Steric Barrier to Bathorhodopsin Decay in 5-Demethyl and Mesityl Analogues of Rhodopsin

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Received March 19, 2001. Revised Manuscript Received June 14, 2001

Abstract: Absorbance difference spectra were recorded from 20 ns to 1 μs after 20 °C photoexcitation of artificial visual pigments derived either from 5-demethylretinal or from a mesityl analogue of retinal. Both pigments produced an early photointermediate similar to bovine bathorhodopsin (Batho). In both cases the Batho analogue decayed to a lumirhodopsin (Lumi) analogue via a blue-shifted intermediate, BSI, which formed an equilibrium with the Batho analogue. The stability of 5-demethyl Batho, even though the C8-hydrogen of the polyene chain cannot interact with a ring C5-methyl group to provide a barrier to Batho decay, raises the possibility that the 5-demethylretinal ring binds oppositely from normal to form a pigment with a 6-s-trans ring-chain conformation. If 6-s-trans binding occurred, the ring C1-methyls could replace the C5-methyl in its interaction with the chain C8-hydrogen to preserve the steric barrier to Batho decay, consistent with the kinetic results. The possibility of 6-s-trans binding for 5-demethylretinal also could account for the unexpected blue shift of 5-demethyl visual pigments and could explain why 5-demethyl artificial pigments regenerate so slowly. Although the mesityl analogue BSI's absorption spectrum was blue-shifted relative to its pigment spectrum, the blue shift was much smaller than for rhodopsin's or 5-demethylisorhodopsin's BSI. This suggests that increased C6−C7 torsion may be responsible for some of BSI's blue shift, which is not the case for mesityl analogue BSI either because of reduced spectral sensitivity to C6−C7 torsion or because the symmetry of the mesityl retinal analogue precludes having 6-s-cis and 6-s-trans conformers. The similarity of the mesityl analogue BSI and native BSI λ_{max} values supports the idea that BSI has a 6-s angle near 90°, a condition which could disconnect the chain (and BSI's spectrum) from the double bond specifics of the ring.

Introduction

The recent X-ray crystal structure of bovine rhodopsin[1] has revealed structural details which are central to understanding the activation mechanism of visual pigments and other Class I G-protein-coupled receptors (GPCRs). Further work is needed to explain how a photoreceptor protein's dark state, inactivated by 11-*cis*-retinal, is transformed by light into the activated form, a process beginning with chromophore photoisomerization and ultimately involving reorganization of several cytoplasmic loops distant from the retinylidene chromophore. Photoaffinity labeling experiments using retinal analogues show that although the chromophore has not moved significantly in bathorhodopsin (Batho), the earliest photointermediate which can be thermally stabilized, a large transformation has occurred after Batho has decayed to lumirhodopsin (Lumi), which is the next trappable intermediate at low temperatures.[2] This chromophore motion is likely to initiate the earliest stage of protein activation in visual pigments and may provide insight into analogous protein activation steps in other Class I GPCRs. The latter are important targets for drug design, but because they are chemoreceptors, their early activation processes are more difficult to study directly. It is therefore important to characterize the relevant rhodopsin processes closer to physiological temperatures where an additional blue-shifted intermediate (BSI) appears between Batho and Lumi.[3]

Artificial visual pigments produced by regeneration of bovine opsin with retinal analogues have proven to be useful tools for probing factors which control early chromophore motions. The absence of chromophore motion reported for Batho is consistent with the previously developed idea that photoisomerization converts the 180° rotation of the C11−C12 bond into smaller torsions distributed over the other bonds in the chromophore's polyene chain. To stabilize this twisted state, some barrier to motion in Batho must prevent release of the torsion. Time-resolved absorbance measurements after photoexcitation of artificial visual pigments show that retinal analogues with increased flexibility in the vicinity of the ring-chain connection destabilize Batho.[4,5] Similar measurements on other artificial pigments where steric bulk was introduced along the retinal polyene chain led to a model in which steric hindrance between the retinal C8-hydrogen and its C5-methyl (see Figure 1 and structures) constitutes the primary barrier to Batho decay.[6] In contrast to Batho decay, where the role of the protein is primarily a passive one, confining the chromophore to the binding pocket where internal rotation barriers are operable, Lumi formation has been attributed to protein change, a view consistent with the photoaffinity labeling results. Although a variety of chromophore modifications can dramatically shorten the lifetime of

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10.1021/ja010724q CCC: $20.00
Published on Web 09/20/2001

