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Early Photolysis Intermediates of the Artificial Visual Pigment 13-Demethylrhodopsin†

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ABSTRACT: Nanosecond time-resolved absorption measurements are reported for the room temperature photolysis of a modified rhodopsin pigment, 13-demethylrhodopsin, which contains the chromophore 13-demethylretinal. The measurements are consistent with the formation of an equilibrium between a BATHO-13-demethylrhodopsin species and a blue-shifted species (relative to the parent pigment), BSI-13-demethylrhodopsin. The results are compared to those acquired after photolysis of native bovine rhodopsin [Hug, S. J., Lewis, J. W., Einterz, C. M., Thorgeirsson, T. E., & Kliger, D. S. (1990) *Biochemistry* (preceding paper in this issue)] and to results obtained after photolysis of several modified isorhodopsin pigments in which the BSI species was first observed. It is concluded that in all of the pigments the results are consistent with the formation of an equilibrium between BATHO and BSI, which subsequently decays on a nanosecond time scale at room temperature to a lumirhodopsin intermediate.

Studies of the products created upon light absorption by rhodopsin (RHO), which consists of an 11-*cis*-retinal chromophore attached via a protonated Schiff base linkage to the protein opsin, are instrumental in elucidating the mechanism underlying the visual transduction process. At liquid nitrogen temperatures, the chromophore of RHO undergoes a photoisomerization to produce a red-shifted absorber, bathorhodopsin (BATHO). When gradually warmed in the dark, BATHO is converted successively into lumirhodopsin (LUMI), metarhodopsin I (META-I), and metarhodopsin II (META-II), and finally the retinal chromophore is released from the protein pocket. It has been shown that BATHO stores about 32 kcal/mol of the photon energy which RHO absorbs (Boucher & Leblanc, 1985; Cooper, 1979; Schick et al., 1987). At physiological temperatures it is believed that this energy is used in the propagation of conformational and structural changes which convert RHO to an activated form which triggers visual transduction.

In order to understand these transitions, many spectroscopic studies have focused on elucidating the structure of BATHO and the mechanism by which BATHO decays to the subsequent intermediates [for recent reviews, see Shichida (1986) and Ottolenghi and Sheves (1987)]. In early studies of the room temperature photolysis products of RHO, a red-shifted absorption was observed on the nanosecond time scale which was attributed to BATHO (Cone, 1972; Busch et al., 1972; Rosenfeld et al., 1972; Bensasson et al., 1975; Birge, 1981; Horwitz et al., 1983). It was believed that BATHO decayed with a lifetime on the order of 10^{-8} s to a blue-shifted intermediate, which was attributed to LUMI.

More recent time-resolved spectral studies have brought this model of a simple BATHO to LUMI transition into question (Einterz et al., 1987; Hug et al., 1988, 1990). The clear shift in the isosbestic point between absorption spectra collected from 20 to 600 ns after photolysis of RHO showed that there was more than one process occurring on the nanosecond time scale. In our original analysis of the multiexponential decay at room temperature in the nanosecond time domain, we suggested that this behavior was consistent with the parallel decay of two BATHO species to either one or two LUMI pigments (Einterz et al., 1987). This was justified in part by previous observations of two BATHO products after low-temperature photolysis of RHO by Sasaki et al. (1980a,b). However, it has recently been suggested that the time de-

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pendence of the spectral data is also consistent with a two-step sequential decay to LUMI. On the basis of the time-resolved spectra measured after excitation at different actinic powers and wavelengths, at different temperatures, and with different light polarizations, it was concluded that the data could be best explained by a sequential decay mechanism for BATHO. This mechanism included an equilibrium[1] between BATHO and a subsequent blue-shifted intermediate (Hug et al., 1990).

The early steps in the mechanism are

$$\mathrm{RHO} \xrightarrow[h\nu]{} \mathrm{BATHO} \underset{105\ \mathrm{ns}}{\overset{75\ \mathrm{ns}}{\rightleftharpoons}} \mathrm{BSI} \xrightarrow{105\ \mathrm{ns}} \mathrm{LUMI} \quad (1)$$

The "blue-shifted intermediate", BSI, has not been seen in low-temperature studies of RHO. However, extrapolation to low temperatures of Arrhenius plots of the equilibrium constant suggests that the BSI concentration at temperatures below −140 °C (the temperature where the BATHO to LUMI transition is induced by warming) becomes so small that BSI would not be observable.

