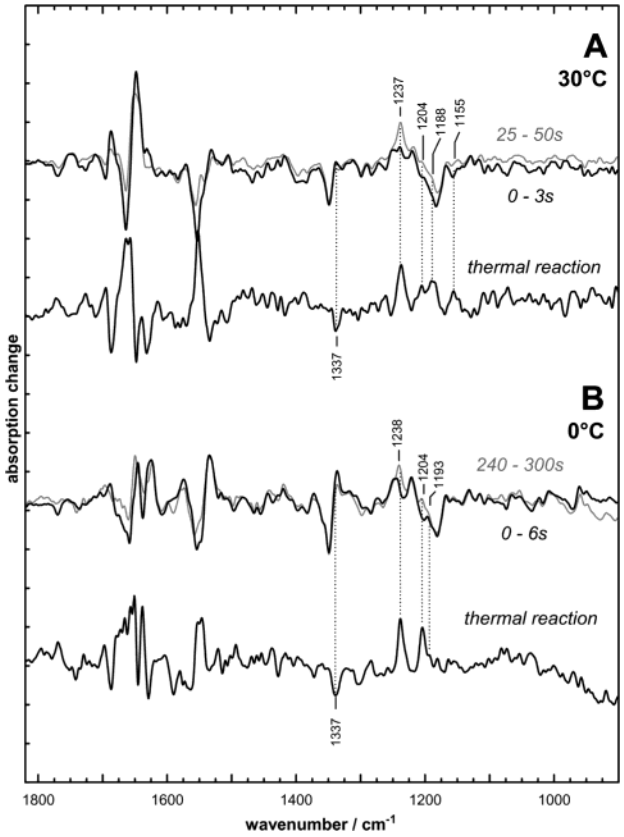
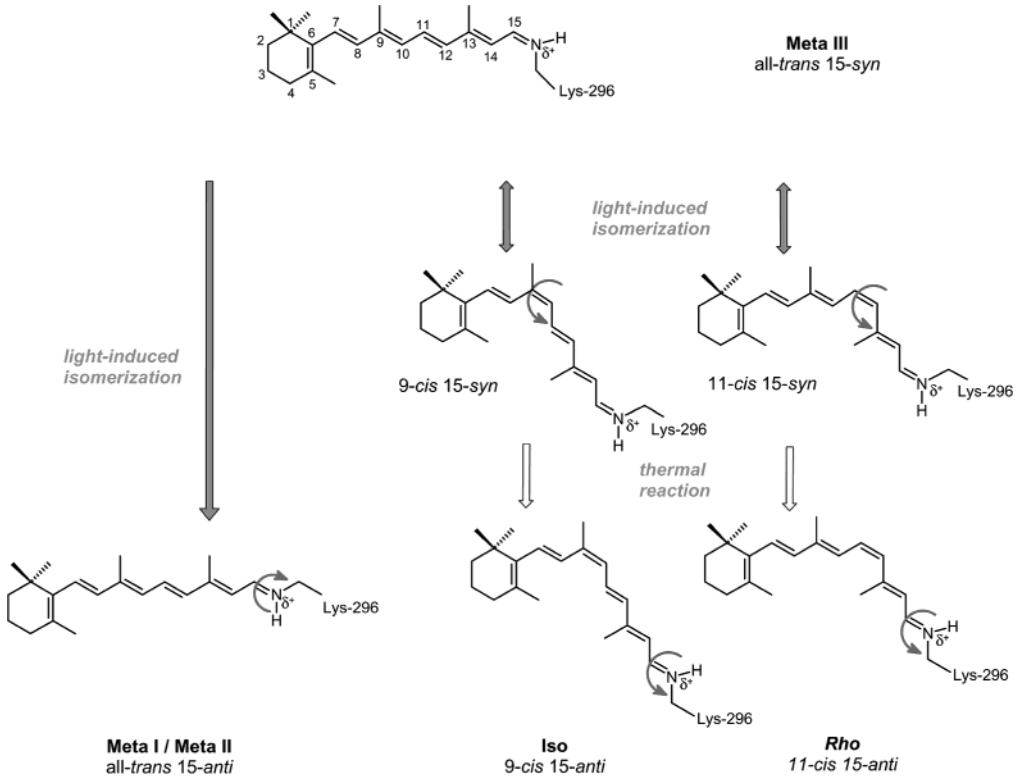


