A Nonbleachable Rhodopsin Analogue with a Slow Photocycle*

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Archaeal rhodopsins, e.g. bacteriorhodopsin, all have cyclic photoreactions. Such cycles are achieved by a light-induced isomerization step of their retinal chromophores, which thermally re-isomerize in the dark. Visual pigment rhodopsins, which contain in the dark state an 11-cis retinal Schiff base, do not share such a mechanism, and following light absorption, they experience a bleaching process and a subsequent release of the photo-isomerized all-trans chromophore from the binding pocket. The pigment is eventually regenerated by the rebinding of a new 11-cis retinal. In the artificial visual pigment, Rh6.10, in which the retinal chromophore is locked in an 11-cis geometry by the introduction of a six-member ring structure, an activated receptor may be formed by light-induced isomerization around other double bonds. We have examined this activation of Rh6.10 by UV-visible and FTIR spectroscopy and have revealed that Rh6.10 is a nonbleachable pigment. We could further show that the activated receptor contains two different isomeric states: one with 9-trans and 9-cis isomers of the chromophore. Both subspecies relax in the dark via separate pathways back to their respective inactive states by thermal isomerization presumably around the C13=C14 double bond. This nonbleachable pigment can be repeatedly photolyzed to undergo identical activation-relaxation cycles. The rate constants of these photocycles are pH-dependent, and the half-times vary between several hours at acidic pH and about 1.5 min at neutral to alkaline pH, which is several orders of magnitude longer than for bacteriorhodopsin.

Rhodopsin is the light receptor molecule in vertebrate rod photoreceptor cells and is involved in vision under dim light conditions. It senses light by its covalently bound chromophore, all-trans retinal, which undergoes a light-dependent cis to all-trans isomerization to trigger conformational changes of the receptor leading to formation of the activated Meta II state and ultimately to an excitation of the photoreceptor cell (1, 2).

In an attempt to create a light-stable rhodopsin, 11-cis-“locked” analogues of retinal were synthesized; the C13 methyl group was bridged to C10 of the polyene of retinal to prevent the activating isomerization around the C11=C12 double bond. Several different bridges were tested, leading to the synthesis of 11-cis-locked retinals with 5-, 6-, 7-, 8-, and even 9-member ring structures (Ref. 3 and references in Ref. 4). Much attention had been drawn to 11-cis ring-constrained retinals with a 6-member ring, Re6.10, as they recombined with opsin to pigments that were capable of at least some rudimentary photochemistry and some marginal activity toward the visual G protein, transducin (5, 6). Re6.10 exists in four isomeric states, which can be grouped as 9-trans and 9-cis, depending on the isomeric state of the C9 double bond (Scheme 1). A recent study characterized the overall shape of these four isomers by their affinities for either 11-cis- or all-trans-retinol dehydrogenase (7). It was revealed that in both the 9-cis and the 9-trans group there is one isomer with an 11-cis-like shape and one with an all-trans-like shape, which are distinguished by the respective isomeric state of the C13 double bond (Scheme 1).

This finding led us to the conjecture that in analogy to native rhodopsin, the 11-cis-like isomers should evoke a pigment with an inactive dark state conformation, whereas the all-trans-like isomers should activate the pigment. It has already been shown that during recombination with opsin the all-trans-like isomers experience a spontaneous isomerization around the C13=C14 double bond to give the respective 11-cis-like isomers, which then form the dark state(s) of the pigment, Rh6.10 (5). It should therefore be possible to induce an activation of the receptor by illumination, as all-trans-like isomers should become populated by light induced isomerization of the chromophores. The invoked protein changes should consequently be detectable by light-induced infrared difference spectroscopy. In a previous attempt to examine Rh6.10 with Fourier-transform infrared (FTIR) spectroscopic techniques, however, no substantial amounts of a putative active state could be detected at neutral pH (7). In a recent study by our group, we were able to detect at more acidic pH the formation of such a photoproduct state, which showed important properties of an active Meta II-like state, namely the characteristic IR difference spectrum of Meta II, as well as binding to and stabilization by G protein-derived peptides (4). In contrast to native Meta II, however, the Schiff base in this photoproduct with active state conformation remained protonated, indicating a different positioning of the Schiff base within the protein compared with native rhodopsin. Similar effects were observed before already for another artificial pigment (8).