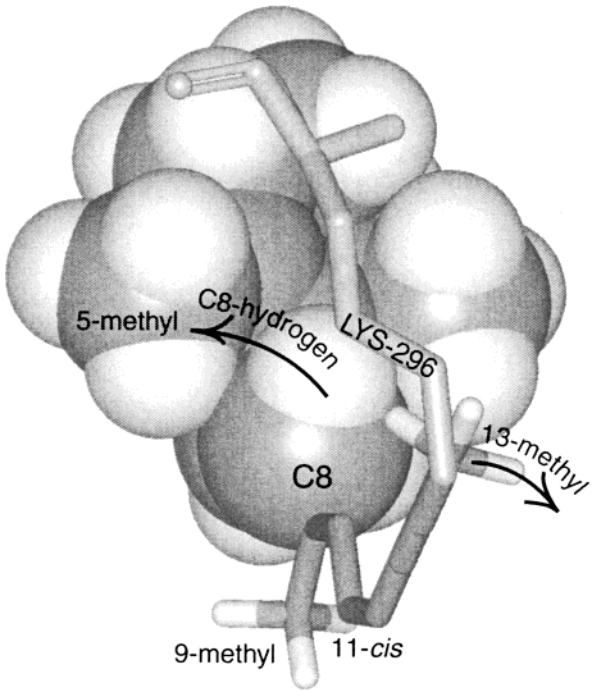

Figure 1. 11-*cis*-Retinylidene structure of rhodopsin determined by X-ray diffraction. Shown in the foreground (rendered in stick style) is the chain of Lys-296 and the carbon backbone up to C9 of the chromophore of rhodopsin (chromophore coordinates taken from molecule B of the X-ray crystal structure of Palczewski et al., 2000 (ref 1)). The ring end of the retinal beyond C9 is rendered in space-filling CPK style to show the steric hindrance that exists between the C8-hydrogen and the C5-methyl group on the β-ionone ring. Because of the pre-existing twist around the C12−C13 single bond, when light isomerizes the 11-cis bond (at bottom), the C13-methyl end of the chain moves as shown, and consequently C8 moves oppositely. This motion creates a stable twist in Batho which requires rotation of the chain C8-hydrogen past the ring C5-methyl in order to relax. This relaxation (which forms BSI) is hindered by the contact between the C8-hydrogen and the C5-methyl. (Except for C8, the main chain hydrogens are not shown in this figure.)

Batho, the subsequent decay of BSI is less affected, particularly when chromophore bulk is unchanged or reduced.[4] The latter observation implicates an intrinsic protein relaxation instead of an intrachromophore barrier as the rate-limiting step in Lumi formation.

The above picture of early events obtained from time-resolved measurements has been difficult to reconcile with low-temperature experiments on 5-demethylrhodopsin where a Batho intermediate can be trapped at liquid nitrogen temperature.[7] Since removal of the retinal C5-methyl group presumably removes the barrier to Batho decay, 5-demethylrhodopsin would have been expected to behave like other artificial pigments where increased ring-chain flexibility prevents Batho from being trapped under these conditions. To investigate the reason for this difference, we conducted time-resolved absorbance measurements at 20 °C on visual pigments derived either from 5-demethylretinal or from a mesityl analogue of retinal.

Experimental Section

Preparation of Retinal Analogues. 9-*cis*-5-Demethylretinal, and 11-*cis*-mesityl retinal were synthesized according to previously described methodologies.[8,9]

Preparation of Artificial Rhodopsins. Rod outer segments (ROS) containing bovine opsin were prepared for regenerations as described previously.[6] Subsequent steps were conducted under dim red light. Regenerations of artificial visual pigments were begun by adding an aliquot of a concentrated chromophore stock solution (freshly prepared in ethanol) to a ROS suspension of opsin. The amount added was sufficient to give a 2−3-fold excess of chromophore over opsin and small enough that ethanol remained less than 3% of the resulting solution. Regenerating mixtures were incubated in the dark at 37 °C, and aliquots were withdrawn periodically to measure the extent of regeneration spectrophotometrically after dilution in 1% Ammonyx LO detergent. Visual pigments typically form within the first hour of incubation as was the case here for the mesityl-11-*cis*-retinal analogue. The pigment from 9-*cis*-5-demethylretinal formed much more slowly, and after the first 2 h, incubation was continued at room temperature for 4 days, at which point pigment formation had ceased. Anomalously slow regeneration of visual pigments derived from 5-demethylretinal has been reported twice previously.[7,10] Several batches of both pigments were made with yields ranging from 20% to 30% based on opsin. After regeneration was complete, excess chromophore was converted to the alcohol form by adding 0.1 mg of NADPH (the cofactor for the endogenous ROS retinol dehydrogenase) per milligram of opsin and continuing the incubation for 1 h. Regenerated ROS were stripped of extrinsic membrane proteins by two cycles of centrifugation (Sorvall SS-34 rotor, 30 min at 17K rpm) followed by resuspension in 1 mM EDTA, pH 7.0. After the final wash, membranes were pelleted and the artificial visual pigments were solubilized in sufficient 2% octylglucoside detergent in buffer (pH 7.0, 10 mM TRIS, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA) to produce a solution whose absorbance was ~0.6 (path length = 1 cm) at the peak of the long-wavelength pigment absorption band (474 nm for 5-demethylisorhodopsin and 480 nm for the mesityl rhodopsin analogue). Octylglucoside suspensions of pigments were centrifuged immediately prior to measurements to remove any unsolubilized material.