Evidence for the additional BSI intermediate comes not only from photolysis studies of RHO but also from nanosecond transient absorption measurements of several different RHO analogues in which the retinal chromophore was replaced by retinals with various modifications in the vicinity of the cyclohexene ring. In these experiments large increases in absorption to the blue of the parent pigments were observed after photolysis on the time scale when one would normally expect to see BATHO-like intermediates. Below we present time-resolved absorption measurements made after photolysis of 13-demethylrhodopsin (13-dm-RHO). These results reveal spectral intermediates consistent with the sequential decay mechanism of RHO photolysis intermediates shown above. For comparison, we will also briefly describe results from other pigments which exhibit similar photolysis intermediates. These pigments include *cis*-5,6-dihydroisorhodopsin (5,6-diH-ISO), 4,5-dehydro-5,6-dihydroisorhodopsin (4,5-deH-5,6-diH-ISO), 7,8-dihydroisorhodopsin (7,8-diH-ISO), and 3,4-dehydroisorhodopsin (3,4-deH-ISO). The results from the modified isorhodopsin pigments will be presented in more detail elsewhere (Einterz et al., in preparation). The chromophores for each of these pigments, as well as for RHO and isorhodopsin (ISO), are shown in Figure 1.

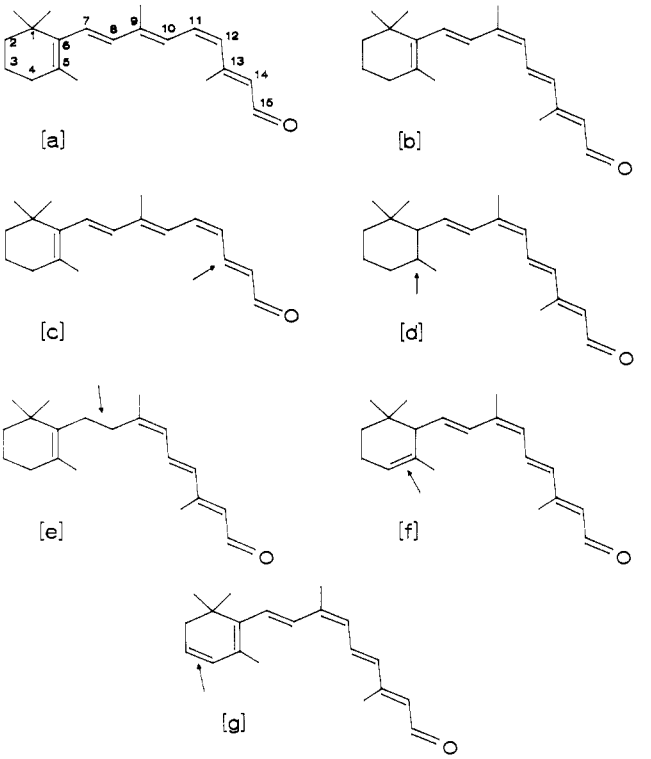


FIGURE 1: Retinal chromophores used to form pigments studied here. Arrows mark the region in which the modification was made. The corresponding pigments are (a) rhodopsin, (b) isorhodopsin, (c) 13-demethylrhodopsin, (d) *cis*-5,6-dihydroisorhodopsin, (e) 7,8-dihydroisorhodopsin, (f) 4,5-dehydro-5,6-dihydroisorhodopsin, and (g) 3,4-dehydroisorhodopsin.

Experimental Procedures

The nanosecond time-resolved absorption apparatus used in these experiments is described in detail elsewhere (Lewis et al., 1987; Hug et al., 1990). Briefly, the sample was excited with a 7-ns (fwhm) pulse from a Quanta Ray DCR-2 Nd:YAG pumped-dye laser (with a wavelength of 477 nm unless otherwise stated). The excitation pulse typically had energies of approximately 0.7 mJ and was focused onto a 1 mm × 10 mm section of the sample, traversing a 2-mm path length. The transient absorption was monitored with a probe beam generated by a xenon flash lamp, which was focused on a 1 mm × 2 mm section of the sample and traversed a 1-cm path length perpendicular to the actinic beam. To avoid artifacts of photoselection, the probe beam was vertically polarized, and the actinic beam was polarized at the magic angle (54.7° from vertical). After each photolysis pulse, new sample was pumped into the irradiated area. Light from the probe beam was analyzed by a gated (10-ns) optical multichannel analyzer, as described previously (Lewis et al., 1987).

Preparation of modified visual pigments followed procedures described previously (Albeck et al., 1989). The 13-demethylretinal chromophore was received as a gift from R. S. H. Liu, and the modified dihydro- and dehydroretinals were synthesized by M. Sheves. All preparations were performed with bovine opsin, and the final sample was detergent-solubilized in 2% octyl β-D-glucopyranoside in pH 7.0 Tris buffer (octyl glucoside, Calbiochem-Behring Cop., La Jolla, CA). Ground-state absorption spectra of the pigments were measured by using an IBM 9420 UV–visible spectrophotometer. In order to measure the amount of pigment photolyzed during a transient absorption measurement, bleaching spectra were obtained as described previously (Albeck et al., 1989), enabling us to calculate absolute time-resolved absorption spectra from the observed difference spectra. While 13-dm-RHO is not completely stable in 1% Ammonyx LO detergent, it has a half-life of approximately 30 min. Thus negligible thermal decomposition of the pigment takes place during the few seconds required to measure the bleaching spectrum.