All-trans-like isomers can, in principle, transform to 11-cis-like isomers by isomerization around the C9 or the C13 double bond.
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bond. As the C13 double bond is prone to thermal isomerization in the bacteriorhodopsins (9, 10) and also in visual pigment analogues (11), it is conceivable that the Rh6.10 photoproducts may relax thermally to their respective ground states in the dark by a thermal isomerization process around C13=C14. In the present study we have shown that the active state of the 11-cis ring-constrained pigment Rh6.10 does not undergo a hydrolysis process and instead relaxes in the dark back to its ground state. The pigment can be repeatedly photolyzed and under the same excitation-relaxation cycle, characterized in this study by UV-visible and IR spectroscopy. Rh6.10 not only constitutes the first visible pigment for which a slow photocycle could be detected, but it may also offer the possibility to apply to rhodopsin those biophysical techniques that require repeated excitation-relaxation cycles.

EXPERIMENTAL PROCEDURES

Retinal and Pigment Preparation—Retinal analogues and pigment in native washed disk membranes were prepared as described previously (4). We started in this study generally from a 50:50 mixture of 9-trans and 9-cis isomers (Scheme 1). Where indicated, pure isomers with more than 90% content (tested by high pressure liquid chromatography) of either group were used.

Sample Preparation—All experiments were performed with ~1 nmol of pigment in sandwich film samples with 4–μm optical path lengths, which allow both UV-visible and IR spectroscopy with the same sample or sample type. Sandwich samples were prepared as described previously (4, 12).

Spectroscopy—Spectroscopy was performed as described in the accompanying paper (4). For illumination we used a 530-nm cut-off filter, as it allowed for maximal yield of the blue-shifted active state photoproduct. Additional illumination with shorter wavelength (e.g. 470- or 440-nm cut-off) reverted formation of this photoproduct by shifting the photochemical equilibrium to the dark state species. In all experiments, illumination did not lead to full conversion into photoproducts but rather to photoequilibria between photoproducts and dark states.

UV-visible Analysis of Decay Components—Half-time constants of the decay components were obtained by first fitting an exponential decay to the long term absorption increase at 530 nm under omission of the short time decay region (five to six times the half-time of the fast decay component). The half-time of the fast decay component was determined by a fit of the 460 absorption decrease to the sum of two exponentials, of which the half-time of the second exponential was fixed to that of the slow decay component.

The decomposition of the complex UV-visible decay difference spectra into the clean difference spectra of the two decay components was achieved as follows. First, the respective spectrum for the slow component ($t_{1/2} = 175$ min) was calculated from the late decay spanning the time frame from 2 to 8 h, thereby covering 47% of the amplitude of this decay component, and was normalized to get the full amplitude. The spectrum of the fast component ($t_{1/2} = 10$ min) was derived from the early decay over the first 20 min (covering 77% of the full amplitude), corrected for the small contribution of the slow component for this time frame, and normalized as well to get the full amplitude of the decay.

For pH values greater than 6.0, the decreasing photoproduct yield hampered an accurate analysis of the decay kinetics by UV-visible spectroscopy only. The fast decay constant was therefore determined alternatively by monitoring the photoinduced decay in the IR using the 1644-cm$^{-1}$ Meta II amide band as a marker.

