Formation of Meta III during the Decay of Activated Rhodopsin Proceeds via Meta I and Not via Meta II†

Reiner Vogel,*,‡ Friedrich Siebert,‡ Xin-Yu Zhang,§ Guibao Fan,§ and Mordechai Sheves§

Biophysics Group, Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Strasse 9, D-79104 Freiburg, Germany, and Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: Thermal isomerization of the retinal Schiff base C═N double bond is known to trigger the decay of rhodopsin's Meta I/Meta II photoproduct equilibrium to the inactive Meta III state [Vogel, R., Siebert, F., Mathias, G., Tavan, P., Fan, G., and Sheves, M. (2003) *Biochemistry 42*, 9863−9874]. Previous studies have indicated that the transition to Meta III does not occur under conditions that strongly favor the active state Meta II but requires a residual amount of Meta I in the initial photoproduct equilibrium. In this study we show that the triggering event, the thermal isomerization of the protonated Schiff base, is independent of the presence of Meta II and occurs even under conditions where the transition to Meta II is completely prevented. We have examined two examples in which the transitions from Lumi to Meta I or from Meta I to Meta II are blocked. This was achieved using dry films of rhodopsin and rhodopsin reconstituted into rather rigid lipid bilayers. In both cases, the resulting fully inactive room temperature photoproducts decay specifically by thermal isomerization of the protonated Schiff base C═N double bond to an all-*trans* 15-*syn* chromophore isomer, corresponding to that of Meta III. This thermal isomerization becomes less efficient as the conformation of the respective photoproduct approaches that of Meta II and is fully absent in a pure Meta II state. These results indicate that the decay of the Meta I/Meta II photoproduct equilibrium to Meta III proceeds via Meta I and not via Meta II.

The visual pigment rhodopsin is the light receptor responsible for dim light vision in the rod photoreceptor cells of vertebrates (*1*). As a G protein-coupled receptor (GPCR),[1] rhodopsin has seven helices that form a membrane-spanning helix bundle. Rhodopsin's function as a light receptor is mediated by an 11-*cis* retinal chromophore, which is covalently bound to a lysine residue on helix 7 and accommodated in a membrane-embedded binding pocket formed by the helix bundle. Absorption of a photon isomerizes the chromophore to an all-*trans* geometry and initiates thereby protein conformational changes that lead within milliseconds to an active receptor species termed Meta II. The active Meta II state (λ_{max} 380 nm) stands in a rapid (millisecond), pH-dependent conformational equilibrium with its still inactive precursor, Meta I (λ_{max} 480 nm), in which Meta II is favored by low pH or high temperature (*2*). A detailed understanding of the mechanisms underlying receptor activation on a molecular level is of particular importance and the topic of current research (*1*, *3*−*8*).

Decay of the Meta I/Meta II pool is much slower compared to its formation and is accomplished on a time scale of minutes mainly by hydrolysis of the retinal Schiff base and dissociation of all-*trans*-retinal from the chromophore binding pocket. The resulting apoprotein opsin was also found to form a conformational equilibrium between an active, low-pH conformation and an inactive, high-pH conformation (*9*). The p*K* of this conformational equilibrium is, however, shifted by more than 4 units from around 8.5 for the Meta I/Meta II pool to around 4.0 for the opsin states at 30 °C. At physiological temperature and neutral pH, the Meta I/Meta II equilibrium is thus largely on the active Meta II side, while the opsin conformational equilibrium is fully on the side of the inactive conformation. Therefore, under these conditions, decay of the Meta I/Meta II pool to the opsin pool by dissociation of retinal corresponds to receptor deactivation (*10*).

In addition to this hydrolysis reaction, another deactivation pathway exists, which contributes predominantly at alkaline pH to receptor deactivation. This pathway leads to formation of a species termed Meta III, which absorbs at 470 nm (*2*, *8*, *11*−*16*). In contrast to opsin as the final product of the Schiff base hydrolysis pathway, the retinal chromophore is still covalently bound in its binding pocket in Meta III, in an orientation similar to that in Meta II or the dark state (*14*, *15*). The triggering event in the transition to inactive Meta III has been shown to be a thermal isomerization of the protonated Schiff base C═N double bond of the all-*trans* chromophore from *anti* (*trans*) to *syn* (*cis*) (*11*). The isomeric

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* Corresponding author. Phone: +49 761 203 5391. Fax: +49 761 203 5390. E-mail: reiner.vogel@biophysik.uni-freiburg.de.

‡ Albert-Ludwigs-Universität Freiburg.

§ Weizmann Institute of Science.

[1] Abbreviations: GPCR, G protein-coupled receptor; FTIR, Fourier transform infrared; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

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state of the chromophore switches therefore from all-*trans* 15-*anti* in the Meta I/Meta II photoproducts to all-*trans* 15-*syn* in Meta III. The latter isomer seems to be more compatible with an inactive protein conformation and allows therefore the protein to relax to an inactive resting state.

This recent discovery of a thermal chromophore isomerization being the triggering event in the transition to Meta III has in our view contributed considerably to a better understanding of the mechanisms of receptor deactivation. Still there are open questions regarding the triggering event itself. The transition to Meta III starts from the Meta I/Meta II photoproduct equilibrium. But in which of these states does the thermal isomerization actually take place? We recently conjectured that the triggering event could be catalyzed by the protein conformation of Meta I such that the decay of the Meta I/Meta II conformational equilibrium proceeds via Meta I (*11*).

This suggestion was based on several observations: First, formation of Meta III as a room temperature decay product is diminished as the pH and hence the contribution of Meta I in the initial Meta I/Meta II photoproduct equilibrium are decreased. Second, virtually no formation of Meta III was detected in rhodopsin prepared in the detergent dodecyl maltoside, which forms fluid protein−detergent micelles that strongly favor Meta II. In the detergent digitonin, on the other hand, under otherwise identical conditions, substantial formation of Meta III could be observed. Digitonin is known to form rather rigid micelles and is the detergent of choice to stabilize Meta I. Third, it was shown that the transition to Meta III is inhibited in the presence of transducin-derived peptides that are known to stabilize the active conformation of Meta II (*13*). These experiments indicated that formation of Meta III correlates with the amount of Meta I present in the photoproduct equilibrium. While these results strongly support our proposal of a Meta I-dependent decay to Meta III, they cannot prove the Meta II independence of this thermal decay.

We therefore sought for an experimental setup by which we could show that the triggering event of the decay, the thermal isomerization of the protonated Schiff base, may occur also under conditions where the transition to the active state Meta II is *completely* inhibited. Formation of Meta II depends on a variety of parameters, such as temperature, pH, presence of ions, hydration, pressure, or properties of the embedding environment (e.g., fluidity and curvature of the lipid membrane bilayer). Changes in pH, pressure, or salts may shift the Meta I/Meta II equilibrium toward Meta I but do not fully inhibit the transition to Meta II at room temperature. This can be achieved, however, as shown in previous studies, in dry films of rhodopsin (*17*, *18*) or by replacing the relatively fluid environment offered by the disk membranes by bilayers of long-chain saturated lipids (*19*−*21*). We show that, under either of the two conditions, the transition to Meta II is completely inhibited even at room temperature and that the inactive photoproducts, that are formed instead, decay by thermal C=N isomerization to a 15-*syn* species, analogous to that in the decay to Meta III.