Results

Time-resolved absorption difference spectra obtained after 477-nm excitation of 13-dm-RHO at 20 °C are presented in Figure 2A. For comparison, the absorption difference spectra obtained after 477-nm excitation of RHO are presented in Figure 2B. The corresponding optical density spectra, calculated by correcting for the photolyzed fraction of parent pigment (Albeck et al., 1989), are presented in panels C and D, respectively, of Figure 2. It should be evident from this comparison that the spectral changes occurring after photolysis of 13-dm-RHO are significantly different from those occurring after photolysis of RHO. The most obvious differences are

[1] Because of the size of the rate constants, a true equilibrium is not reached after photolysis of RHO. For semantic simplicity, we will refer to the reversible reaction defined in eq 1 as an equilibrium regardless of the relative size of the equilibrium rate constant and subsequent rate constants (which vary according to the pigment photolyzed).

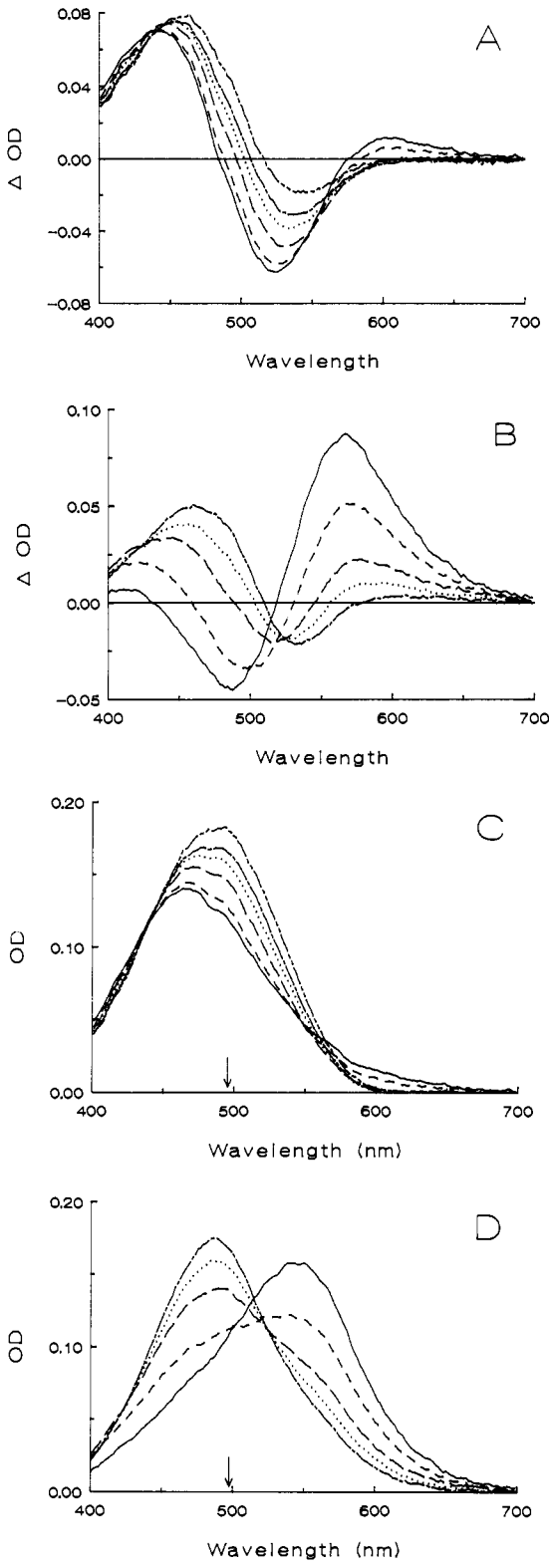


FIGURE 2: Time-resolved spectra of rhodopsin and 13-demethylrhodopsin. (A) Absorption difference spectra measured 15 ns (—), 60 ns (---), 300 ns (--), 600 ns (···), 1000 ns (-·-), and 2000 ns (-··-) after 477-nm photolysis of 13-demethylrhodopsin at room temperature. (B) Absorption difference spectra measured 20 ns (—), 60 ns (---), 170 ns (--), 300 ns (···), and 600 ns (-·-) after 477-nm photolysis of rhodopsin at room temperature. Parts C and D show the absolute absorptions at corresponding times following photolysis of 13-dm-RHO and RHO, respectively, obtained by correcting the difference spectra for the photolyzed fraction of the parent pigment as described under Experimental Procedures. Note that in 13-dm-RHO the major contribution at early times is blue-shifted from the parent pigment (the maximum absorption of which is marked on the wavelength axis of the absorption plot) with only a small contribution from a red-shifted absorber. The opposite is true for rhodopsin.

at early times, when there is a much smaller transient absorption to the red of the parent pigment in 13-dm-RHO relative to that in RHO and a larger transient absorption which is blue-shifted from the ground-state pigment after photolysis of 13-dm-RHO.