Determination of Photoproduct Composition—The relative contributions, $A_f$ and $A_s$, of the Meta II species with fast and slow decay, respectively, to the total amount of activated pigment, $A_f + A_s$, was determined by performing two illuminations on a photoequilibrated sample spaced in time by a time interval $t_{1/2}$ sufficiently long to allow an almost full decay of the Meta II component with fast decay. The amplitude of the FTIR spectrum of the second photolysis, $Q$, normalized relative to that of the first photolysis, therefore reflects the relative amount of photoproduct that has decayed up to $t_{1/2}$. With the decay constants $t_f$ and $t_s$ determined from the exponential fast and slow decay in UV-visible spectra, we can decompose $Q$ into $Q = A_f \cdot (1 - e^{-t_f/t_{1/2}}) + A_s \cdot (1 - e^{-t_s/t_{1/2}})$. With the normalization $A_f + A_s = 1$, we are able to express this from the relative contributions, $A_f$ and $A_s$. These contributions, which are relative to the amount of the photoproduct at the given pH, can be converted into absolute contributions normalized to the total amount of pigment by weighting it with the photoproduct yield. The photoproduct yield as a function of pH was reported previously (4) and was determined from the amplitude of the $\sim 1768 / 1747$ cm$^{-1}$ Meta II marker band of the Rh6.10 photoproduct normalized to the absolute amide II absorption as a measure for pigment content of each sample.

RESULTS

In the present study we regenerated bovine opsin with an 11-cis ring-constrained retinal analogue, Re6.10, to obtain the artificial pigment Rh6.10. A previous study (5) reported that upon regeneration with opsin membranes, absorption peaks at $\lambda_{max}$ 510 and 494 nm were obtained for the 9-trans and 9-cis isomers, respectively, which were confirmed by the present study. For most of our experiments, we used pigment obtained from a 50:50 isomeric mixture, which gave a peak absorption around 507 nm. Despite the 11-cis lock imposed on the retinal, the pigment showed photochemistry, yet longer illumination times were needed to reach photostationary states compared with native rhodopsin. Because of the strong spectral overlap of dark and photoproduct states, we never achieved full conversions in the photostationary equilibria but only shifts in the relative contributions of dark states and photoproduct states (see “Experimental Procedures”).

The Photoprodut of Rh6.10 Relaxes Thermally to the Ground State—Photolysis of native rhodopsin in membranes at 20 °C and pH 5.0 produces the active state, Meta II, absorbing at 380 nm, which decays then by hydrolysis of the Schiff base ($t_{1/2}$ 7 min) and dissociation of the receptor into all-trans retinal and opsin (not shown). At pH 5.0, Rh6.10 produces a photoproduct with a much smaller blue-shift, indicating a protonated Schiff base also in the photoproduct, which is shown in Fig. 1A. This photoproduct of Rh6.10 is likewise not stable, but in contrast to Meta II of native rhodopsin it does not decay by hydrolysis of the retinal Schiff base and dissociation of the receptor. Instead it relaxes thermally in the dark within 24 h to a species absorbing again close to 500 nm. The absorbance of the initial state before the first illumination is, however, only partially recovered (Fig. 1A). This is different after subsequent illuminations, which were applied in 24-h intervals. Importantly, for all subsequent illumination and relaxation cycles, the respective initial state before illumination was almost fully recovered within 24 h (Fig. 1B).

In Fig. 2A, we compare the light-induced difference spectra of six subsequent illuminations of the same sample recorded in 24-h intervals, reflecting the blue-shift of the photoproduct state compared with the dark state. Apart from the first illumination, the spectra of the five following illuminations are virtually identical. This may indicate that the pigment is undergoing a slow photocycle and returns to the initial state before illumination during the 24 h in the dark following photolysis. To test this hypothesis, we studied the light-induced conformational changes of the pigment by FTIR spectroscopy. As reported previously (4), under the given conditions the mod-
ified pigment forms a photoproduct similar to the active state of native rhodopsin, Meta II, yet with a protonated Schiff base. Like Meta II, this state is recognized by peptides derived from the rhodopsin cognate G protein, transducin, and therefore presumably possesses an active conformation as well.