MATERIALS AND METHODS

Pigment Preparation. Rhodopsin in washed disk membranes was prepared from cattle retinae according to standard procedures (*22*) and stored in 1 mM phosphate buffer at pH 6.5 at −80 °C. Disk membranes used for Meta III experiments were adjusted to pH 8.0 in 1 mM BTP (bis-tris-propane).

For pigment reconstitution in lipids of defined composition, rhodopsin was purified and delipidated on concavalin A in 1.2% *n*-octyl β-D-glucoside (β-OG) (*23*) and mixed with the respective lipid (Sygena, Liestal, Switzerland), dissolved in 1.2% β-OG, at a molar ratio of 1:200. After short sonication and incubation for 2 h on ice, this mixture (10 nmol of rhodopsin in a total volume of 100−400 μL) was dialyzed over a period of at least 36 h against a volume of 40 mL of 1 mM phosphate buffer, pH 6.5, at 4 °C in a microflow-through system using microdialyzer units with a 7 kDa MWCO (Pierce Biotechnology, Rockford, IL) and a buffer flow rate of 150 mL/h. After dialysis, samples were pelleted and stored at −80 °C.

Sample Preparation. Unless stated differently, we used fully hydrated sandwich samples throughout this study. These samples were prepared as described elsewhere (*6*, *9*) with 0.5 nmol of pigment (disk membranes or reconstituted membranes) and 20 μL of 200 mM buffer. We used citric acid, MES (2-*N*-morpholinoethanesulfonic acid), and BTP, in overlapping ranges. Dry films were prepared as described in Results.

FTIR Spectroscopy. FTIR spectroscopy was performed with a Bruker IFS 28 spectrometer with an MCT (mercury cadmium telluride) detector and a temperature-controlled 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. Samples were photolyzed for 20 s by a 150 W slide projector equipped with a heat filter, through a fiber optics and color or cutoff filters as specified.

Time-resolved experiments were done at 4 cm^{-1} resolution in rapid-scan mode with a scanner velocity of 15 (160 kHz) with, on average, 65 ms per scan. Spectra used for evaluation comprised at least 48 scans per experiment. The samples were photolyzed either by a short 2 s illumination through a GG495 filter or by a 30 ns laser pulse at 477 nm 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.

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. Illumination was similar to that in the IR experiments. Spectra were analyzed using Bruker's Opus software for OS/2 (Bruker, Ettlingen, Germany) and Statistica (StatSoft, Hamburg, Germany).

G Protein Activation Assay. Transducin was isolated from illuminated, osmotically shocked rod outer segments from bovine retinae and purified by hexylagarose chromatography essentially as described previously (*24*, *25*). Purified transducin was dialyzed against 10 mM phosphate buffer, pH 7.0, containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 50% (w/v) glycerol, and stored at −20 °C. Transducin activation was assayed by monitoring its intrinsic tryptophan fluorescence changes upon nucleotide exchange (*26*) in a custom-made instrument with excitation by a deuterium lamp and a 290−

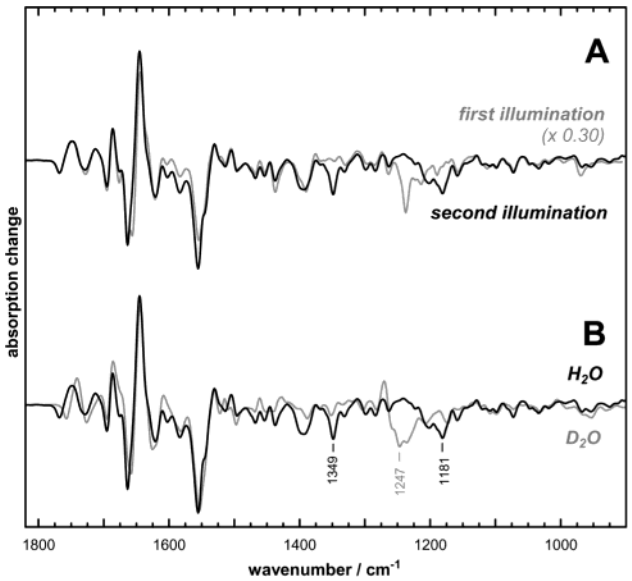


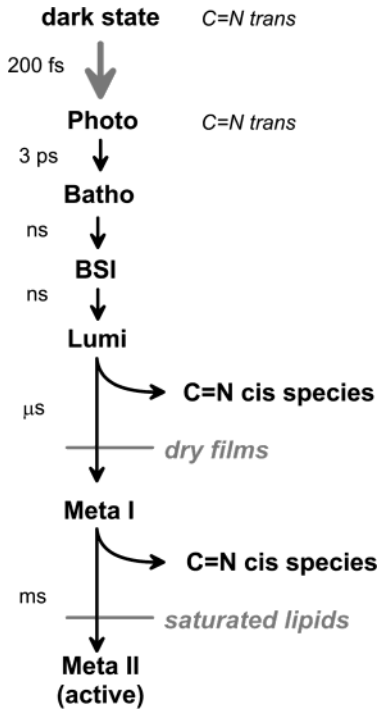
FIGURE 1: Meta III difference spectra. (A) An FTIR difference spectrum *after* minus *before illumination* was recorded of the photoreaction from the initial dark state to the Meta I/Meta II photoproduct pool (gray, scaled by a factor of 0.30), induced by a 30 s illumination with a >530 nm long-pass filter at 30 °C and pH 8.0. Under these conditions, Meta I contributes about 20% to the photoproduct equilibrium. 20 min later, after full decay of the photoproducts to opsin and Meta III, another difference spectrum of the photoreaction from Meta III back to the Meta I/Meta II pool was recorded (black), induced by a second 30 s illumination with a >475 nm long-pass filter. (B) Comparison of difference spectra of the transition from Meta III to the Meta I/Meta II pool obtained in H_2O [black, as in (A)] and D_2O (gray), showing the H/D sensitivity of the NH bending mode at 1349 cm^{-1} and of the C14−C15 stretching mode at 1181 cm^{-1} (both in H_2O).