FIGURE 3: Time-evolution of Meta III photoproducts at 30 and 0 °C after laser excitation. Photoproduct minus Meta III difference spectra were obtained by time-resolved rapid-scan IR spectroscopy after laser excitation of Meta III at 477 nm and 30 (A) and 0 °C (B). In part A, we show the spectrum obtained in the first 3 s after laser photolysis (48 scans, black), the spectrum obtained in the time range between 25 and 50 s after photolysis (384 scans, gray), and the difference between both (thermal reaction, enlarged by a factor of 2). The spectra in part B were obtained accordingly with Meta III samples cooled to 0 °C in the time range from 0 to 6 s and from 240 to 300 s after photolysis. All spectra are averages of at least three experiments to increase the signal-to-noise ratio; tickmarks are 0.1 mOD.

step involves the C═N isomerization because the intrinsic energy barrier for thermal isomerization is lower in the C═N bond than in any of the C═C bonds of the chromophore (*21*, *22*). We could therefore assign the initial light-induced step to C9═C10 and C11═C12 isomerizations, which yield 9-cis and 11-cis 15-syn isomers, respectively. These isomers relax subsequently by thermal reaction to Iso and Rho, as depicted in Scheme 1. While making sense, this proposition should be tested experimentally. Theoretical calculations employing simple force fields (*23*) and more elaborate DFT calculations (*5*) have convincingly shown that C14−C15 stretching modes of retinal chromophores may serve as an indicator for the isomeric state of the Schiff base, as this stretching frequency should respond sensitively to H/D exchange at the Schiff base in the 15-syn, but not in the 15-anti isomer.

We will therefore try a crude analysis of the spectra of the thermal reactions shown in Figure 3. Laser photolysis of Meta III yields only little photoconversion, and therefore the signal-to-noise ratio is quite small, particularly if we employ time-resolved FTIR techniques with only a limited number of scans. At 0 °C, the thermal reaction is sufficiently

Scheme 1



slow (time constants of 80 and 120 s, as shown above) to replace the laser flash by a short 2 s illumination delivered by a 150 W tungsten lamp, which yields a higher photoconversion of Meta III without photolyzing the thermal reaction products to a significant extent. The corresponding spectra are shown in Figure 4A, in which photoproduct minus Meta III spectra are presented that were recorded immediately after illumination and about 5 min later, after completion of the thermal reaction. The spectra correspond to those obtained by flash photolysis (presented in Figure 3B) but have a significantly better signal-to-noise ratio.

In Figure 4B,C, we show the complete temporal evolution of the thermal dark reaction in H_2O and in D_2O with bands of the decay products being positive in this representation. The bands evolving at 1239 and 1204 cm^{-1} of Rho and Iso, respectively, are clearly observed. The 1204 cm^{-1} band of Iso consists of the polyene C14−C15 stretching vibration, together with the C8−C9 stretch (*19*). In Rho, contributions of the C14−C15 stretching vibration are distributed in the 1238 cm^{-1} band and in a band around 1190 cm^{-1} (*19*, *24*). The latter vibration possibly causes the asymmetric broadening at the low wavenumber side of the 1204 cm^{-1} vibration of Iso in Figure 4B. As evident from a comparison of the spectra obtained in H_2O in Figure 4B with the spectra obtained in D_2O in Figure 4C, neither of these positive bands shows a strong H/D shift. The band at 1239 cm^{-1} shifts slightly up to 1243 cm^{-1}, while the others are insensitive. A similar behavior of the corresponding infrared bands is observed in samples of dark state rhodopsin and of isorhodopsin (*20*, *24*, and our own experiments). This lack of a pronounced H/D sensitivity of the bands representing the C14−C15 stretching vibration indicate that the chromophore geometry in the final decay products is 15-anti. These results confirm that the thermal reaction products are identical to the 11-cis 15-anti and 9-cis 15-anti isomers, Rho and Iso. The evolving HOOP bands at 968 and 958 cm^{-1}, being at the same position as those in Rho and Iso, further support this notion.