Absorption spectra of photolysis intermediates of ISO and several other synthetically modified ISO pigments at early (nanoseconds) and late (microseconds) times following photolysis have also been measured. In the case of 5,6-diH-ISO, the product present 20 ns after photolysis is blue-shifted by about 40 nm from the ground-state pigment and decays within 4 μs to a product that absorbs near the parent pigment, as one might expect for the LUMI intermediate. When these results for 5,6-diH-ISO were first presented (Albeck et al., 1989), it was suggested that the blue-shifted intermediate present 20 ns after photolysis was a decay product of BATHO-5,6-diH-ISO (the BATHO intermediate of 5,6-diH-ISO). It was proposed that BATHO-5,6-diH-ISO had decayed too rapidly to be observed on a nanosecond time scale. Furthermore, we suggested that the rapid decay of BATHO to this blue-shifted intermediate (BSI) was made possible by the increased flexibility of the ring due to saturation of the 5,6-double bond (Albeck et al., 1989).

Similar results have been found in two other synthetic pigments, 4,5-deH-5,6-diH-ISO and 7,8-diH-ISO. In neither of these pigments was a BATHO-like product seen on a nanosecond time scale. Instead, in the 4,5-deH-5,6-diH-ISO pigment the photoproduct present 20 ns after photolysis is blue-shifted by about 30 nm from the parent pigment. By 5 μs this intermediate has decayed to a LUMI-like product (i.e., red shifted by about 10 nm from its parent ISO pigment). The intermediate present 20 ns after 416-nm photolysis of 7,8-diH-ISO absorbs maximally near the parent pigment (whose absorption maximum lies at 421 nm at room temperature). No intermediate red shifted by about 30 nm, as would be the case for a BATHO-like pigment, is seen. At 1 μs after photolysis the maximum absorption still lies near that of the parent pigment. On the other hand, 3,4-deH-ISO, in which the chromophore conjugation has been extended farther into the ring than in RHO or ISO, exhibits normal photolysis behavior (i.e., the initial product on our time scale is red-shifted by about 40 nm from the parent pigment).

The results presented here for 13-dm-RHO are analogous to those of the dihydro pigments in several respects. That is, at 15 ns after photolysis we see an increase in absorption about 20 nm to the blue of the parent pigment, which by 2 μs has decayed to a LUMI-like product, with a wavelength of maximum absorption near that of the parent pigment. It is, however, the only pigment discussed thus far which clearly shows a large blue-shifted absorption as well as a red-shifted absorption which decays on this time scale. Because we were able to prepare larger quantities of the 13-dm-RHO pigment than the other modified pigments, we obtained higher quality data which warranted a more quantitative analysis than the other rhodopsin analogues. As is seen in the data from RHO, the isosbestic shift in the absorption difference spectra of 13-dm-RHO indicates that more than one process occurs during the time scale of our observations. A quantitative analysis enabled us to determine the rate constants of these processes.

To analyze the data, a global exponential fitting routine using singular-value decomposition (SVD), described in detail elsewhere (Nagle et al., 1982; Hofrichter et al., 1985; Hug et al., 1990), was used. Briefly, this approach enables one to simultaneously analyze absorption measurements at all

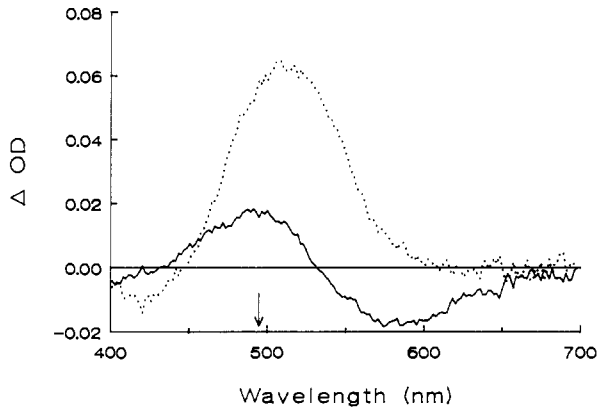


FIGURE 3: Difference spectra of 13-dm-RHO photolysis intermediates calculated by assuming a parallel decay of two species, I_F and I_S, with lifetimes of 100 and 1200 ns, respectively, to the corresponding products, P_F and P_S. Absorptions are normalized to the total amount of I_F and I_S present immediately after photolysis. The absorption maximum of the parent pigment is marked in the figure. Note that while P_F–I_F (—) represents a red-shifted species decaying to a blue-shifted species (typical of the BATHO to LUMI decay in RHO), P_S–I_S (···) represents the decay of a blue-shifted species to an intermediate with a spectral maximum near that of the parent pigment. This is contrary to what one predicts from a parallel analysis of the decay of RHO photolysis, in which both initial species are red-shifted from the parent pigment (Einterz et al., 1987). As shown in Figure 4, this conflict can be resolved by analyzing the decay processes in both RHO and 13-dm-RHO with a sequential mechanism.