310 nm band-pass filter. Fluorescence was detected in 90° geometry from a 1 mL thermostated fluorescence cuvette under continuous stirring through a >340 nm long-pass filter by a photomultiplier in single-photon counting mode. The assay was performed at 20 °C in 900 μL of 20 mM MES, pH 6.0, 150 mM NaCl, and 2 mM $MgCl_2$ with 400 nM transducin and catalytic amounts of pigment (either in disk membranes or as lipid-reconstituted pigment). The pigment was photolyzed for 10 s (>530 nm long-pass filter), and after having recorded a fluorescence baseline for 100 s, nucleotide exchange was started by addition of 50 μL of nonhydrolyzable GTPγS to a final concentration of 10 μM. The fluorescence traces were corrected for background intensity of the system and the dilution effect and then normalized to the initial fluorescence intensity.

RESULTS

Formation and Photoreaction of Meta III. Illumination of the dark state of rhodopsin (λ_{max} 500 nm) at 30 °C leads to the Meta I/Meta II photoproduct equilibrium (λ_{max} 480 and 380 nm, respectively), which is at pH 8.0 with about 80% Meta II more on the Meta II side (Figure 1A, gray spectrum). With a half-time of about 2 min, this photoproduct equilibrium decays thermally, yielding mainly opsin and all-*trans*-retinal (λ_{max} 380 nm), as well as about 30% Meta III (λ_{max} 470 nm). Meta III can be selectively reverted to Meta II by a second illumination after 20 min with a >475 nm long-pass filter (Figure 1A, black spectrum), allowing thus the exclusive study of Meta III, despite the fact that Meta III

Scheme 1



cannot be obtained as a pure species, but only in combination with opsin (*11*, *13*).

In the difference spectrum of the second illumination (Figure 1A, black spectrum) the bands corresponding to Meta III are negative, and those of the Meta I/Meta II photoproducts are positive. The negative bands at 1349 cm^{-1} and at 1181 cm^{-1} (Figure 1B, black spectrum) are characteristic bands of Meta III and have been assigned to the NH bending vibration of the protonated Schiff base and the C14−C15 stretching mode of the chromophore, respectively (*11*). Both bands are altered following H/D exchange at the Schiff base (Figure 1B), which can serve as a marker for the configuration of the protonated Schiff base C15═N bond (*27*) and which was used to assign an all-*trans* 15-*anti* chromophore geometry for Meta III (*11*).

Blocking Rhodopsin's Sequence of Photointermediates. In the following, we will examine two possibilities to block rhodopsin's sequence of photointermediates, either between Lumi and Meta I (in dry films) or between Meta I and Meta II (in rigid lipid bilayers; Scheme 1). In both cases, the transition to the active state will be fully prevented even at 30 °C, allowing us to study the decay of the resulting inactive photoproduct state at or close to room temperature.

The Meta I−Meta II Block in Rigid Lipid Bilayers. Previous studies have shown that formation of Meta II may be inhibited upon replacing the fluid environment of the disk membrane by the more rigid bilayer formed by lipids with long saturated acyl chains (see, e.g., refs *19*−*21*). We reconstituted purified rhodopsin into dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) with saturated acyl chains with 14, 16, and 18 carbons, respectively. The gel to liquid-crystalline phase transitions of pure lipid bilayers formed by these lipids were reported to be at 24.0, 41.5, and 54.3 °C, respectively (*28*), and may be shifted to slightly higher temperatures for recombinant membranes containing rhodopsin (*29*).

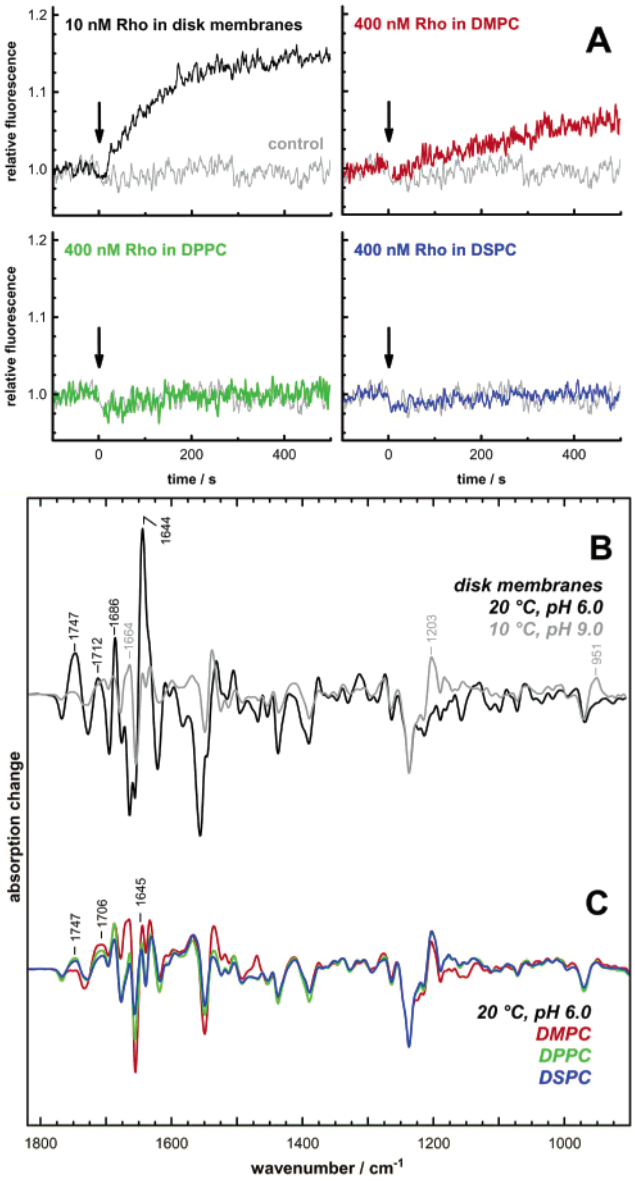


FIGURE 2: Reconstitution into long-chain saturated PC lipids blocks the transition to the active state, Meta II. (A) A fluorescence-based G protein activation assay was performed at 20 °C and pH 6.0 for a 10 nM suspension of rhodopsin in disk membranes (black) and 400 nM suspensions of rhodopsin reconstituted into DMPC (red, 14 carbons), DPPC (green, 16 carbons), or DSPC (blue, 18 carbons). The arrow indicates the start of the reaction by addition of nonhydrolyzable nucleotide; the control experiment (gray) was performed in the absence of pigment. (B) FTIR reference spectra of the transition from the dark state to active Meta II with characteristic marker bands (black, obtained from disk membranes at 20 °C, pH 6.0) and, in addition, from the dark state to inactive Meta I (gray, obtained from disk membranes at 10 °C, pH 9.0). (C) Corresponding difference spectra of rhodopsin reconstituted into DMPC (red), DPPC (green), and DSPC (blue) reveal a photoproduct, where typical Meta II marker bands are missing or less intense.