The negative bands in Figure 4B,C, on the other hand, which correspond to the initial photoproduct states, show a pronounced H/D sensitivity. The negative band at 1337 cm^{-1} disappears in D_2O, while the bands at 1142, 1173, and 1215 cm^{-1} are replaced by bands at 1214 and 1229 cm^{-1} in D_2O. This is a strong indication that the initial photoproduct states have a 15-syn chromophore geometry and correspond therefore to the 11-cis and 9-cis 15-syn isomers. This was further corroborated in experiments employing pigment ^{13}C-labeled at C14 and C15 of the retinal chromophore, performed both in H_2O and D_2O (see Supporting Information). In these experiments, the bands at 1173 cm^{-1} in H_2O and at 1229 cm^{-1} in D_2O clearly responded to labeling, indicating the contribution of the C14−C15 stretch to these bands. For the other fingerprint bands, a similar analysis was hampered by interfering negative or positive bands. The 1337 cm^{-1} band was also sensitive to labeling, indicating that it corresponds to the NH bending vibration coupling to the C14−C15 stretch in the 15-syn chromophore. Its position is at a lower frequency in these 15-syn chromophores with 11-cis or 9-cis polyene compared to the chromophore with all-trans polyene of Meta III (*5*).

We thus arrive at the sequence of single events depicted in Scheme 1 for the transition from Meta III to Rho and Iso, involving 9-cis and 11-cis 15-syn species as initial photoproducts that decay by a subsequent thermal reaction in the dark. In the following, we will now focus on the other branch of the photoreaction that produces Meta II by a single light-induced isomerization.