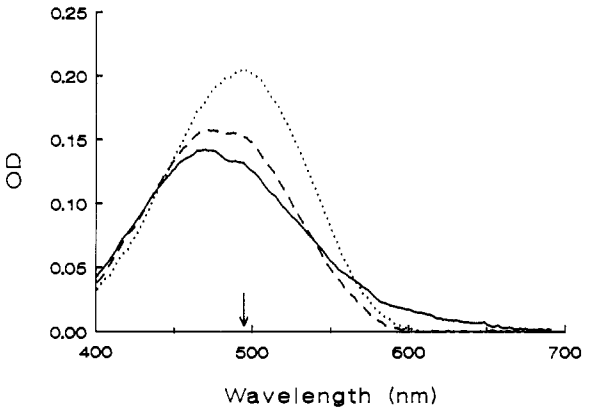


FIGURE 4: Absorption spectra of intermediates after photolysis of 13-dm-RHO calculated by assuming a sequential mechanism for decay of the intermediates. In this mechanism we assume that I_1 (—) decays to I_2 (- - -) with a lifetime of 100 ns, and I_2 decays to I_3 (···) with a lifetime of 1200 ns. Note the broad red tail on I_1. This indicates that I_1 is actually the composite of two (at least) absorbers, one of which is blue-shifted relative to 13-dm-RHO (marked in the figure) and one of which is red-shifted and is thus responsible for the tail. By analogy with RHO, we suggest that these two intermediates are in equilibrium and assign them to be BATHO-13-dm-RHO and BSI-13-dm-RHO. I_2 then represents the spectrum of LUMI-13-dm-RHO, and I_3 represents the spectrum of META-I-13-dm-RHO.

wavelengths and at all times. The fitting routine finds the apparent rate constants and the corresponding preexponential factors ("*b* spectra"). The real rate constants and, often, the actual absorption spectra of individual intermediates can be obtained from the *b* spectra and the observed rate constants once a mechanism has been proposed.

When applied to the transient difference spectra obtained after photolysis of 13-dm-RHO, the SVD analysis showed that two processes were occurring between 15 ns and 2 μs, with rate constants corresponding to lifetimes of 100 and 1200 ns. The two simplest mechanisms based on two rate constants are the parallel decay of two intermediates to the same or different products or the sequential decay of two intermediates to a third product.

With the data available, assuming a parallel mechanism, absolute spectra of the intermediates cannot be determined. Instead, only the difference spectra between the product(s) and the corresponding preceding intermediates can be calculated. These difference spectra are shown in Figure 3. Here the solid-line spectrum corresponds to the difference between the faster decaying species (I_F) and its decay product (P_F), and the dotted-line spectrum corresponds to the difference between the slower decaying species (I_S) and its decay product (P_S). The relevant mechanism is

$$\text{13-dm-RHO} \xrightarrow[h\nu]{} I_F + I_S + \text{prod}$$
$$I_F \xrightarrow{100\text{ ns}} P_F \qquad I_S \xrightarrow{1200\text{ ns}} P_S$$

Here "prod" symbolizes any stable photolysis products such as 13-dm-isorhodopsin (13-dm-ISO). Note that having two rate constants puts no constraints on P_F and P_S, so that they may or may not be identical. Also important to understand is that, in this context, I_F and I_S represent the absorption of the fast and slow intermediates appropriate for their initial concentrations, while P_F and P_S represent the absorptions of the products appropriate for their final concentrations during the time of observation.

It is clear from Figure 3 that if one assumes the parallel decay of two intermediates on this time scale, the fast (100-ns) process corresponds to the decay of an intermediate that is red-shifted from the parent pigment to one that is blue-shifted from or lies near the parent pigment. Qualitatively, this is not unlike what one would expect for the decay of BATHO to LUMI. The slow process, on the other hand, corresponds to the decay of an intermediate that is blue-shifted from the parent pigment to one that lies near the parent pigment. This is not consistent with the original parallel model proposed for RHO, in which two BATHO products decayed to LUMI-like products (Einterz et al., 1987).[2] Here we are observing a red shift at later times, and in the two-BATHO model of RHO both BATHO's decayed to blue-shifted products. Thus, if we accept the parallel mechanism as being the best model for our data, we must introduce the presence of a blue-shifted intermediate that decays in parallel with a BATHO-like intermediate, the analogue of which is not observed after photolysis of rhodopsin.