The activity of photolyzed reconstituted rhodopsin was tested by a real-time transducin fluorescence assay at 20 °C and pH 6.0. Control experiments were carried out with 10 nM photolyzed rhodopsin in disk membranes as positive control and in the absence of pigment as negative control (upper left panel in Figure 2A). We tested the activity of the reconstituted pigment at a 40-fold higher concentration and found marginal activity (<1%) for rhodopsin in DMPC and no detectable activity at all for rhodopsin in either DPPC or DSPC (Figure 2A, other panels). In additional experiments, rhodopsin photoproducts in DPPC or DSPC furthermore revealed no extra Meta II effect upon addition of a transducin high-affinity peptide analogue, peptide 23 (*30*), despite the fact that binding of the peptide was not inhibited (*4*; unpublished data).

We further examined the photoproduct produced in the reconstituted membranes at 20 °C and pH 6.0 by FTIR difference spectroscopy. In Figure 2B, we show a control spectrum obtained from disk membranes that produce Meta II under these conditions and, for comparison, a Meta I spectrum obtained at lower temperature and more alkaline pH. The reconstituted membranes yield a photoproduct minus dark state difference spectrum with photoproduct bands that are rather Meta I-like than Meta II-like (Figure 2C). Particularly, the positive Meta II marker bands at 1747 and 1644 cm^{-1} are considerably reduced in the reconstituted samples. The positive fingerprint band at 1203 cm^{-1} indicates that the Schiff base is largely protonated in these samples, in agreement with UV−visible spectra (not shown). The DMPC sample differs from the other two reconstituted samples in the range above 1700 cm^{-1}, in the amide I range around 1650 cm^{-1}, and in the amide II range around 1550 cm^{-1}. Similar difference spectra were obtained during the partial unfolding of rhodopsin at very low pH, leading to the formation of an only loosely packed helix bundle (*31*, *32*). Together with the time evolution of the DMPC photoproduct spectra (not shown), this could indicate that the modified lipid environment severely perturbs the native fold of the protein in the intrinsically less stable photoproduct state, leading to a partial loss of the tertiary structure of the protein. The very weak but detectable activity of the DMPC-reconstituted pigment could be due to a very small fraction of the photoproduct being present in the natively folded Meta II state. It could, however, as well be fully accounted for by the partially unfolded photoproduct state, as presentation of two of rhodopsin's cytoplasmic loops on a rather floppy scaffold was shown to be sufficient for at least some activity (~1% of Meta II) toward G protein (*33*).

In the case of the DPPC and DSPC samples, the photoproduct does not unfold during decay. Exemplary, we followed the decay of the photoproduct in DPPC at 20 °C and pH 7.0 (Figure 3A). Experiments carried out with DSPC-reconstituted pigment yielded similar results (not shown). Besides changes in the amide range and above 1700 cm^{-1}, indicating conformational changes during the decay, decay bands can be observed at 1348 and 1180 cm^{-1} that evolve with a time constant of 4.0 min (Figure 3C; note that decay bands are negative in this representation). Similar bands at 1349 and 1181 cm^{-1} were found to be characteristic fingerprint bands of the all-*trans* 15-*syn* chromophore of Meta III (compare Figure 1B) and represent the NH bending and C14−C15 stretching vibration, respectively (*11*). This would imply that at least part of the photoproduct obtained in DPPC decays by a thermal isomerization of the protonated Schiff base C=N double bond.

To further corroborate this, we first examined the photoreversibility of this decay. We applied a second illumination (30 s, >475 nm) both to a sample 30 s after the first illumination (i.e., after the time needed to record a first photoproduct spectrum) and to a sample that had decayed

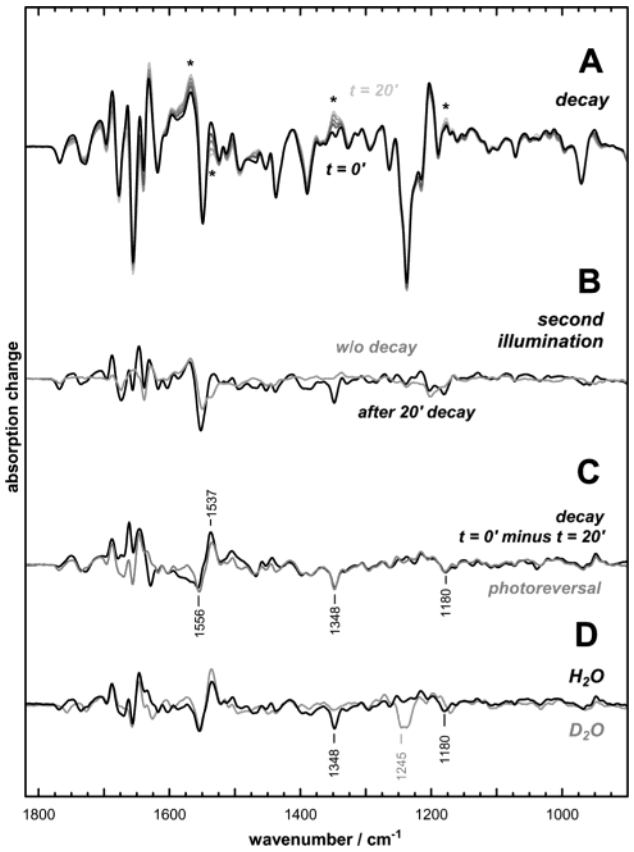


FIGURE 3: The inactive, blocked photoproduct formed by rhodopsin in rigid lipid bilayers decays by a thermal C=N isomerization step. (A) Rhodopsin reconstituted into DPPC was illuminated (30 s, >530 nm) at 20 °C and pH 7.0 and *photoproduct* minus *initial dark state* spectra were recorded at $t = 0$, 2, 4, 10, and 20 min. (B) After 20 min decay, the decay product was illuminated again (30 s, >475 nm, black spectrum). In a control experiment, this second illumination was applied immediately after having recorded the first spectrum after the first illumination (termed w/o decay; gray). (C) The gray spectrum in (B) was subtracted from the black spectrum. The correspondence between the resulting difference spectrum (*photoreversal* in gray) and the decay spectrum ($t = 0$ minus $t = 20$ min, in black) indicates that the thermal decay is photoreversible. (D) The H_2O/D_2O sensitivity of the 1348 and 1180 cm^{-1} bands in the photoreversal spectra show that the decay of the initial photoproducts involves a C=N isomerization step to a 15-*syn* chromophore geometry.

for 20 min after the first illumination and recorded the respective light-induced difference spectra of these second illuminations. By subtracting the two difference spectra for each sample, we can select the changes that are due only to photoreversal of the decay product (Figure 3B). From the coincidence of the characteristic chromophore fingerprint bands in the resulting photoreversal spectrum and in the decay spectrum ($t = 0$ minus $t = 20$ min after photolysis), we can conclude that the thermal decay can be photoreverted, similarly as the decay of the Meta I/Meta II pool to Meta III (*2*, *11*, *13*, *34*).