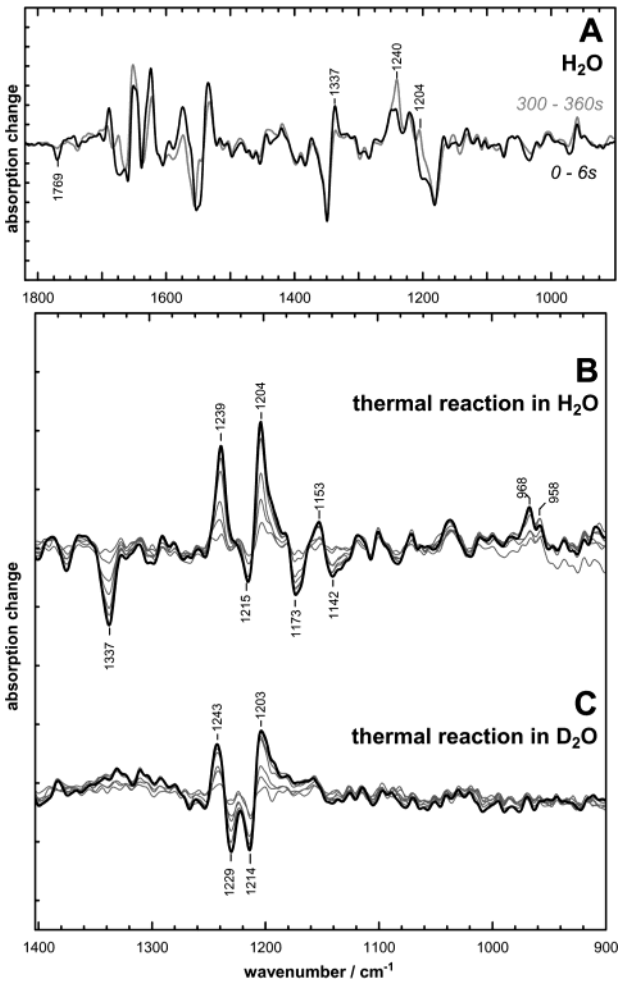


FIGURE 4: Time-evolution of Meta III photoproducts at 0 °C after short (2 s) illumination. Photoproduct minus Meta III difference spectra were obtained as in Figure 3B but after photolysis by a short 2 s steady illumination between 460 and 495 nm. Part A presents spectra obtained in the time range from 0 to 6 s and from 300 to 360 s after end of illumination in H_2O. Part B presents the evolution of the spectra in the fingerprint range during the thermal reaction calculated by subtraction of the photoproduct spectra obtained between 0 and 6 s after illumination from the photoproduct spectra obtained at 18, 37, 75, 150, 210, 270, and 330 s after illumination (midpoint of data acquisition). Part C presents evolution spectra as described in part B but obtained in D_2O. All spectra are averages of at least three experiments to increase the signal-to-noise ratio; tickmarks are 0.1 mOD.

Direct Light-Induced Formation of Meta II from Meta III by Light-Induced C═N Isomerization. As was mentioned above, laser photolysis of Meta III at 30 °C yields besides Rho and Iso roughly 40% Meta II, which is formed by light-dependent C═N isomerization. This was concluded from an analysis of Meta II marker bands in the range of amide I and carboxylic acid vibrations (Figure 2A). Using a different approach, we can calculate the amount of Rho and Iso being formed in the dark reaction after flash photolysis of Meta III by analysis of the amplitudes of fingerprint marker bands of Rho and Iso at 1237 and 1204 cm^{-1}, respectively, using the NH bending marker band of Meta III at 1349 cm^{-1} as reference (see Materials and Methods). This analysis yields a value of about 58% of Meta III being converted to Rho (37%) and Iso (21%), consistent with 40% of Meta III being directly converted to Meta II, as derived above (we use the term "direct" in the sense that the light-induced chromophore isomerization is followed by thermal relaxation of the protein, possibly via several intermediates that are not resolved but not by an additional thermal isomerization of the chromophore). Direct formation of Meta II from Meta III can be further followed in time-resolved UV−visible experiments that reveal formation of a stable species absorbing at 380 nm already within 100 ms after flash photolysis at 30 °C (see Supporting Information).

We analyzed the contributions of the different branches of the photoreaction also at 0 °C. We found that approximately 28% of the photolyzed Meta III is converted to Rho and 25% to Iso. The remaining 47% should then be accounted for by the branch leading to all-trans 15-anti via light-dependent C═N isomerization. Because all experiments were performed at pH 8.0 (a requirement for production of Meta III samples suitable for experiments, see Material and Methods) and because the p*K* of the Meta I/Meta II equilibrium is 6.3 at 0 °C (*25*), Meta I instead of Meta II will be formed by the C═N isomerization.

Low-Temperature Photoproducts of Meta III. As the thermal dark reaction becomes very slow already at 0 °C (rate constants of 120 and 80 s for the 11-cis and the 9-cis isomer), the 15-syn photoproducts of Meta III with cis polyene should become stable at somewhat lower temperature and be thus accessible by conventional steady-state FTIR spectroscopy. Figure 5 presents photoproduct minus Meta III spectra obtained at −20, −95, and −183 °C (these are the temperatures that allow the cryotrapping of the photoproduct states Meta I, Lumi, and Batho after illumination of dark state rhodopsin). In comparison, the spectrum obtained by time-resolved techniques directly after photolysis at 0 °C is presented (reproduced from Figure 4A). The spectrum for −20 °C was obtained by a 2 s illumination with the same 455−500 nm filter combination to have similar conditions as in the time-resolved spectrum. Both spectra exhibit a close similarity and have almost identical fingerprint bands. Only the negative band at 1202 cm^{-1} is less pronounced in the 0 °C time-resolved spectrum, which may indicate interference with a positive product band in this spectrum. At −95 °C, the fingerprint band positions are largely preserved but have an increased intensity. The negative band at 1769 cm^{-1}, which likely corresponds to the Asp83 C═O band observed in Rho, has almost disappeared at this low temperature. At −183 °C, the fingerprint band pattern changes noticeably. This can be due to the nonrelaxed chromophore structures at this temperature. It may, however, also indicate a shift in the relative contributions of the single photoreactions, the C11═C12, C9═C10, or C═N isomerizations, for example, due to specific interactions of the chromophore with the more rigid chromophore binding pocket or due to the energetic barriers during the isomerization.