Time-Resolved Spectroscopy. Individual samples were photolyzed by 7-ns (fwhm) laser pulses. The changes in absorbance at particular time delays after photolysis, ranging from 20 ns to approximately 1 μs, were measured using a gated optical multichannel analyzer.[11] For measurements on 5-demethylisorhodopsin, samples were excited using 477-nm light from a dye laser pumped by the 355-nm third harmonic of a Nd:YAG laser. To reduce secondary photolysis of the unusually blue-shifted mesityl rhodopsin analogue that could produce the even more blue-shifted mesityl isorhodopsin analogue, the dye laser was adjusted to produce 440-nm excitation. In both cases the energy delivered to the sample was 80 μJ/mm^2. Absorbance changes due to rotational diffusion were eliminated either by averaging the absorbance changes measured using parallel and perpendicularly polarized (with respect to the laser polarization axis) light, $\Delta A = (\Delta A_{\parallel} + 2\Delta A_{\perp})/3$, or by measuring absorbance changes with light linearly polarized at 54.7° relative to the laser polarization axis. The path length for probe light in the sample was either 2 mm (5-demethylisorhodopsin) or 1 cm (mesityl rhodopsin analogue), and sample temperatures were maintained at 20 °C. Fresh sample was pumped into the optical path from a computer-controlled syringe after each photolysis pulse. The spectrum of the pigment bleached by the laser was determined as described previously.[12]

Data Analysis. The sets of experimental difference spectra, $\{\Delta A(\lambda,t)\}$, were fit as previously described[3] to a function whose form was a sum of exponential decays:

$$\Delta a(\lambda,t) \equiv b_0(\lambda) + b_1(\lambda)\exp(-t/\tau_1) + b_2(\lambda)\exp(-t/\tau_2) + \cdots$$

The apparent lifetimes, τ_i, and the difference spectra associated with

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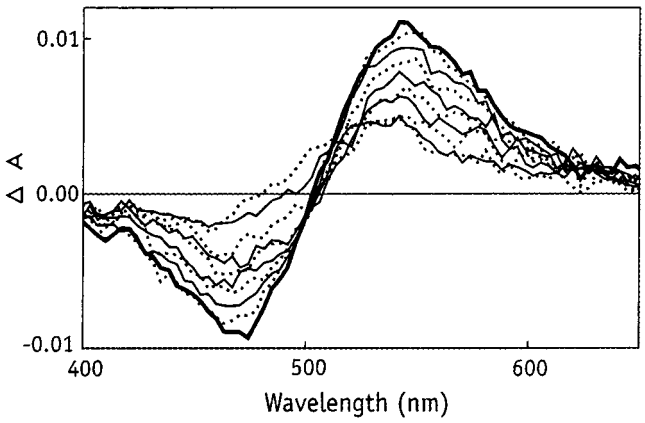

Figure 2. Absorbance changes occurring after 477-nm photoexcitation of 5-demethylisorhodopsin at 20 °C. Difference spectra shown were collected 25 (heavy line), 35, 50, 70, 100, 150, 200, 400, and 800 ns and 1.6 μs (alternating dotted and solid lines) after the 7-ns photoexcitation pulse. Negative absorbance below 500 nm (bleaching) and positive absorbance above that wavelength clearly show conversion of the visual pigment into a bathorhodopsin analogue at early times.

the individual lifetimes, or *b*-spectra, $b_i(\lambda)$, were determined for fits involving one, two, and three exponential terms. The residuals from these fits were compared to determine the best choice using as criteria the spectral structure of residuals relative to the noise level and reproducibility of the fit. For data on the time scale studied here, the following mechanism describes results for rhodopsin:[3]

Scheme 1

$$\cdots \text{Batho} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{BSI} \xrightarrow{k_3} \text{Lumi} \cdots$$

Using the experimentally determined *b*-spectra and the spectrum of the pigment bleached by the laser, absolute spectra of the initial and final species (Batho and Lumi) can be directly calculated. These spectra, like those of visual pigments themselves, typically have broad wavelength profiles with roughly Gaussian shape (the red half of the long-wavelength absorption bands of rhodopsin, isorhodopsin, Batho, and Lumi are very well represented by Gaussians), and this character can be used to estimate the equilibrium constant for the equilibrium. By assuming that the spectrum of BSI has reasonable properties, e.g., no negative bands etc.,[13] the equilibrium constant can be estimated by variation until the best BSI spectrum is obtained. Conversely, the presence of an equilibrium between Batho and BSI can be deduced from the fact that an unacceptable shape is calculated for the BSI spectrum when the data are fit with a straight sequential model (i.e., no back reactions).