Second, we wanted to determine explicitly the C=N isomeric state by examining the H/D effect on the 1348 and 1180 cm^{-1} bands of the decay product. We have therefore repeated this experiment in D_2O (Figure 3D) and observed the same H/D effects on these bands as on those of Meta III, in particular a disappearance of the band at 1348 cm^{-1} and an upshift of the band at 1181 cm^{-1} to 1245 cm^{-1} in D_2O (compare Figure 3D to Figure 1B). The isomeric state

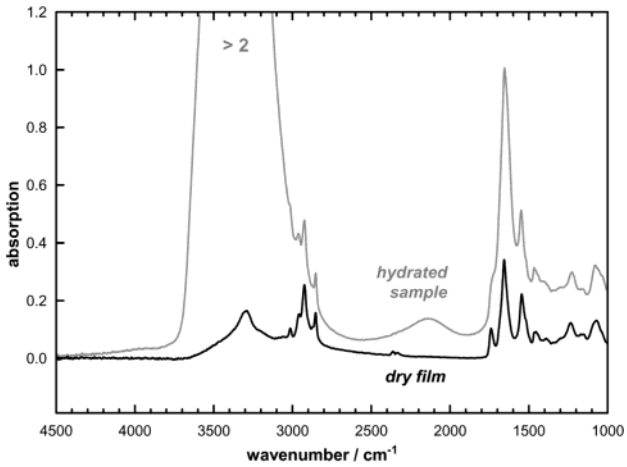


FIGURE 4: Dry films of rhodopsin. Rhodopsin in disk membranes (0.5 nmol of pigment) was dried under a flow of nitrogen onto IR windows and mounted into the airtight sample holder without hydration. Resulting IR absorption spectra of the dry film (black) lack the strong water absorption bands observed in our fully hydrated standard sandwich samples (see text for details).

of the Schiff base is therefore *syn* (or *cis*), and the decay of the photoproduct in the rigid lipid bilayers proceeds by a thermal C=N isomerization to all-*trans* 15-*syn*, similarly as in the transition to Meta III.

The Lumi−Meta I Block in Dry Films. It was reported previously that dry films of rhodopsin do not support formation of the active state Meta II, but only that of Lumi, which decays further on, accompanied by a small blue shift (*17*, *18*, *35*). We have investigated this decay by both UV−visible and FTIR spectroscopy. We prepared dry films of rhodopsin by drying 10 μL of a 50 μM stock suspension of disk membranes (in 0.5 mM phosphate buffer, pH 6.5) under a stream of nitrogen and mounted the films in airtight sample holders. Dry films that were prepared after an additional washing step in distilled water prior to drying behaved identically in initial control experiments. In Figure 4, we show a representative absorption spectrum of such a dry film in comparison to that of a fully hydrated sandwich sample, which we use as standard samples for FTIR and UV−visible spectroscopy. As evidenced by the water OH stretching vibration around 3300 cm^{-1} and the OH bending vibration at 1650 cm^{-1}, as well as the Fermi resonance band at 2150 cm^{-1}, the water content in the dry film is extremely reduced.

Photolysis of such a dry film at −95 °C and similarly at −50 °C (Figure 5A,C) yields a photoproduct which can be considered as a regular Lumi state. At higher temperatures, the IR difference spectra change only marginally, and even at 30 °C, the structural marker bands of Meta II are still completely lacking. A closer examination reveals a small structural transition below 10 °C, such that the spectrum obtained at 10 °C is slightly different from the spectra obtained at lower temperatures and different as well from a Lumi spectrum obtained from hydrated samples (Figure 5B,D). Subtle band shifts can be observed for the HOOP mode at 946 cm^{-1} and in the amide II range around 1550 cm^{-1}, which are both more Meta I-like. In addition, small changes in the band pattern around 1700 cm^{-1} are detected, which are partially due to Glu122 on helix 3 interacting with His211 on helix 5 (*36*). The photoproduct obtained in the dry films at 10 °C is therefore no longer a pure Lumi state. However, does it already correspond to a Meta I state? Not

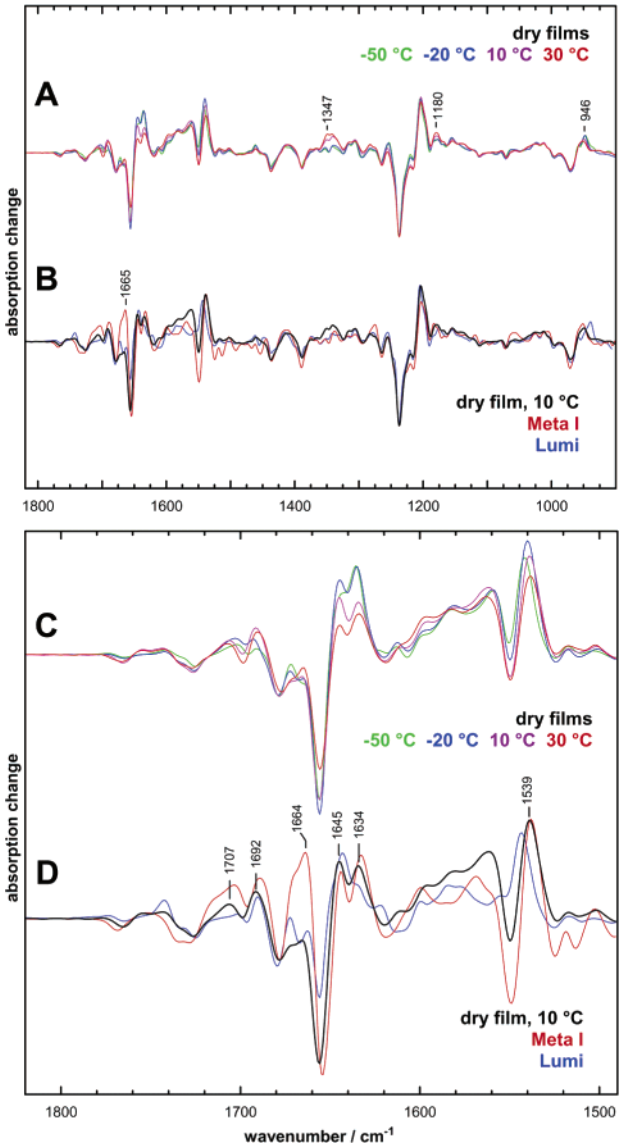


FIGURE 5: Rhodopsin's photocascade is blocked between Lumi and Meta I in dry films. (A) Light-induced difference spectra obtained from rhodopsin in dry films at −50, −20, 10, and 30 °C, induced by a 30 s illumination (>530 nm). The spectra obtained at 10 °C or higher deviate only slightly from the spectra obtained at lower temperature. (B) Comparison of the spectrum obtained at 10 °C to Lumi (−95 °C, pH 7) and Meta I (10 °C, pH 9.0) reference spectra obtained from (fully hydrated) sandwich samples reveals that the reaction cascade does not fully proceed to Meta I in the dry films, as evident, e.g., from the lack of the characteristic Meta I band at 1664 cm^{-1}. (C) and (D) are enlarged views of (A) and (B), respectively, to allow a closer examination of the carboxylic acid and amide I bands.