A quantitative determination of the relative contributions of 15-syn and 15-anti isomers in the photoproduct equilibria, as performed at 30 and 0 °C, is unfortunately not possible in the low-temperature experiments because quantification of the 15-syn isomers requires their complete thermal decay. However, in the spectra presented in Figure 5, the positive photoproduct band at 1337 cm^{-1} of the NH bending mode may serve as a marker band for the formation of 15-syn products and thus for light-dependent C═C isomerizations. While the amplitude of this band remains approximately constant between 0 and −95 °C, it is markedly decreased at

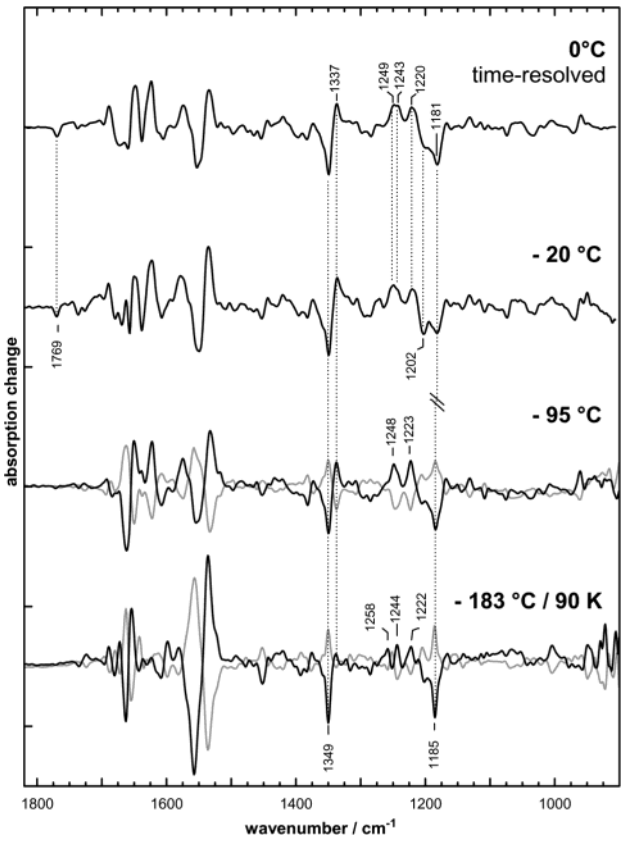


FIGURE 5: Low-temperature photoproducts of Meta III. Meta III samples were obtained at 30 °C as described in Materials and Methods, then frozen to the desired temperature and illuminated at the indicated temperature. The spectrum for −20 °C was obtained by a 2 s illumination in the wavelength range from 455 to 500 nm to allow comparison with the time-resolved spectrum; the spectra for −95 and −183 °C were obtained by a 30 s illumination in the 455−480 nm range. The latter reaction products were illuminated again for 30 s with orange light (>530 nm, gray spectra) to examine the reversibility of the light reaction. For comparison, we show the time-resolved spectrum obtained at 0 °C between 0 and 6 s after a 2 s illumination of Figure 4A.