Results

The absorbance difference spectra collected after photolysis of 5-demethylisorhodopsin are shown in Figure 2. Those data were best fit by two exponential processes with apparent lifetimes 80 and 860 ns. Absolute spectra of the intermediates obtained under the assumption of a straight sequential model are shown in Figure 3. As can be seen there, the initial photoproduct is a red-shifted Batho intermediate. The second intermediate has a broad absorption profile with substantial tailing toward the red edge where the Batho intermediate absorbs, indicating the presence of a Batho−BSI equilibrium. Figure 4 shows the spectra of intermediates obtained using $K = 1.8$ for the Batho−BSI equilibrium constant. The λ_{max} values of these intermediates and the microscopic rate constants obtained using that equilibrium constant in Scheme 1 are given in Tables 1 and 2, respectively.

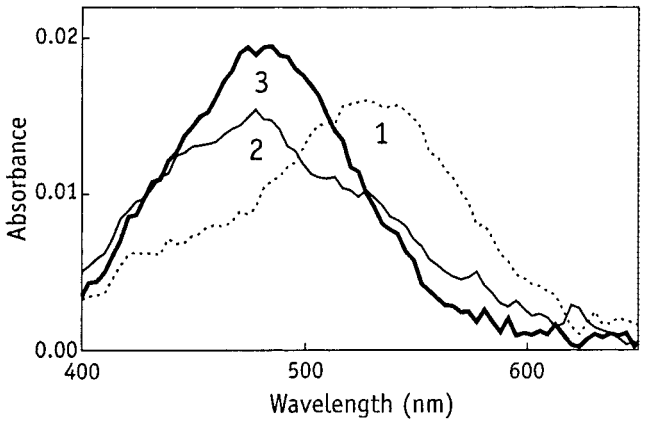

Figure 3. Absolute spectra of intermediates which result from fitting the data of Figure 2 to a straight sequential (no back reactions) scheme. The dotted line shows the earliest intermediate (Batho), the thin solid line shows the second intermediate, and the thick line shows the spectrum of the 5-demethyl Lumi analogue. Because back reactions are not considered in this fit, the spectrum resulting for the second intermediate is a mixture of BSI and Batho, accounting for the tailing seen on the red edge of the second intermediate.

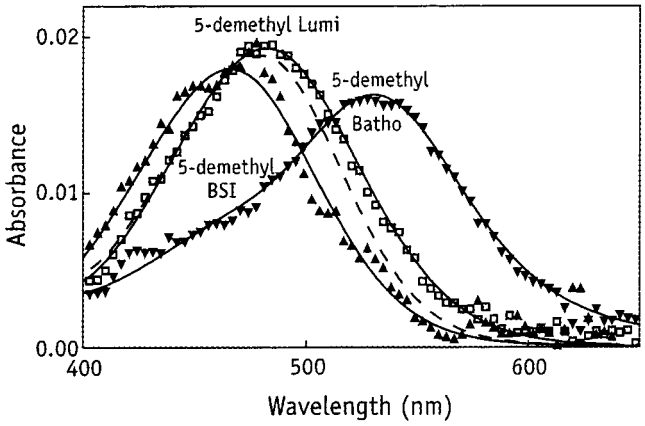

Figure 4. Absolute spectra of 5-demethylisorhodopsin intermediates resulting from Scheme 1 with a Batho−BSI equilibrium constant, $K = 1.8$. Symbols show the data points obtained by averaging four data points. Solid lines show the spectra of intermediates obtained from the fit, and the dashed line shows the spectrum of the material that was bleached to form these intermediates ($\lambda_{max} = 474$ nm).

Table 1. Spectra of Intermediates Determined from Fitting Scheme 1 to Absorption Difference Spectra

	5-demethylisorhodopsin	mesityl rhodopsin analogue	rhodopsin[a]
Batho	530 nm	506 nm	531 nm
BSI	466 nm	464 nm	473 nm
Lumi	482 nm	458 nm	488 nm
pigment[b]	474 nm	480 nm	500 nm

[a] Data at 22 °C from Hug et al., 1990.[3] [b] Determined from the pigment bleached by one laser pulse.