In Fig. 2B, we show the IR difference spectra corresponding to the UV-visible spectra of Fig. 2A. In the upper part, we compare the spectra of the first and the second illumination, of which the first was scaled by a factor of 0.82 for clarity. Both spectra are very similar with small deviations only at 1205, 1294, 1525, and 1558 cm⁻¹, which are marked by asterisks and related to vibrational modes of the chromophore in the pre-photolysis state as will be shown below. There are also subtle changes in the range of amide I and Schiff base C=N vibrations around 1640 cm⁻¹. The spectra of the second to fourth illuminations are shown in the lower part of Fig. 2B and are virtually identical, not only in shape but also in amplitude.

To further investigate the influence of isomer composition in the initial state on the IR difference spectra, we used samples regenerated with pure 9-trans and 9-cis isomers. The corresponding difference spectra for the first illumination are shown in Fig. 3A and have clear deviations (Fig. 3B). Spectra of subsequent illuminations, however, are again virtually identical (Fig. 3C) and correspond to those obtained for subsequent illuminations of samples with an initial 50:50 isomeric composition (Fig. 2B). The first 2-min illumination of the samples is sufficient to reach a photostationary state in which an isomeric composition of the chromophores is produced, which is independent of the initial composition. This isomeric mixture then decays in the dark reproducibly to a dark-adapted chromophore composition, which again remains independent of the initial composition.

We will term this process "photoequilibration" and will refer to samples that have undergone at least one excitation-relaxation cycle back to their dark-adapted state as "photoequilibrated samples." The isomeric composition of these samples depends only on parameters influencing the photoproduct equilibrium, and therefore particularly on the filter combination used for illumination, and to some extent probably also on pH and temperature. In turn, the differences observed persistently between the first and any subsequent illumination, both in the UV-visible and the IR spectra, reflects the difference between initial and photoequilibrated isomeric composition. In the IR, the resulting difference bands correspond to changes in chromophore related bands and have the shape of the spectrum in Fig. 3B, which reflects the transition from the 9-trans to the
Fig. 3. Influence of initial chromophore composition on the IR spectra. The initial chromophore composition of the samples was varied by using samples regenerated with pure 9-trans (black) and 9-cis (gray) isomers (instead of a 50:50 mixture). The samples were photolyzed as described in the legend for Fig. 2A; the asterisks are reproduced from Fig. 2A to allow a better comparison. We further subtracted from the spectrum of the 9-trans isomer that of the 9-cis isomer to obtain a spectrum reflecting the differences between the respective dark states (black spectrum in B). In comparison, the double-difference spectrum "first minus second illumination" of the spectra in Fig. 2B is shown in gray (scaled by a factor of 2.4). The correspondence of the two spectra indicates that the difference between first and subsequent illuminations generally reflects a non-photoequilibrated chromophore composition in the samples prior to the first illumination, leading to a shift of this composition from the 9-trans to the 9-cis species during the first excitation-relaxation cycle. C, spectra as described in A with pure chromophores but for the second illumination after a 24-h recovery in the dark. The differences between the two samples have largely vanished, indicating that both samples have adjusted to the same photoequilibrated isomer composition during the first excitation and relaxation cycle.

9-cis dark state, i.e. from the 11-cis to the 9,11,13-tricis isomer of Rh6.10 (Scheme 1).

We can therefore conclude that, after an initial photoequilibration process, Rh6.10 can be repeatedly excited to undergo identical illumination and relaxation cycles. Following, we will focus on the properties of photoequilibrated samples and examine in more detail the relaxation kinetics of the photoproducts.