An alternative to this parallel decay model would involve the sequential decay of one initial intermediate to another. In this case, one can calculate from the SVD analysis the difference spectrum between each intermediate plus any stable products formed upon photolysis, such as 13-dm-ISO, and the photolyzed fraction of 13-dm-RHO. By subtracting out the absorption due to the stable photoproducts, and adding back the appropriately scaled 13-dm-RHO absorption [both of which are obtained from the bleaching spectrum (Albeck et al., 1989)], one can obtain the absorption spectrum of the species corresponding to each rate constant. We define this mechanism as

$$\text{13-dm-RHO} \xrightarrow{h\nu} I_1 + \text{prod} \xrightarrow{100\text{ ns}} I_2 \xrightarrow{1200\text{ ns}} I_3$$

Assuming this mechanism, the calculated spectra of I_1, I_2, and I_3 are presented in Figure 4. In this figure the maximum

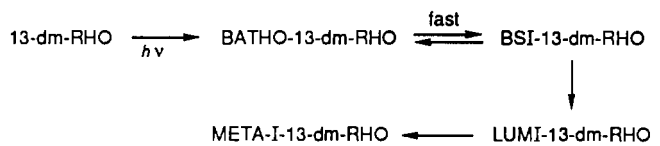
[2] Note that in the preceding paper (Hug et al., 1990) we present evidence which supports the sequential model for the decay of RHO photolysis intermediates in preference to the parallel model.

absorption of each intermediate is normalized to the total amount of that species which is created.

Clearly, the spectra of I_2 and I_3 are qualitatively similar to known absorption spectra of visual pigments. That is, they are smoothly varying spectra with only one maximum and can be described by a log-normal curve (Metzler & Harris, 1978). However, the spectrum of I_1 is skewed significantly toward the red, very much unlike the shape of other visual pigment spectra. It appears, therefore, that when one assumes a sequential decay of intermediates on this time scale, the first intermediate is actually composed of two different absorbers, one that is red-shifted relative to the parent pigment (and is thus responsible for the broad red tail in the spectrum of I_1) and one that is blue-shifted from the parent pigment. Given only two apparent rate constants, this is possible if these two absorbers reach an equilibrium by the time we measure the first transient spectrum. Then the first rate constant is related to the decay of the equilibrated mixture to I_2.

This result is especially intriguing when compared to results on RHO. The spectrum of I_1 is easily decomposed into two log-normal components, one with a maximum near 530 nm and the other with a maximum near 470 nm, which contribute 20% and 80%, respectively, to the total spectrum. These maxima correspond very closely to the absorption maxima proposed for BATHO (530 nm) and BSI (477 nm) when RHO photolysis results are analyzed by assuming a sequential decay with the mechanism defined in eq 1 (Hug et al., 1990). At room temperature the equilibrium constant in RHO (eq 1) is 1.4, which would result in about 58% BSI and 42% BATHO if the decay of BSI to LUMI were blocked (Hug et al., 1990).

Given the correspondence with photolysis intermediates of RHO, it is tempting to choose the sequential model with equilibrium in preference to the parallel model to explain the 13-dm-RHO results. We thus propose that the following mechanism is consistent with our results for 13-dm-RHO:



There are, however, discrepancies in the time domains of the various spectral changes after photolysis of RHO and 13-dm-RHO. In RHO, a process occurring with a lifetime of 36 ns has been attributed to the "approach to equilibrium" of BATHO and BSI (Hug et al., 1990). In 13-dm-RHO, on the other hand, this model suggests that an equilibrium between BATHO-13-dm-RHO and BSI-13-dm-RHO is already established by the first time observed in these experiments, 15 ns. (We also measured spectra with a gate beginning 0 ns after photolysis. While the laser scatter was quite large, we were able to determine that there were no significant differences in the red between the 15-ns data and the 0-ns data.) Furthermore, by analogy with RHO, the spectrum of I_2, representing the decay product of the equilibrated mixture, would correspond to LUMI-13-dm-RHO. This absorption decays with a lifetime of 1200 ns, as opposed to LUMI from RHO, which is stable on this time scale. The absorption maximum of this intermediate, at 480 nm, does correspond reasonably well to the absorption maximum of LUMI from RHO, which lies at 492 nm. To continue the analogy with RHO, the spectrum of I_3 would then correspond to 13-dm-metarhodopsin I (META-I-13-dm-RHO), with a maximum absorption at about 490 nm. As discussed below, it is possible that the rapid decay of LUMI-13-dm-RHO and appearance of META-I-13-dm-RHO are due to relative instabilities of these species in the detergent-solubilized preparations used in our experiments.

Discussion

To summarize thus far, we are proposing that 13-dm-RHO photolysis yields intermediates that are analogous to those formed upon photolysis of RHO, but which are formed more rapidly than the corresponding intermediates in RHO. Thus, photolysis of 13-dm-RHO yields an equilibrated mixture of about 20% BATHO and 80% BSI on a time scale faster than the time resolution of our apparatus. This mixture then decays with a lifetime of 100 ns to a LUMI-like intermediate, which itself decays with a lifetime of 1200 ns to a META-I-like intermediate. It is interesting to compare these results both to the low-temperature results obtained by Shichida et al. (1981) for 13-dm-ISO and to the room temperature results for the other pigment analogues described above.