Absorbance difference spectra collected after photolysis of the mesityl rhodopsin analogue are shown in Figure 5. Those data were again best fit by two exponential processes with apparent lifetimes 40 and 145 ns. As would be expected when apparent lifetimes are close together, the precision in determining the lifetimes was not as great as it was for 5-demethylisorhodopsin or for bovine rhodopsin where the lifetimes differ by a factor of ~5. However, the residuals obtained from a two-exponential fit were better than those from a single-exponential

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Table 2. Microscopic Rate Constants Determined from Fitting Scheme 1 to Absorption Difference Spectra

	5-demethylisorhodopsin	mesityl rhodopsin analogue	rhodopsin[a]
k_1 (10^6 s^{-1})	7.4	16.5	13.9
k_2 (10^6 s^{-1})	4.2	4.1	9.2
k_3 (10^6 s^{-1})	1.9	9.9	9.3

[a] Data at 22 °C from Hug et al., 1990.[3]

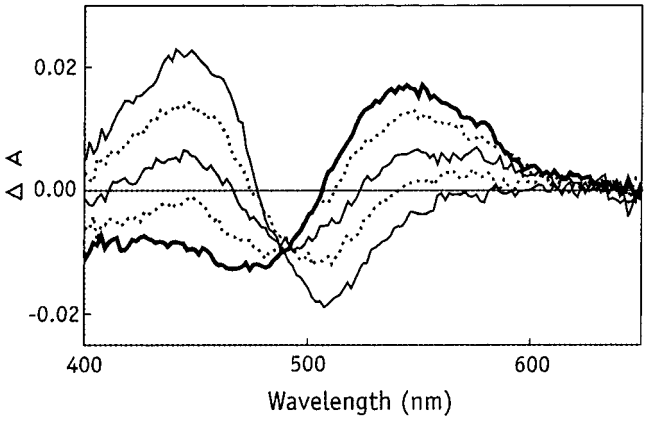

Figure 5. Absorbance changes occurring after 440-nm photoexcitation of the mesityl rhodopsin analogue at 20 °C. Difference spectra shown were collected 20 (heavy line), 40, 80, 160, and 640 ns (alternating dotted and solid lines) after the 7-ns photoexcitation pulse. Negative absorbance below 500 nm (bleaching) and positive absorbance above this wavelength clearly show conversion of the visual pigment into a bathorhodopsin analogue at early times.

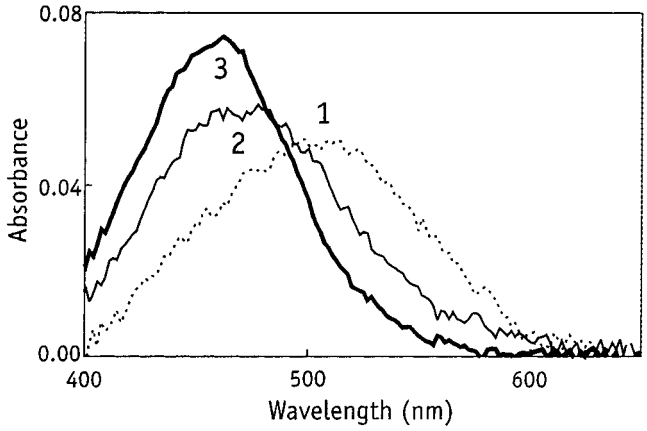

Figure 6. Absolute spectra of intermediates which result from fitting the data of Figure 5 to a straight sequential (no back reactions) scheme. The dotted line shows the earliest intermediate (Batho), the thin solid line shows the second intermediate, and the thick line shows the spectrum of the 5-demethyl Lumi analogue. Because back reactions are not considered in this fit, the spectrum resulting for the second intermediate is a mixture of BSI and Batho, accounting for the tailing seen on the red edge of the second intermediate.

fit. The absolute spectra of the intermediates obtained under the assumption of a straight sequential model are shown in Figure 6. Note that the second intermediate is not blue-shifted relative to the Lumi analogue, a factor which also contributes to the reduced precision in the determination of the apparent lifetimes. Although not as pronounced as for 5-demethylisorhodopsin or for rhodopsin itself (where K= 1.4), the spectrum of the second intermediate produced by the straight sequential model shows tailing to the red which signals the presence of a Batho−BSI equilibrium. Figure 7 shows the spectra of the intermediates obtained using K = 4 for the Batho−BSI