−183 °C. This may indicate that C═C isomerizations are inhibited at very low-temperature such that light-dependent C═N isomerization becomes the predominant path of the photoreaction. This is further supported by experiments in D_2O, which showed no obvious H/D sensitivity of the photoproduct fingerprint bands at −183 °C, in contrast to −95 °C (spectra not shown). In principle, to avoid the concept of light-induced C═N isomerization, the formation of Meta II at room temperature could be formed by a light-induced C═C isomerization, followed by a very rapid thermal C═C,C═N double isomerization, instead of a single light-induced C═N isomerization. Such a model is, however, rebutted by the presence of C═N isomerized photoproducts even at very low temperatures, which would inhibit thermal isomerizations.

The spectra shown for −95 and −183 °C employed a 30 s illumination with a 455−480 nm filter combination and produced due to the longer illumination time a photostationary equilibrium. These photostationary equilibria were subsequently probed by a second illumination in a different wavelength range (30 s with orange light >530 nm). Remarkably, at −95 and −183 °C, the photoproducts could be photoreverted specifically to the initial Meta III state by this orange illumination. The resulting difference spectra of this back reaction are almost perfect mirror images of the corresponding photoproduct minus Meta III spectra of the forward reaction and are shown as gray spectra in Figure 5. In contrast, the corresponding photoproducts of dark state rhodopsin at these temperatures, Lumi and Batho, are converted by a second orange illumination largely to Iso (not shown). The almost complete reversibility of the photoreaction of Meta III at −95 and −183 °C indicates therefore a Meta III binding pocket that is substantially different from those of Rho or Iso or their low-temperature photoproducts, despite the fact that Meta III bands, which are related to the conformation of the protein, show many similarities with those of the dark state. At −20 °C, this reversibility of the photoreaction is gradually lost (data not shown), indicating a structural rearrangement of the protein after the photoreaction at this temperature, in agreement with previous studies on rhodopsin photoproducts (*26*).

DISCUSSION

Meta III is formed as an inactive decay product of the Meta I/Meta II photoproduct equilibrium of rhodopsin. As shown previously, its formation is triggered by a thermal isomerization of the C═N double bond of the retinal protonated Schiff base (*5*) and catalyzed by the protein conformation of Meta I (*6*). Early work by Wald and coworkers indicated that the thermal reaction leading from Meta I/Meta II to Meta III can be reverted by photolysis of Meta III, which restores the initial Meta I/Meta II photoproducts (*4*). These findings had been reproduced recently (*5*, *8*, *12*). On the other hand, it was reported that photolysis of Meta III produces 11-cis dark state rhodopsin (Rho) and 9-cis isorhodopsin (Iso) (*13*, *14*). The photoreactions of Meta III seemed therefore more complex.

To avoid multiphoton processes, we studied the photoreactions of Meta III by laser flash photolysis. While Rho and Iso are highly specific in their photoreactions by channeling the excitation energy exclusively into the respective C11═C12 and C9═C10 cis to trans isomerizations, the photoreactions of Meta III are much less specific (Scheme 1). The all-trans 15-syn chromophore of Meta III may respond to photon absorption by cis (15-syn) to trans (15-anti) isomerization of the C15═N double bond of the protonated Schiff base, as well as by trans to cis isomerizations of either the C11═C12 or the C9═C10 double bonds of the retinal polyene. Isomerization of the protonated Schiff base yields the regular all-trans 15-anti chromophore of the photoproducts of rhodopsin and thus, at room temperature, the Meta II state, presumably via its precursor Meta I. The other two pathways, involving isomerizations of the polyene, lead to 11-cis 15-syn and 9-cis 15-syn isomers, which are unstable. These intermediates rapidly thermally decay by isomerization of the C═N double bond to the stable 11-cis 15-anti and 9-cis 15-anti isomers of Rho and Iso with time constants of 9 and 3 s, respectively, at 30 °C. Of the three branches of the photoreaction, the C═N and the C11═C12 isomerizations are the most predominant, accounting for about 40% and 37%, respectively, of the photoconverted Meta III, while the C9═C10 isomerization accounts for only 22% at 30 °C. At 0 °C, the C═N isomerization becomes slightly more predominant at the expense of the C11═C12 branch, and also the rates of the thermal reactions of the 15-syn

photoproduct isomers, leading to Rho and Iso, become slower (120 and 80 s, respectively).

