Photolysis intermediates of the artificial visual pigment *cis*-5,6-dihydro-isorhodopsin

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ABSTRACT The photolysis intermediates of an artificial bovine rhodopsin pigment, *cis*-5,6-dihydro-isorhodopsin (*cis*-5,6,-diH-ISORHO, λ_{max} 461 nm), which contains a *cis*-5,6-dihydro-9-*cis*-retinal chromophore, are investigated by room temperature, nanosecond laser photolysis, and low temperature irradiation studies. The observations are discussed both in terms of low temperature experiments of Yoshizawa and co-workers on *trans*-5,6-diH-ISORHO (Yoshizawa, T., Y. Shichida, and S. Matuoka. 1984. *Vision Res.* 24:1455–1463), and in relation to the photolysis intermediates of native bovine rhodopsin (RHO). It is suggested that in 5,6-diH-ISORHO, a primary bathorhodopsin intermediate analogous to the bathorhodopsin intermediate (BATHO) of the native pigment, rapidly converts to a blue-shifted intermediate (BSI, λ_{max} 430 nm) which is not observed after photolysis of native rhodopsin. The analogs from lumirhodopsin (LUMI) to meta-II rhodopsin (META-II) are generated subsequent to BSI, similar to their generation from BATHO in the native pigment. It is proposed that the retinal chromophore in the bathorhodopsin stage of 5,6-diH-ISORHO is relieved of strain induced by the primary *cis* to *trans* isomerization by undergoing a geometrical rearrangement of the retinal. Such a rearrangement, which leads to BSI, would not take place so rapidly in the native pigment due to ring–protein interactions. In the native pigment, the strain in BATHO would be relieved only on a longer time scale, via a process with a rate determined by protein relaxation.

INTRODUCTION

Initiation of visual pigment activation (Fig. 1) in rhodopsin (RHO, containing an 11-*cis* retinal chromophore bound to the protein via a protonated Schiff base linkage) is associated with the early red-shifted intermediate, bathorhodopsin (BATHO) (for reviews, see Packer, 1982). BATHO, which at room temperature decays to the next photointermediate, lumirhodopsin (LUMI), over a 10^{-8}–10^{-7} s time scale, appears to be formed in the subnanosecond (10^{-12}–10^{-10} s) range from a precursor denoted as prebathorhodopsin (PBATHO; Dinur et al., 1981), based on the low temperature experiments of Peters et al. (1977), or photorhodopsin (PHOTO), based on the room temperature data of Yoshizawa et al. (1984) and Shichida et al. (1984; Shichida, 1986). PBATHO and PHOTO are either precursors of BATHO, or are primary products formed in parallel with BATHO which decay back to ground state pigments.

Much of the attention devoted to BATHO, the first transient species produced by photolysis which can be trapped at low temperatures, is due to its property of storing 36 kcal/mol of the photon energy for use in subsequent stages of the transduction process (Boucher and Leblanc, 1985; Cooper, 1979; Honig et al., 1979*a,b;* Schick et al., 1987). The exact mechanism of energy storage in BATHO and the origin of its bathochromic spectral shift are presently unclear (for recent discussions see Birge et al., 1988; Ottolenghi and Sheves, 1987; Palings et al., 1987). The relative contributions of factors such as electrostatic interactions between the chromophore and the protein or surrounding environment, and conformational distortions of the polyene chromophore are still open questions (Honig et al., 1979*a,b;* Palings et al., 1987; Birge and Hubbard, 1980; Warshel and Barboy, 1982).

Artificial pigments in which the native retinal chromophore is substituted by a synthetic retinal analog are potential tools for elucidating the nature of photolysis intermediates (for a review, see Derguini and Nakanishi, 1986). For example, as shown in the case of bacteriorhodopsins, the photocycles of artificial pigments can be used to discriminate between light-induced changes associated with the Schiff base moiety, bond rotations and isomerizations, interactions with protein charges, etc. (Ottolenghi and Sheves, 1987; Sheves et al., 1987). The lack of photoreaction in the case of visual pigments with a blocked C_{11}=C_{12} bond has confirmed the role of the 11-*cis* to all-*trans* isomerization in the primary photochemical event (Akita et al., 1980; Mao et al., 1981; Fukada et al., 1984; Hubbard and Kropf, 1958; Rosenfeld et al., 1977). Additionally, studies performed on a pigment with blocked C_9—C_{11} bonds has shown that C_{10}—C_{11} bond rotation is not needed for photoreactions to proceed as in the native pigment (Sheves et al., 1986).