Shichida et al. irradiated 13-dm-ISO at liquid nitrogen temperatures to produce a thermally stable, red-shifted product which they assigned to BATHO-13-dm-ISO (the BATHO product from 13-dm-ISO), with an absorption maximum at 532 nm. This species decayed when warmed above −175 °C to a blue-shifted intermediate which they called BL-13-dm-ISO, with an absorption maximum at 475 nm. BL was stable only in a narrow range of temperatures and decayed to an intermediate assigned as LUMI-13-dm-ISO above −140 °C. LUMI had an absorption maximum at 517 nm and decayed at temperatures greater than −35 °C to META-I-13-dm-ISO, which had its maximum absorption at 512 nm (Shichida et al., 1981).

Shichida et al. based their assignments of LUMI-13-dm-ISO and META-I-13-dm-ISO largely on the decay temperature of LUMI-13-dm-ISO, at −35 °C. This compares well with LUMI from ISO, which decays to META-I at temperatures greater than −40 °C. The assignment of BATHO-13-dm-ISO appears to have been based mainly on the red-shifted spectrum, which is expected for BATHO from ISO or RHO. BATHO-13-dm-ISO absorbs maximally at 532 nm, while BATHO from ISO absorbs maximally at 543 nm at low temperatures. Thus, on the basis of these assignments, they proposed the existence of a new intermediate after photolysis of 13-dm-ISO, BL-13-dm-ISO, which is blue-shifted from the parent pigment.

We propose that we are seeing the same intermediates as Shichida et al., except that the intermediate they refer to as BL corresponds to an equilibrium between BSI and BATHO, which is largely shifted toward BSI. Thus we suggest that BATHO-13-dm-RHO is stable at liquid nitrogen temperature and thermally equilibrates with BSI-13-dm-RHO only when the temperature is raised above −175 °C. The final equilibrium mixture, which is comprised mainly of BSI-13-dm-ISO, is not established until about −150 °C, where Shichida et al. see a plateau which they attribute to BL. As does BATHO from RHO, this mixture then decays to LUMI-13-dm at temperatures above −140 °C. This is in keeping with the observation at room temperature that the lifetime of decay of the equilibrated mixture in 13-dm-RHO is very close to the decay rate of BSI to LUMI in RHO (both about 100 ns).

The wavelengths of maximum absorption for our LUMI-13-dm-RHO (480 nm) and META-I-13-dm-RHO (490 nm) are very different from those of Shichida et al. (LUMI-13-dm-ISO = 517 nm, META-I-13-dm-ISO = 512 nm). It is unclear why Shichida's values differ significantly from the maximum absorption wavelengths of LUMI and META-I from ISO at low temperatures (497 and 478 nm, respectively).

However, the fact that our measured values differ so much from the values of Shichida requires further explanation. There are several factors here that might be significant. First, some of the difference can be attributed to the different temperatures at which the spectra were measured. For instance, the wavelength of maximum absorption of the parent pigment at liquid nitrogen temperature is shifted 8 nm to the red of the room temperature value.

Second, our results are for 13-dm-RHO, whereas Shichida et al. worked with 13-dm-ISO. This is not likely to be a large effect, since our own measurements on 13-dm-ISO gave qualitatively similar results to those presented here for 13-dm-RHO. However, differences with respect to stability in hydroxylamine are observed between the 11-cis and 9-cis forms of the 13-dm parent pigment (Nelson et al., 1970; Shichida et al., 1981). It is plausible that structural features causing this difference may also be responsible for quantitative differences in absorption spectra of photolysis intermediates.

Third, and perhaps most significantly, is the fact that we performed our experiments on detergent-solubilized pigments in order to avoid problems with light scattering normally encountered in membrane suspensions. It is known that detergents drastically affect the equilibrium between META-I and META-II in RHO (Stewart et al., 1977). In a pigment such as 13-dm-RHO, which is not particularly stable in harsh environments (such as in the presence of hydroxylamine), it is thus not surprising that the detergent would affect intermediates as early as the LUMI stage, when the chromophore might be exposed to the solvent. Such environmental perturbations might easily shift the spectra of the intermediates. Perhaps of equal importance is the possibility that the detergent has a significant effect on the stability of the later intermediates (Lewis et al., 1981). This might explain the rapid decay of LUMI-13-dm-RHO as compared to LUMI from RHO.

An important question that still remains is why we are able to observe the approach to equilibrium between BATHO and BSI in RHO over a 35-ns time period, while an equilibrium is already established in 13-dm-RHO by 15 ns after photolysis. Possibly related to this is the difference in equilibrium constants for BATHO $\rightleftarrows$ BSI vs BATHO-13-dm-RHO $\rightleftarrows$ BSI-13-dm-RHO. Recall that we find in RHO an equilibrium mixture consisting of approximately 65% BSI, while for 13-dm-RHO the equilibrated mixture consists of about 80% BSI. Some insight to this answer may be gained by considering the dihydro pigment analogues along with the 13-dm-RHO analogue.