Biphasic Relaxation Behavior and Existence of Two Photoprodut Relaxation Pathways—As already discernible in Fig. 1, photoproduct relaxation is not a homogeneous process. This becomes more obvious in Fig. 4A, where we plotted besides the photolysis spectrum (after minus before illumination) the decay spectra of the photoproduc (decay product at a certain time minus photoprodut at t = 0), and where the absence of an isosbestic point is clearly evident. Indeed, we observe a rapid decrease of absorbance around 460 nm on the time scale of minutes and a much slower increase around 530 nm on the time scale of hours. The separation of these time constants by more than one order of magnitude allows a straightforward analysis by fitting exponentials to the short term decrease at 460 and to the long term increase at 530 (see “Experimental Procedures” for details), yielding \( t_{460} = 10 \) and 175 min for the two components, respectively, at pH 5.0 and 20 °C. With these data in hand, we can decompose the complex decay pattern into the pure decay spectra of each component, as described in more detail under “Experimental Procedures.” The pure decay spectra thus obtained are shown in Fig. 4B. Both components involve a red-shift of the absorption band during the decay. The asymmetrical shape of the spectra also suggests a decrease of the absorption coefficient of the product in the case of the fast component, whereas in the case of the slow component the absorption coefficient increases.

The Decay Process Is Parallel Rather Than Sequential—Which of the two components observed in the UV-visible spectra reflects the conformational changes involved in the relaxation of the active state to the ground state? To address this question, we performed the following FTIR experiment. We illuminated a photoequilibrated, dark-adapted sample of Rh6.10 for 2 min and obtained the gray spectrum shown in Fig. 4C. The illumination was sufficient to reach a photoequilibrium with maximal conversion, as longer illumination led to no further spectral changes (not shown). We incubated the sample for 40 min in the dark, such that about 95% of the fast decay component had been completed, whereas the slow component had proceeded only by 15% according to the derived half-times. We then illuminated the sample again and obtained thus two difference spectra: the first corresponding to the transition from the fully dark-adapted state to the photoprodut state and the second corresponding to the transition from the photoproduct state after having completed the fast decay component back to the photoprodut state. We plotted the two spectra in Fig. 4C for comparison. Surprisingly, the second spectrum is very close to the first one (with smaller differences arising mainly from the chromophore-related fingerprint bands as at 1205 cm\(^{-1}\) but with only ~50% of its amplitude.

This phenomenon can be accounted for by two scenarios: (a) the homogenous Meta II-like photoprodut decays rapidly to an exactly semi-active conformation and then slowly to the fully dark-adapted inactive state; or (b) the Meta II-like photoprodut consists of two different subspecies, namely the two all-cis-like isomers 9,11-dicis and 11,13-dicis (Scheme 1), that decay independently with differing time constants back to their respective dark-adapted states. The existence of a semi-active state with exactly the same IR band pattern but only half the amplitude in (a) seems extremely unlikely. We must therefore assume a parallel decay of two distinct Meta II-like states. Based on the previous observation that the C\(_{13}\) double bond, but not the C\(_9\) double bond, of Re6.10 may isomerize thermally during pigment regeneration (5), it is likely that the decay of the photoprodut states back to the dark states involves a similar thermally activated isomerization step around C\(_{13}\) = C\(_{14}\). With this assumption, the single components of the decay correspond to the transitions 11,13-dicis → 11-cis in the 9-trans group and 9,11-dicis → 9,11,13-tricis in the 9-cis group (Scheme 1). With the visible absorption bands of the 11-cis isomer (\( \lambda_{\text{max}} \) 510 nm) and the 9,11,13-tricis isomer (\( \lambda_{\text{max}} \) 494 nm), we may unequivocally assign the slow decay component, which restores the 510 nm absorption band (Fig. 1), to the 9-trans process and the fast component to the 9-cis process. Both processes involve a spectral red-shift and a decrease/ increase of the absorption coefficient for the 9-cis/9-trans process, respectively. The latter also explains the irreversible loss of absorption intensity observed during the first excitation-relaxation cycle (Fig. 1A), which was shown to be due to a shift from the 9-trans (11-cis) to the 9-cis (9,11,13-tricis) isomer for the samples characterized by an initial 50:50 isomeric composition (Fig. 3B).