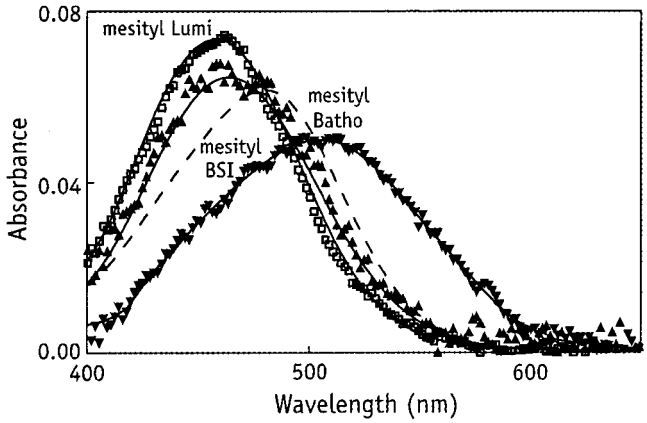

Figure 7. Absolute spectra of mesityl rhodopsin analogue intermediates resulting from Scheme 1 with a Batho−BSI equilibrium constant, K = 4. Symbols show the data points obtained by averaging four data points. Solid lines show the spectra of intermediates obtained from the fit, and the dashed line shows the spectrum of the material that was bleached to form these intermediates (λ_{max} = 480 nm).

equilibrium constant. The λ_{max} values of these intermediates are given in Table 1, and the microscopic rate constants are given in Table 2.

Discussion

Both artificial visual pigments studied here form Batho intermediates displaying red-shifted spectra and decaying with lifetimes comparable to what is seen after photolysis of bovine rhodopsin. This is in contrast to artificial pigments with increased ring-chain flexibility[4,5] or to certain rhodopsin gene mutations which disrupt amino acid side chain packing in the retinal binding pocket,[14] modifications that both result in pigments where Batho decays to BSI much more rapidly. Similarly, red cone pigments display rapid Batho decays which are unresolvable on the time scale studied here.[15,16] These chromophore and protein modifications affect the rate of BSI decay to Lumi much less than they affect the Batho decay rate, and that pattern holds for the artificial pigments from 5-demethylretinal and the mesityl retinal analogue studied here, supporting the idea that protein change, as opposed to binding pocket/chromophore specifics, is rate determining in Lumi formation.

Ring Conformation in 5-Demethylisorhodopsin. The stability of the mesityl analogue Batho is consistent with previously developed ideas about the barrier to Batho relaxation. However, the fact that 5-demethylisorhodopsin forms a Batho intermediate at room temperature which has stability comparable to that formed after photolysis of unmodified bovine rhodopsin is unexpected because removal of the retinal C5-methyl should remove the steric interaction with the C8-hydrogen which is believed to stabilize Batho. Resonance Raman results for 5-demethylrhodopsin have shown that the Batho formed at low temperatures displays HOOPs vibrations similar to those of native Batho, indicating that 5-demethyl Batho has a torsionally strained chromophore.[10,17] Since similar strain likely exists in the room-temperature 5-demethyl Batho chomophore as well,

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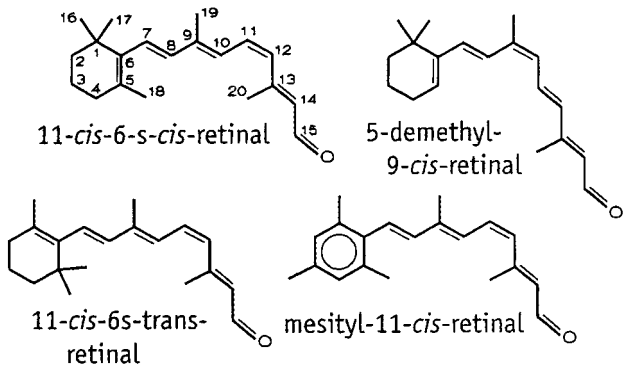
it is of interest to consider what barrier prevents relaxation of Batho after photoisomerization of 5-demethyl pigments. One possible answer is that the ring of 5-demethylretinal binds oppositely to that of the unmodified pigment such that the C1-methyls replace the C5-methyl in its interaction with the C8-hydrogen, reconstituting the barrier.