At temperatures far below 0 °C, the thermal relaxations of the 11-cis and 9-cis 15-syn intermediates to the 15-anti isomers of Rho and Iso are frozen, and the 15-syn isomers are, together with the product of the light-dependent C=N isomerization of the first branch, stable photoproducts. The band pattern of the chromophore fingerprint bands of these products is stable at −20 °C and shows close similarity to that observed at 0 °C immediately after flash photolysis in the time-resolved experiments. At −95 °C and lower, an interesting behavior is observed: the photoproducts obtained from Meta III after a blue-green illumination (455−480 nm) can be specifically reverted to the initial Meta III state by a subsequently applied orange illumination. This is in contrast to the photoreactions of dark state rhodopsin, as its low-temperature photoproducts, Batho and Lumi, are converted to 9-cis Iso by orange illumination rather than to the initial dark state. The almost complete reversibility of the Meta III photoreaction below the protein glass transition temperature highlights therefore the structurally different binding pocket of Meta III in comparison to that of the dark state, that can accommodate 11-cis, 9-cis, and all-trans chromophore isomers in their 15-anti forms.

Our experiments have shown, that flash photolysis of Meta III at room temperature leads to formation of Meta II, Rho, and Iso. How can we reconcile this result with the finding that continuous illumination produces only Meta II? Two possibilities can account for that: First, the light-induced formation of the 11-cis 15-syn and 9-cis 15-syn products is presumably a photoreversible reaction (we account for this possibility by the double arrows in Scheme 1). Under continuous illumination, part of these photoproducts will therefore be photoconverted back to Meta III before relaxation to Rho and Iso. Back in the Meta III state, they are available again for light-dependent C=N isomerization, which produces Meta II, such that the photoproduct pool will eventually be drained to Meta II (which due to its absorption maximum at 380 nm does not participate in the photoequilibrium). Alternatively, such a photoreversion may be slower than the thermal reaction to Rho and Iso due to a very low quantum yield of the isomerization or possibly be completely inhibited. Rho and Iso will then be formed as transient species that are photoconverted to Meta II under continuous illumination, as had been suggested before (*13*). Because the photoproduct ratios obtained at 0 °C are the same after flash photolysis as after a 2 s photolysis, the latter mechanism seems to be predominant. Importantly, this formation of Meta II by a two-photon process occurs in parallel to the Meta II formation by the single-photon C=N isomerization described above and increases therefore the Meta II yield under continuous illumination.

The photoreactions of Meta III are interesting because they give insight into reaction pathways and chromophore isomers that have not been described before. We will focus first on the light-induced C=N isomerization. Formation of Meta III occurs by thermal isomerization of the C=N double bond of the protonated Schiff base in Meta I, being present in the Meta I/Meta II photoproduct equilibrium of rhodopsin (*5*, *6*). A similar thermal isomerization is observed in the transition of bacteriorhodopsin (BR) to its dark-adapted state, which involves thermal isomerizations of both the C13=C14 and C15=N double bonds. Probably isomerization of only one double bond leads to an isomer that is not stable in the protein binding site of BR and the concomitant isomerization of the second double bond allows the chromophore to fit into the protein environment. Thermal isomerization of the C=N double bond seems therefore to be a common phenomenon in retinal proteins.

A light-induced C=N isomerization has been shown to be possible in theoretical models of retinal protonated Schiff bases (*27*). However, it has been never observed in the protein environment of rhodopsin or other retinal proteins. Interactions of their binding pockets with the chromophore seem to inhibit this photoisomerization path efficiently. The binding pocket of Meta III must therefore be substantially different from that of dark state rhodopsin because it does allow for several light-dependent isomerization pathways, including isomerization of the protonated Schiff base. The same holds probably for Meta II, which had been shown to be photoconvertible to Meta III, Rho, and Iso (*4*, *12*, *28*, and our own observations).