According to this parallel relaxation model, the fast decay component depletes 9-cis Meta II (9,11-dicis) from the isomeric equilibrium being formed during illumination and enriches it with 9-cis dark state (9,11,13-tricis). Subsequent illumination restores the photoprodut equilibrium, and the corresponding difference spectrum therefore reflects the transition from the 9-cis dark state to the 9-cis Meta II-like state (black spectrum...
in Fig. 4C). We can obtain the corresponding spectrum for the 9-trans species by subtracting this difference spectrum from that obtained from the fully dark adapted sample. Both spectra are shown together in Fig. 4D. Their similarity indicates that the isomeric state of the C13 double bond has only little influence on the chromophore-related bands specific for the 9-trans or 9-cis isomers.

Both Fast and Slow Decay Constants Are pH-dependent—Up to now, we have focused on the decay kinetics at pH 5.0. What happens at higher pH? With increasing pH we observe an acceleration of the kinetics of both the 9-cis (fast) and 9-trans (slow) decay component. In Fig. 5A, we compare the kinetic constants obtained as a function of pH by UV-visible and FTIR spectroscopy. The half-times decrease from more than 50 min at pH 4.0 to 2 min at pH 7 or higher for the fast component, whereas the decrease is not as pronounced for the slow component. We further analyzed the pH dependence of the decay kinetics of the fast decay component by fitting the rate constants to a Henderson-Hasselbalch equation (Fig. 5B). We thereby obtained a pK value of 6.3, splitting the 9-cis Meta II-like photoproduction into a thermally stable (for the most part) low-pH species and an unstable high-pH species decaying with a half-time of 1.4 min. The nature of the residue involved, which becomes deprotonated in the high-pH species and which catalyzes the thermal isomerization process, is presently unclear, particularly as its deprotonation shows no pronounced effects on the spectral properties of the dark and photoproduction states.

As reported in the accompanying paper (4), there is a steady decrease of photoproduction yield with increasing pH at room temperature (Fig. 5C). We extend that result by analyzing the relative contributions of the two all-trans-like isomers to the total photoproduction as a function of pH. The clear separation of the rate constants for the decay of the 9-trans and the 9-cis isomer allows an evaluation of the single contributions by an FTIR spectroscopic, two-photolysis method similar to that in Fig. 4C, which is detailed under “Experimental Procedures.” The contributions of the two isomers to the photoproduction at 2°C as well as the pH dependence of the total amount of photoproduction are shown in Fig. 5C in a cumulative plot. By this two-photolysis method, contributions of the 9-trans isomer to the photoproduction were no longer detectable at pH 7.0 or higher, whereas 9-cis contributions persisted up to very alkaline pH. In native rhodopsin, the decrease of the light-dependent formation of Meta II with increasing pH is compensated for by a concomitant reciprocal increase of Meta I. Rhb-10 shows a clearly different behavior, as the pH-dependent decrease of the active state photoproduction at room-temperature is not counterbalanced by formation of a Meta I-like species. This indicates that other mechanisms operate that give a high photoproduction yield only for active state photoproducst and that complicate a straightforward interpretation of these results.

**DISCUSSION**

Archaeal rhodopsins, which function as ion pumps or signal transducers, all show photocycles that are initiated by light-dependent isomerization of the covalently bound chromophore all-trans retinal around its C13=C14 double bond, thereby trig-
complicated process involving hydrolysis of the Schiff base and enzymatic reduction of the retinal to all-trans retinol, back-isomerization and further processing outside the photoreceptor cell in the molecular machinery of the pigment epithelium, and subsequent regeneration of opsins with this newly formed 11-cis retinal (2).