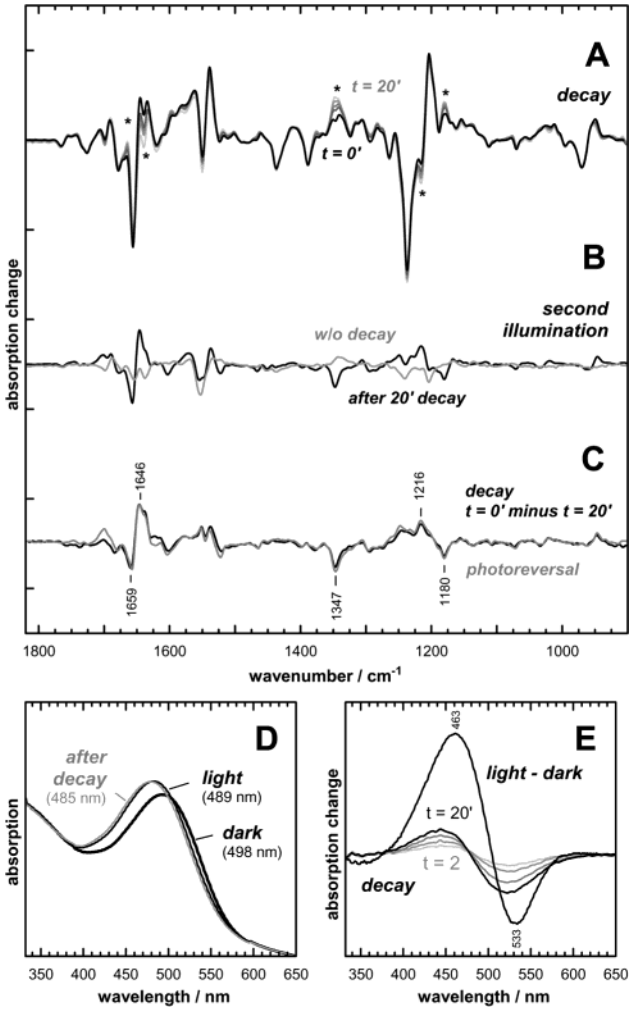


FIGURE 6: The inactive blocked photoproduct obtained in dry films decays as well by a thermal C=N isomerization step. (A) A dry film of rhodopsin was illuminated at 10 °C (30 s, >530 nm), and photoproduct minus dark state difference spectra were recorded at $t = 0$, 2, 4, 10, and 20 min during the subsequent decay process. (B) After 20 min decay, the decay product was illuminated again (30 s, >475 nm, black spectrum). In a control experiment, this second illumination was applied without decay, immediately after having recorded a single photoproduct spectrum (termed w/o decay; gray). (C) The gray spectrum in (B) was subtracted from the black spectrum. The correspondence between the resulting difference spectrum (*photoreversal* in gray) and the decay spectrum ($t = 0$ minus $t = 20$ *min*, in black) and the characteristic fingerprint bands indicates that the thermal decay involves a C=N isomerization step, which is photoreversible, similar to that shown in Figures 1 and 3. (D) UV−visible absorption spectra of an identical sample before (thick black) and after (thin black) illumination, as well as after 20 min of decay (thin gray), involving a slight blue shift of the absorption peak. Peak positions were assigned after subtraction of the scattering background. (E) The initial *after* minus *before illumination* difference spectrum was calculated for the experiment shown in (D). The decay of this photoproduct is presented in the form of decay spectra (spectra obtained at $t = 2$, 4, 10, and 20 min minus the spectrum obtained immediately after illumination), showing the transition to the blue-shifted final product.

entirely, as the spectrum completely lacks the amide I marker band of Meta I at 1664 cm^{-1}, which is observed neither at −10 or 0 °C (not shown) nor at higher temperature (Figure 5C, red spectrum at 30 °C). Furthermore, the HOOP band difference band of the chromophore at −971/+951 cm^{-1} is reduced compared to native Meta I. Thus, dry films do not allow the complete structural transition to Meta I even at room temperature. Instead, a photoproduct state with features corresponding to both Lumi and Meta I is formed above 0 °C.

The small positive bands at 1347 and 1180 cm^{-1} in the spectra obtained at 10 °C and even more pronounced at 30 °C in Figure 5A indicate already that this photoproduct is not stable. This has been closer examined in Figure 6A at 10 °C, in which we have indeed observed a decay of the photoproduct, producing chromophore marker bands similar to those described in the previous sections for the all-*trans* 15-*syn* chromophore. We correspondingly assign the bands at 1347 and 1180 cm^{-1} to the NH bending and the C14−C15 stretching vibrations of this chromophore, respectively.

The time constant of the decay to this all-*trans* 15-*syn* species was 3.9 min (at 10 °C). Similarly as with the DPPC samples, we have examined whether the thermal decay is photoreversible (Figure 6B). Comparison of the decay and the photoreversal spectra in Figure 6C indicates that this is indeed the case. This decay of the photoproduct to a 15-*syn* species can be observed over a wide temperature range already from −10 °C on, where it is a very slow process (data not shown), up to 30 °C, where a large part of the 15-*syn* species is already formed within the 90 s required for illumination and data acquisition (compare Figure 5A). In DPPC samples, on the other hand, no significant decay was observed at 0 °C, but only at higher temperature (not shown).

In Figure 6D,E, UV−visible spectra of the photoreaction and of the subsequent decay of the dry film photoproduct at 10 °C are presented. These spectra reveal that the initial dry film photoproduct is blue shifted by 9 nm from the dark state, from 498 to 489 nm. The decay product obtained after thermal isomerization of the protonated Schiff base C═N is further blue shifted by 4 nm to 485 nm. The absorption maxima have been determined from the spectra in Figure 6D following subtraction of the scattering background. The assignment does however not account for rhodopsin and isorhodopsin contributions, which are present to some extent in the photoequilibrium mixture. The UV−visible data agree very well with earlier spectra obtained from dry gelatin films of rhodopsin published by Wald and co-workers (*17*), who reported a 6 nm blue shift and 10% absorption increase of the photoproduct, followed by a further 5−10 nm blue shift and slight decrease of the absorption during the decay, as well as with spectra obtained by Rothschild and colleagues (*35*). Maeda and co-workers published more recently difference spectra (*18*), which allowed a more detailed comparison, revealing slight differences to our UV−visible data, indicating that their sample conditions were not exactly similar to ours. In this latter publication, the authors found a thermal decay of an initially produced Lumi-like intermediate to an intermediate featuring some, but not all, characteristics of Meta I.