Discussion of this hypothesis begins with the dark conformation of the retinal. Significant evidence points to a 6-s-cis (C5═C6 and C7═C8 double bonds on the same side of the C6−C7 single bond, see structures, below) form of the retinal in rhodopsin, including previous artificial pigment studies of 6-s-cis-locked bicyclic rhodopsin analogues which displayed λ_{max} values and photochemical behavior similar to those of rhodopsin.[18] Deuterium NMR measurements have suggested a



6-s-trans conformation,[19] but the most recent determination from the rhodopsin X-ray structure has supported the more commonly reported 6-s-cis conformation. For practical purposes, the difference between these two conformations is not as great as it might seem, since in neither case can a planar 6-s conformation be achieved because of steric interaction between the retinal C8-hydrogen and the ring C5-methyl or C1-methyls. The 6-s-trans form most consistent with the ^{2}H NMR data was reported to have a 70° twist away from planarity,[19] and the typical torsion angle expected for 6-s-cis forms is 45°.[20,21] Torsion of the C6−C7 bond away from planarity has important consequences for the absorption spectrum because it blue shifts the spectrum of either 6-s conformation.[18,22]

The λ_{max} of 5-demethylisorhodopsin (474 nm) is significantly blue-shifted relative to that of isorhodopsin (485 nm). This is surprising since removal of the C5-methyl group should allow the ring's C5═C6 double bond to become more planar with the chain's C7═C8 double bond, bringing them into conjugation and red shifting the spectrum. The observed blue shift of 5-demethyl visual pigments is only unexpected, however, under the assumption that the native retinal is in the twisted 6-s-cis form and that 5-demethylretinal binds in the same conformation. If 5-demethylretinal were to bind in the twisted 6-s-trans form it would be expected to be significantly blue-shifted relative to a native 6-s-*cis*-retinal conformation since s-trans conformations have long been known to be blue-shifted relative to the corresponding s-cis forms,[23] although such rules must be applied with caution when comparing twisted conformers. Such an effect might also explain the anomalously slow regeneration observed for 5-demethylretinal pigments. Because β-ionone competitively inhibits rhodopsin regeneration, it has been assumed that the ring end of retinal binds to opsin first.[24] This initial binding step is likely to be favored by a transient conformation where the ring adopts the same twist relative to the polyene chain as occurs in rhodopsin. The presence of the 5-methyl group could act to increase the temporal probability of such a conformation since it defines a turning point for torsional vibrations of the chain relative to the ring. When it is absent, as in 5-demethylretinal, the ring-chain orientation required for native rhodopsin to form may be quite transient, reducing the probability of an important regeneration step. In that case, the C1-methyls could provide an alternative turning point with similarly high temporal probability, but resulting in a chromophore conformation which only approximates the native one, being instead 6-s-trans. That less optimal form might bind at the observed much slower rate to produce a blue-shifted 6-s-trans pigment.

Reduced Mesityl Analogue BSI Blue Shift. From the standpoint of the 6-s question, the mesityl analogue of retinal is interesting since its symmetry precludes its having different 6-s-cis and 6-s-trans forms. The mesityl rhodopsin analogue and its intermediates except for BSI are at least 20 nm blue-shifted relative to rhodopsin and its intermediates. Apparently the extension of conjugation which red shifts the spectra of 3,4-dehydroretinal-based visual pigments and their photointermediates[4] does not have a similar effect in this aromatic pigment. The blue shifts observed for the mesityl rhodopsin analogue and its intermediates indicate reduced conjugation which could be caused by effects intrinsic to the mesityl retinal analogue or external to it, arising from interaction with opsin. One intrinsic factor could be a stronger interaction between the two mesityl methyls and the polyene chain which could reduce ring-chain planarity. Another possible intrinsic blue shifting factor would be a reduced spectral sensitivity to torsion of the aromatic ring. Alternatively the general blue shift of the mesityl pigment and its intermediates could result from factors external to the chromophore, such as a misfit of the aromatic ring into the binding pocket which could induce a more twisted initial ring-chain conformation. If an intrinsic blue shifting factor is at work in the mesityl chromophore, the fact that the mesityl analogue BSI is not blue-shifted by ~20 nm relative to native rhodopsin's BSI suggests that a contribution to native BSI's blue shift comes from a feature which is absent from the mesityl analogue chromophore. One way this could occur is if part of native BSI's blue shift comes from a transient 6-s-cis to twisted 6-s-trans conversion which, because of symmetry, does not have a spectral effect in the mesityl rhodopsin analogue case. Such a transient 6-s-trans species could remain highly twisted, and the fact that the mesityl analogue BSI has a λ_{max} relatively similar to that of native BSI suggests a ring conformation at BSI which electronically disconnects the polyene chain from the double bond specifics of the ring, i.e., a 6-s angle near 90°. This increase in the ring-chain angle could be the distinctive feature of BSI which triggers protein change leading to Lumi formation. It should be emphasized that we only propose 6-s torsional effects to account for a portion of the blue shift of BSI. The mesityl analogue BSI remains blue-shifted relative to its parent pigment spectrum although much less so than in the case of rhodopsin and its BSI. Therefore, other factors must also contribute to the blue shift of BSI, such as the change in Schiff base environment on BSI formation seen in FTIR measurements on 5,6-dihydroisorhodopsin.[25] The possibility that the C6−C7