As already noted, in all of the dihydro pigments a blue-shifted absorption (or, in the case of 7,8-diH-ISO, little or no shift) was obtained immediately after photolysis on our time scale. In 13-dm-RHO a blue-shifted absorption as well as a smaller red-shifted absorption was seen immediately after photolysis. In RHO and 3,4-deH-RHO a large red-shifted absorption is observed as well as a smaller blue-shifted absorption. We propose that in all of these pigments we are simply observing variations of the same basic mechanism, as written in eq 1. By analogy to RHO, we suggest that the earliest spectra which we observed after photolysis of the dihydro pigments are due to the equilibrated BATHO $\rightleftarrows$ BSI mixtures but that in each pigment the equilibrium has been shifted largely toward BSI. In 13-dm-RHO we observe similar behavior, but the equilibrium is shifted less toward BSI than in the dihydro pigments, and more toward BSI than in RHO. In all cases this equilibrated mixture decays to the LUMI intermediate, which has an absorption maximum nearer to the parent pigment than either BATHO or BSI.

It is difficult to elucidate the molecular mechanism that might be responsible for the BATHO $\rightleftarrows$ BSI equilibrium. It is likely that the structural properties of BATHO which are responsible for its red-shifted spectrum are associated with the twisted nature of the polyene, which gives rise to the HOOP modes observed in the BATHO Raman and IR spectra (Eyring et al., 1980, 1982; Bagley et al., 1985). One possibility is that the transition from BATHO to BSI involves a relaxation of the chromophore twists in order to relieve strain induced by these twists in the ring and chain regions. Steric or electrostatic interactions within a fairly constrained protein pocket, however, may push the molecule back into the BATHO conformation, thereby establishing the BATHO $\rightleftarrows$ BSI equilibrium. The transition to LUMI would then involve the relaxation of the protein accompanied by chromophore motions made possible by the protein relaxation.

That the equilibrium is established more rapidly in the dihydro pigments than in RHO and is shifted more toward BSI may be due to the increased flexibility in the ring region of the chromophore. Saturation of the 5,6- or 7,8-double bonds should decrease geometric and electronic constraints arising from conjugation between the ring and polyene portions of the chromophore. Removal of the 5,6-double bond is likely to increase flexibility at least in the ring. Structural readjustments to relieve strain induced by the cis to trans isomerization to form BATHO might then occur more readily in the dihydro pigments than in RHO itself. If the back-reaction from BSI to BATHO is indeed triggered by protein constraints on the chromophore, this rate may be affected very little by the double-bond saturation. Thus, one would expect to see a faster approach to equilibrium as well as a shift of the equilibrium toward BSI.

The same may be true of 13-dm-RHO, but to a lesser extent. That is, removal of the methyl group may increase chromophore flexibility, perhaps because of fewer steric constraints with the protein, which then increases the rate of the BATHO to BSI transition. If, however, the increased degree of flexibility is not as great as in the dihydro pigments, one might expect that the equilibrium, while shifted more toward BSI than in RHO, would not be shifted as much as in the dihydro pigments. The 3,4-dehydro pigment, in which one would not necessarily expect any more chromophore flexibility than in RHO, correspondingly exhibits behavior similar to that observed after photolysis of RHO.

In all of the pigments discussed here, we estimate that the decay to the LUMI intermediate occurs with a lifetime near 100 ns. This is consistent with our proposal that in all of the pigments the rate-determining step of the transition from BSI to LUMI involves protein rearrangement to further relieve chromophore strain induced by the initial photoisomerization. These protein changes would likely be accompanied by changes in the chromophore as it readjusts to the new protein conformation.

Conclusions

We have presented room temperature measurements of transient absorption on a nanosecond time scale after photolysis of 13-demethylrhodopsin. Quantitative analysis of these results shows that the decay processes occurring after photolysis of 13-dm-RHO are consistent with those that occur after photolysis of rhodopsin on our time scale. That is, we observe an equilibrium between a BATHO intermediate and a blue-shifted intermediate (BSI) which then decays to a LUMI intermediate. Results from photolysis of several modified ISO pigments are also consistent with this decay mechanism. It

is suggested that replacement of the 13-methyl group by hydrogen, or saturation of the 5,6- or 7,8-double bonds in the retinal chromophore, results in increased chromophore flexibility, giving rise to a faster approach to the equilibrium between BATHO and BSI, as well as a shift of the equilibrium toward BSI. The observation of an additional blue-shifted intermediate after photolysis of 13-dm-RHO is consistent with low-temperature measurements of 13-dm-ISO, in which a blue-shifted intermediate was also observed.

The structure of BSI is currently undetermined, as are the processes by which it is formed from BATHO and by which it decays to LUMI. It is proposed that the transition from BATHO to BSI involves mainly chromophore changes within a relatively constrained protein, while the transition to LUMI involves protein changes accompanied by further chromophore movements. Recent polarization measurements on RHO photolysis intermediates suggests that the spectral shifts of these intermediates can be correlated with the chromophore orientation (Lewis et al., 1989). Such studies, along with investigation of other modified pigments, should clarify the nature of these early photolysis intermediates.

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