Comparison between Dry Films and Rigid Bilayers. For a more exhaustive analysis of the kinetics of the thermal isomerization in dry films and rigid bilayers, we used time-resolved FTIR spectroscopy in the rapid-scan mode. To allow a reasonable time resolution, the samples were photolyzed at 20 °C by a short 2 s illumination (>495 nm), which also minimized contributions of isorhodopsin being otherwise present under photostationary conditions. Alternatively, the samples were photolyzed by a laser pulse from a dye laser at 477 nm. Both methods gave similar results, yet the degree of photoconversion was considerably lower with laser excitation.

For an analysis of the decay, we monitored the absorption increase at 1348 cm^{-1} as a function of time (Figure 7). In DPPC samples, the kinetics of the decay could be reproducibly fitted by a single exponential with a time constant of 4.0 min. In dry films, on the other hand, the results varied between 25 and 60 s, presumably depending on the precise water content in the sample. Furthermore, the quality of the fit for the dry films was considerably improved by using two exponentials with time constants of 9.1 ± 2.5 and 101 ± 30 s and about equal amplitudes (mean values ± standard deviation of three independent experiments), implying that the initial photoproduct might be not homogeneous. Such an inhomogeneity might be due to a phase separation in the lipid−protein system induced by the dehydration. For comparison, formation of Meta III from the Meta I/Meta II equilibrium in well-hydrated native membranes proceeds with an apparent time constant of 4.8 min at 20 °C and pH 8.5.

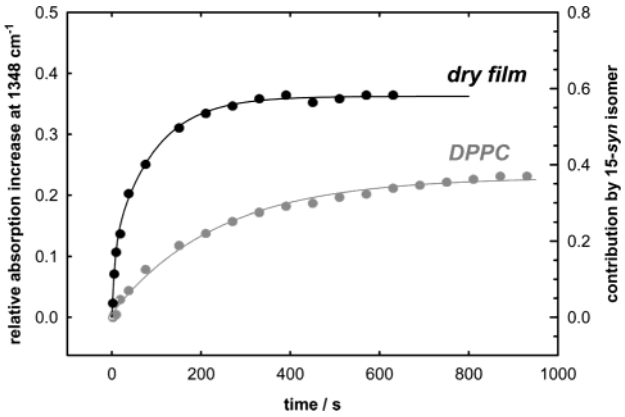


FIGURE 7: Kinetics of the thermal isomerization and position of the C═N isomeric equilibrium in dry films and rigid bilayers. Dry films of rhodopsin and rhodopsin in DPPC samples were photolyzed for 2 s (>495 nm) at 20 °C, and the time evolution of the photoproduct spectra was recorded by time-resolved FTIR spectroscopy. Evaluation of the relative absorption increase at 1348 cm^{-1}, normalized by the intensity of the depletion band of rhodopsin at 1238 cm^{-1} (left ordinate), yields an apparent time constant of 4.0 min for the thermal isomerization in the case of the rigid bilayer sample. Using a suitable scaling factor, the contribution of 15-*syn* species in the final equilibrium is estimated as approximately 35% (right ordinate; see text for details). In the case of the dry films, the thermal isomerization was considerably faster, and the contribution by the 15-*syn* decay product in the final equilibrium was increased to about 60%.

To quantitatively assess the amount of 15-*syn* product being produced during the thermal decay of the initial 15-*anti* photoproduct, we compared the intensities of marker bands of Meta III at 1348 cm^{-1} and dark state rhodopsin at 1238 cm^{-1} in spectra of their respective transitions to Meta II, using Meta II bands as internal calibration (compare Figure 1A or Figure 2 in ref *11*). This analysis yielded a scaling factor of 0.63 for the ratio of the intensity of the 15-*syn* marker band of Meta III to that of the dark state band. By normalizing the intensity increase at 1347 cm^{-1} during the decay by the intensity of the 1238 cm^{-1} depletion band of rhodopsin and multiplying it with this scaling factor, we obtain a rough estimate of the relative amount of the 15-*syn* species present at the end of the decay. This analysis indicates that the decay leads to a thermal equilibrium between 15-*anti* and 15-*syn* species, implying that the thermal isomerization is reversible, in keeping with previous results on Meta III (*15*, *16*, and our own unpublished observations). In the dry films, the 15-*syn* isomer contributes 64 ± 6% to the final isomeric equilibrium (mean ± SD of three experiments) (Figure 7). In DPPC samples, on the other hand, only about 34% of the photoproduct adopts the 15-*syn* configuration in the final equilibrium. This equilibrium mixture is stable, such that no irreversible decay of the pigment by hydrolysis to opsin and all-*trans*-retinal is observed during the time scale of the experiments.

DISCUSSION

Already in 1963, George Wald and co-workers conjectured that the deactivating transition from rhodopsin's active state Meta II to Meta III (termed by them "465 mμ dark product of metarhodopsin II") may be triggered by a thermal isomerization of one of the double bonds of the retinal chromophore (*2*). Their proposal was based on the observations that Meta III can be photoreverted to Meta II and that Meta II, on the other hand, can be photoconverted to a mixture consisting of mainly Meta III and some contributions of isorhodopsin (9-*cis*) and dark state rhodopsin (11-*cis*). They proposed therefore an isomerization of a double bond different from C9═C10 and C11═C12 to trigger the transition to Meta III. To our knowledge, this proposition had neither been confirmed nor really challenged since then. In the past years, an increasing interest in rhodopsin's deactivation reactions emerged, which have as well been studied more intensely by biophysical methods (e.g., refs *9* and *12*−*14*). Recently, we were able to characterize the chromophore structure in Meta III by combining FTIR spectroscopy, isotopic labeling, quantum chemical calculations, and retinal extraction experiments (*11*). We have shown that the transition from the active state Meta II, which forms together with still inactive Meta I the pH- and temperature-dependent Meta I/Meta II photoproduct pool, to Meta III is triggered by a thermal isomerization of the protonated Schiff base C15═N double bond. The chromophore isomeric state changes therefore from all-*trans* 15-*anti* in rhodopsin's direct photoproducts, including Meta II (*11*), to all-*trans* 15-*syn* in Meta III. As Meta III is an inactive state, the 15-*syn* isomer seems to be more compatible with an inactive protein conformation compared to the 15-*anti* isomer of the Meta I/Meta II pool, which represents, in a pharmacological sense, an agonist.

In our previous characterization of Meta III, we proposed a model in which the formation of Meta III from the Meta I/Meta II photoproduct pool proceeds via the Meta I state and conjectured that the thermal isomerization of the protonated Schiff base is catalyzed by the inactive protein conformation of Meta I (*11*). This proposition is based on several experimental results that all showed that the yield of Meta III as a decay product correlates with the amount of Meta I present in the initial photoproduct equilibrium, as detailed in the introduction. These results implied that the presence of Meta I is required for the transition to Meta III. In this study, we provide evidence that, on the other hand, the presence of Meta II is not required to induce the thermal isomerization of the chromophore. We show this by means of two interesting examples in which rhodopsin's sequence of photointermediates is blocked before the transition to the active state, Meta II.