Why is the process so complicated? The answer to this question is not entirely clear, but it may be that nature chose 11-cis retinal as the chromophore for the inactive dark state, as it allows for a pigment with an exquisitely low rate of dark activity (15, 16). This absence of constitutive activity in the dark state enables the photoreceptor cell to work as a single photon detector. One can imagine that such an extreme sensitivity would be out of reach for a pigment in which spontaneous isomerization around the crucial C11 double bond is possible in the dark. However, in principle, dark isomerizations around double bonds other than C11=C12 are possible. They were reported for instance for the C13=C14 double bond in the pigment analogue 9,13-dicis rhodopsin (but not for the C9=C10 double bond) (11). This preference for thermal isomerization around the C13=C14 double bond instead of C9=C10 may reflect a lower energy barrier for C13=C14 isomerization since more positive charge is delocalized along C13=C14 relative to the C9=C10 bond, as was demonstrated by model studies of retinal protonated Schiff bases in solution (17, 18). In a previous study (5) on the same 11-cis ring-constrained retinal analogues as used in our study, it was shown that dark isomerization around the C13=C14 double bond (but not around C9=C10) may be invoked by interaction of the retinal with opsin during recombination of the pigment. This notion was corroborated in a recent study that reported considerable dark isomerization as well in the pigment around C13=C14 but again not around C9=C10 (7).

That study also confirmed that in pigment, the 9-trans species accumulates almost fully as 11-cis-like isomer (13-trans), whereas there seemed to be an equilibrium distribution for the two 9-cis species, at least in detergent-solubilized pigment.

Based on these ideas, we propose the following scheme for the illumination and relaxation cycles observed in our experiments with Rh6.10. In the dark state of the pigment, the chromophores adopt 11-cis-like states, as shown previously (5). Photolysis invokes an equilibrium distribution of the chromophore composition, which is independent of the initial composition but which depends on the spectral distribution of the illuminating light and also on other factors such as pH and presumably temperature. In this photoequilibrium, the two all-trans-like isomers, 11,13-dicis and 9,11-dicis, are likely to form the active Meta II-like photoprodut states. These two isomers decay subsequently in the dark by two separate pathways (the 9-trans and 9-cis pathway, representing the slow and fast decay components, respectively). The two decay pathways involve spontaneous isomerization around the C13=C14 double bond regenerating the 11-cis-like isomers, 11-cis and 9,11,13-tricis, respectively, which reconstitute the dark-adapted states.

According to previous studies (5, 7), spontaneous isomerization around the C9=C10 double bond seems very unlikely and can therefore be excluded. After each illumination-relaxation cycle, the same isomeric composition of the dark-adapted pigment is regenerated. This distinguishes the initial illumination-relaxation step of freshly reconstituted samples from subsequent cycles, as the initial isomeric composition (determined by retinal composition used for pigment preparation) may differ from that obtained after a first illumination-relaxation cycle. We termed the underlying process, which takes place during the first cycle only, photoequilibration.

How does the back-isomerization in the dark proceed in detail? Analysis of the kinetics of the 9-cis photoprodut (fast
decay) indicates the existence of a pH-dependent equilibrium between two Meta II-like species for this isomer with a pK of 6.3; only in the species formed at higher pH does thermal isomerization around the C13 double bond proceed at an appreciable rate. The nature of these residue(s) is presently unclear.

In previous studies on rhodopsin it was conjectured that the rare events of thermal chromophore isomerization depend on a transitory deprotonation of the Schiff base (15). Such a mechanism seems very unlikely in our case, as the spectral changes we observed are not consistent with the rates of deprotonation of the protonated Schiff base. It is known that certain polar residues of the binding pocket, e.g., Glu-122 and Glu-181, strongly modulate the rate of hydrolysis (20, 21). A perturbed interaction of these residues with the Schiff base in the modified pigment may therefore be involved as well.

In summary, we have shown that the visual pigment rhodopsin, modified with an 11-cis ring-constrained chromophore, undergoes a light-induced activation/thermal deactivation cycle similar to those observed for archaeal rhodopsins, yet with a deactivation step that is slower by several orders of magnitude. Together with previously published results (4, 5, 7), our studies indicate that the orientation of the C13=C14 double bond is critical in triggering receptor activation and that the relaxation of the resulting active state in the dark is due to a thermal back-isomerization around this double bond.

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