At very low temperature (−183 °C), light-dependent C=N isomerization becomes the dominant reaction path of Meta III, indicating an inhibition of the competing C=C isomerizations under these conditions. Previous studies suggested that in dark state rhodopsin the quantum yield of isomerization is independent of temperature and that the excited state of the C11=C12 torsional surface is barrierless (*29*). Due to differing wave packet dynamics the quantum yield for isorhodopsin isomerization is considerably lower (*30*), and its temperature dependence is thought to reflect a small activation barrier of 0.2 kcal/mol on the torsional surface of the C9=C10 cis to trans isomerization (*29*, *31*). The excited-state torsional potential surface of both C11=C12 and C9=C10 in Meta III is probably characterized by a relatively high activation barrier, which leads to decreasing quantum yield of C=C isomerizations at lower temperature. In contrast, the excited state of the C=N torsional surface of Meta III is characterized by a lower barrier or is even barrierless such that at low temperatures the C=N isomerization becomes the predominant photoreaction path.

A second interesting point is the formation of the 15-syn isomers of the 11-cis and 9-cis chromophores, which are described here for the first time. Their thermal isomerization to the respective 15-anti isomers indicates that they are energetically unfavorable in the protein environment, implying a pronounced incompatibility with the protein binding pocket, even under conditions that would allow the transition of the protein to the more flexible Meta II conformation. Thermal C=N isomerization seems therefore to be a general pathway to relieve unfavorable chromophore−protein interactions in the binding pocket of rhodopsin isomers.

An intriguing difference between rhodopsin as a visual pigment and the proton pump bacteriorhodopsin is the thermal isomerization of the chromophore of bateriorhodopsin to recover the ground state after photolysis, thereby establishing a photocycle (*32*). The physiological necessity for this difference is quite obvious, yet the molecular mechanisms by which it is achieved are still not completely understood. In the bacteriorhodopsin photocycle, the 13-cis 15-anti isomer, which is produced following irradiation of the all-trans 15-anti isomer of the ground state, is not stable and experiences a deprotonation process to produce the M

intermediate. Bacteriorhodopsin differs from rhodopsin in its ability for a fast Schiff base reprotonation process, which subsequently leads to the N/O intermediates, which are significantly red-shifted compared to the photoproducts of rhodopsin (*33*). The thermal reisomerization to the all-trans isomer is thought to coincide with the N (550 nm) to O (640 nm) transition in wild-type bacteriorhodopsin, while studies on the L93A mutant even suggest an isomerization after the transition to the more red-shifted O state (*34*), implying the possible existence of two O states (*35*). In the regular photoproduct cascade of rhodopsin, such reprotonation and formation of equivalent red-shifted intermediates are not observed, which may be the reason for lack of a thermal C11═C12 back isomerization. However, even for rhodopsin photoproducts with protonated Schiff bases, such as Meta I or Meta II_{480} (a Meta II species with protonated Schiff base that is formed in the presence of suitable anions (*36*)), thermal C═C back isomerization to the 11-cis isomer of the initial dark state does not take place (*5*, *6*), indicating that protonation of the Schiff base may be a requirement yet is not sufficient for this step. This can probably be accounted for by the already intrinsically high energy barrier for C11═C12 trans to cis isomerization and the lack of a red-shifted intermediate that would lower this barrier. In addition, specific protein−chromophore interactions certainly play an important role in tuning the energy barriers for thermal isomerizations.

ACKNOWLEDGMENT

We would like to thank B. Mayer for the protein preparations and W. Sevenich, W. D. Schielin, P. Merkt, and K. Zander from our shops.

Supporting Information is available on chromophore band assignment and on flash photolysis of Meta III in the UV−visible region. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI049182Q