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connection has an increased deviation from planarity in BSI is consistent with the observation of the blue-shifted intermediate, BL, after batho decay in an artificial visual pigment containing a chromophore with a seven-membered ring-locked 6-s-cis conformation, whereas no BSI was observed in the much more restricted case where a five-membered locking ring was used.[18]

5-Demethyl Lumi Forms Slowly. Unlike the relatively normal kinetic behavior of mesityl analogue BSI, the decay of the 5-demethyl BSI seems quite slow compared to that of BSI from rhodopsin and most other artificial pigments. That may be a consequence of the ring binding in a non-native 6-s-trans conformation, since similarly slow rates have previously only been observed in cases where bulk has been added along the polyene chain,[6] which is not the case here. Given that Batho decay involves movement of the C8-hydrogen past the C5-methyl,[6] so that subsequently C8 pushes the C5-methyl of the ring in the opposite direction, it is likely that BSI formation involves β-ionone ring relaxation to accommodate the new relationship of these two groups. This relaxation, and hence competent BSI formation with respect to progression to Lumi, may suffer interference by the extra methyl at the position normally occupied by C5 and the absence of both methyls at the position normally occupied by C1. Given that the mesityl rhodopsin analogue results suggest a BSI structure with a ring-chain angle near 90°, it is possible that the native C5-methyl stabilizes BSI and that its absence permits a range of unproductive BSI conformations. These ideas remain speculative since currently, with the exception of the recent photoaffinity labeling experiments, there are few structural details known about events following BSI decay, and in the case of a 6-s-trans artificial pigment like 5-demethylisorhodopsin, even those may be of limited applicability. Thus, the unusual initial state of a 6-s-trans chromophore in 5-demethylisorhodopsin makes it difficult to conclude which structural features account for the somewhat slowed rate of BSI formation and the spectral shifts of the later intermediates relative to that of the unphotolyzed pigment. Interpretation of these latter properties is complicated by the question of when the ring reverts to its normal conformation relative to the chain, which is a distinct possibility at some point. Indeed, rotation of the ring might contribute to slow 5-demethyl BSI decay, but because other factors besides ring conformation can affect spectra and kinetics, firm conclusions are difficult to make. From that standpoint, the symmetry of the mesityl rhodopsin analogue and the similarity of the decay rates to those of native rhodopsin simplify the interpretation of its photointermediate's decay.

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Conclusion

Prior to the publication of the X-ray crystal structure of bovine rhodopsin, a good deal of progress had been made in using synthetic analogues of retinal to explore the activation mechanism of this prototypical G-protein-coupled receptor. Many aspects of what had been found previously were confirmed by details of the X-ray structure, while other aspects of the X-ray results were quite unexpected, such as the proximity of extracellular loop II, strand 4 to retinylidene. This new information opens the way for more direct approaches using synthetic retinal analogues and promises to considerably reduce ambiguity in the interpretation of results. Rhodopsin has become a valuable tool for understanding early stages of activation in drug candidate GPCR's whose chemoreceptor function makes them less amenable to rapid kinetic study. Although excellent time resolution has been achieved in characterizing the rhodopsin photointermediates, interpretation of rhodopsin photolysis results has been made difficult by the fact that the spectra of the photointermediates are influenced by a combination of factors which are difficult to resolve. Here we have studied two retinal analogues with modified rings which form Batho intermediates with normal stability. Interpretation of the results was simplified by the minimally perturbed kinetics and the presence of all intermediates which normally appear after rhodopsin photolysis. In the case of 5-demethylisorhodopsin, the observed kinetics and blue-shifted pigment spectra suggest that the ring binds in a 6-s-trans conformation in that pigment. The mesityl analogue of rhodopsin produced a BSI with an unusually small blue shift relative to that of its parent pigment. The reduced blue shift suggests that either increased ring-chain twist or transient formation of a 6-s-trans conformer contributes to the native BSI blue shift since the symmetry of this chromophore does not allow formation of a 6-s-trans conformer to blue shift its spectrum. The relative similarity of the mesityl analogue BSI's λ_{max} to that of native BSI suggests a structure having a ring-chain angle near 90°. That chromophore conformation may be the structural feature which triggers the protein change leading to lumirhodopsin.

Acknowledgment. This work was supported by Grant EY00983 from the National Eye Institute of the National Institutes of Health (to D.S.K.), the A.M.N. Fund for the Promotion of Science, Culture and Arts in Israel (to M.S.), and the Fund for Basic Research (administered by the Israel Academy of Sciences and Humanities) (to M.S.). We thank Professor W. Atom Yee for reading this manuscript and making helpful suggestions.

JA010724Q