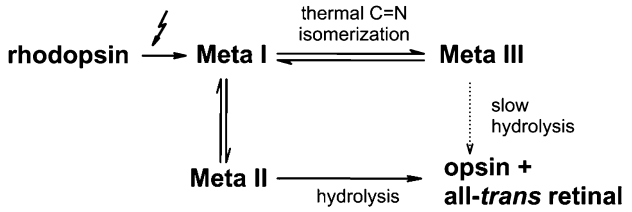
In the first example, in dry films, rhodopsin's sequence of photointermediates is perturbed during the transition from Lumi to Meta I (Scheme 1) (*17*, *18*, *35*). Infrared difference spectra indicate that the resulting photoproduct state possesses already many features of Meta I but completely lacks an amide I difference band at 1664 cm^{-1}, which is a structural marker band of Meta I. It is interesting that dry conditions can influence already the transition to Meta I. Possibly, the recently proposed counterion switch from Glu113 to Glu181 (*7*) during the transition from Lumi to Meta I, which is thought to involve a partially water-mediated hydrogen-bonding network between the two residues, is perturbed in the dry films. On the other hand, removal of structural water, as achieved previously by vigorous drying (*37*, *38*), results in severe changes of the spectral properties of the pigment, in particular in a partial deprotonation of the retinal Schiff base already in the dark state, which was not observed after our apparently milder drying procedure. Alternatively, the perturbed Lumi to Meta I transition in the dry films may be due to the effects of dehydration on the surrounding native lipid bilayer. Dehydration of lipid bilayers may result in a transition from the liquid-crystalline to the solid gel phase, often via a pretransition to an additional intermediate phase (*39*, *40*). Such a phase transition will of course affect the embedded receptor protein and may severely restrict its conformational freedom. In keeping with that, we showed previously that the transition from Lumi to Meta I involves small structural changes that couple to the matrix surrounding the protein (*4*).

In the second example, we blocked the transition to Meta II by replacing rhodopsin's native membrane environment with artificial lipid environments of higher rigidity. Rhodopsin reconstituted in lipids with long saturated acyl chains, such as dipalmitoyl-PC (C-16, DPPC) or distearoyl-PC (C-18, DSPC), remains completely inactive toward transducin after illumination. This is further supported by corresponding IR spectra, which indicate that the transition to Meta II is completely inhibited even at higher temperature and acidic pH. In contrast to dry films, formation of Meta II's inactive precursor Meta I is not blocked in these reconstituted samples, although the transition temperature from Lumi to Meta I is increased compared to rhodopsin in native disk membranes (*4*; unpublished observation). The resulting room temperature photoproduct is therefore a state with a conformation sharing features of both Meta I and Meta II, which remains, however, fully inactive. Interestingly, a similar room temperature photoproduct state is obtained after illumination of rhodopsin in 2D crystals ($P22_12_1$ form), which shows as well no detectable activity toward transducin (*4*). In both cases, the rigid environment does not allow for helix rearrangement required for receptor activation.

In both examples, in dry films and in the rigid bilayers, the chromophore configuration after photolysis is all-*trans* 15-*anti*, similar to rhodopsin's photoproducts in the native system, in fully hydrated disk membranes. In the native system, this initial chromophore isomerization is sufficient to drive the protein to its active Meta II conformation. In the reconstituted samples and in the dry films, the conformational freedom of the receptor is, however, substantially limited, such that the protein cannot overcome the barrier to the activated Meta II state or even to its precursor, Meta I, respectively. Our experiments show that the all-*trans* 15-*anti* chromophore is not stable in these blocked states but isomerizes thermally to the 15-*syn* geometry, as evidenced by the all-*trans* 15-*syn* marker bands of the coupled NH bending mode around 1348 cm^{-1} and the C14−C15 stretching mode at 1180 cm^{-1}, which appear during the decay (*11*).

In the dark state (11-*cis* configuration) the C═N *syn* configuration is higher in energy relative to C═N *anti*, whereas following isomerization to 11-*trans* the protein probably raises the energy of the C═N *anti* isomer and possibly lowers that of the *syn* configuration. The energy

Scheme 2



gap between the two isomers is sensitive to the protein conformation such that, both in the irradiated dry films and in rigid bilayer samples, an equilibrium between both Schiff base isomers, 15-*anti* and 15-*syn*, is achieved. Remarkably, the 15-*syn* is favored more in the dry films than in the rigid bilayer samples. In addition, the isomeric equilibrium is attained at a considerably faster rate in the dry films compared to the rigid bilayer samples. From an energetic point of view, this means that the thermal isomerization to 15-*syn* is energetically more favorable in the dry film than in the rigid bilayers. Furthermore, the activation energy required for the thermal isomerization step is lower in the dry films compared to the rigid bilayer samples, implying, in other words, that the protein catalyzes the reaction more efficiently in the dry film photoproduct. The photoproduct obtained in the dry films can be considered an intermediate that is "earlier" than the photoproduct obtained in the rigid bilayer samples, such that less structural transitions have occurred after the initial photoisomerization. This leads to the conclusion that the energy barrier for the protein-dependent chromophore thermal isomerization is increased as the conformation of the protein resembles closer that of Meta II. It should be further noted that the photoproduct states obtained in dry films and in rigid bilayers represent artificially stabilized states that are not observed as stable intermediates in the native sequence of rhodopsin photoproducts. Instead, they correspond to states that are intermediate between the native photoproduct states Lumi and Meta I and between Meta I and Meta II, respectively. In structural terms, the native Meta I state is therefore intermediate between the photoproducts obtained in dry films and rigid bilayers.

Our results imply that the thermal C═N isomerization, which is also the triggering step for the formation of Meta III, does not depend on the presence of the active state Meta II but is even inhibited in its presence. In the introduction, we have detailed several lines of evidence which show that the thermal isomerization to 15-*syn* and thus formation of Meta III are completely inhibited under conditions where the position of the Meta I/Meta II photoproduct equilibrium is fully shifted to the active Meta II side. Combining those findings with the results derived in this paper, we arrive at a new scheme for Meta II decay and concomitant formation of Meta III (Scheme 2) (*11*). In this new picture, the triggering event of the transition from the Meta I/Meta II photoproduct equilibrium to Meta III, the thermal isomerization of the Schiff base, is catalyzed not while the protein is in the active Meta II conformation but rather while it is in the inactive Meta I conformation. Therefore, there are two pathways for Meta I to relax thermally: either via the conformational changes that lead to the active state Meta II or via thermal isomerization of the protonated Schiff base, leading to Meta III. The latter pathway, however, can contribute substantially only under conditions where the transition from Meta I to Meta II is blocked (or at least slowed considerably) or where appreciable Meta I is formed in stable equilibrium with Meta II. Previous models, in which Meta III was thought to be formed directly from Meta II, should therefore be reconsidered and if necessary be replaced by this more refined scheme, in which the transition to Meta III proceeds via Meta I.

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