To date, no systematic study has been carried out on

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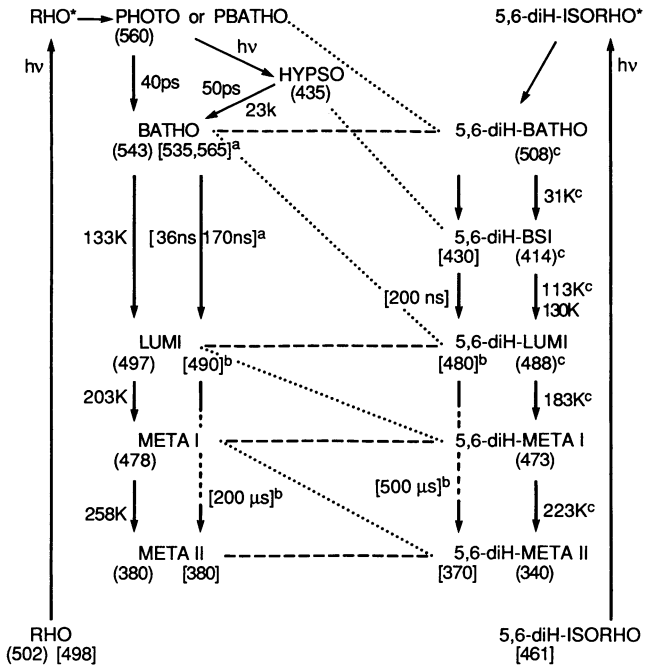
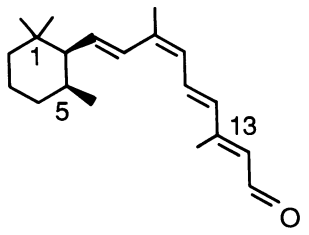


FIGURE 1. Correlation diagram between the photolysis intermediates of native rhodopsin (RHO) or isorhodopsin (ISORHO), and 5,6-dihydro-isorhodopsin (5,6-diH-ISORHO). Data for RHO are adapted from Yoshizawa et al. (1984), Shichida (1986), and Einterz et al. (1987). Data for 5,6-diH-ISORHO are from Yoshizawa et al. (1984) and this work. Temperatures denote stability limits of transitions to subsequent intermediates. Time notations are approximate lifetimes at room temperature. Numbers in parentheses denote wavelengths of maximum absorption at low temperature (Yoshizawa et al., 1984), and those in square brackets denote wavelengths of maximum absorption at room temperature (this work and Einterz et al., 1987). Dotted lines show the correlation between the phototransients of the two pigments as suggested by Yoshizawa et al. Dashed lines show the correlations suggested in this work. [a]The values given for the absorption maxima, 565 and 535 nm, and the lifetimes, 170 and 36 ns, refer to the two BATHO species observed after room temperature photolysis (Einterz et al., 1987). [b]In detergent-solubilized RHO the transition from LUMI to META I is not resolved at room temperature. Thus the wavelength given refers to the wavelength of maximum absorption after 5 µs, and the time refers to the decay of a broad absorption centered near 490 nm in RHO and 480 nm in 5,6-diH-ISORHO. Microsecond and millisecond kinetics on sonicated rod outer segment suspensions of RHO are considerably more complicated. For this reason, and because increased scattering in ROS preparations of the 5,6-diH-ISORHO pigment leads to poorer signal-to-noise ratios than in detergent-solubilized samples, we have chosen to compare only the intermediates from the detergent preparations. [c]Values are from Yoshizawa et al., 1984.

the effects of retinal modifications of the spectra and kinetics of the photointermediates of visual pigments at room temperature (close to physiological conditions). Here we present data on the room temperature intermediates obtained after photolysis of an artificial bovine rhodopsin pigment, *cis*-5,6-diH-ISORHO, derived from *cis*-5,6-dihydro-9-*cis*-retinal, structure I (Spudich et al., 1986). (The *cis* notation denotes a "*cis*" configuration with respect to the 5-methyl/chain structure.)



I

We will compare these results to those obtained by Yoshizawa et al. (1984) at low temperatures for *trans*-5,6-diH-ISORHO. Substantial differences between *cis*-5,6-diH-ISORHO and RHO are observed 10–100 ns after photolysis, providing insight to the nature of the BATHO photointermediate of native rhodopsin.

GLOSSARY

cis-5,6-diH-ISORHO	*cis*-5,6-dihydro-isorhodopsin
RHO	bovine rhodopsin
BATHO	bathorhodopsin
LUMI	lumirhodopsin
PBATHO	prebathorhodopsin
PHOTO	photorhodopsin
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
ROS	rod outer segments
HYPSO	hypsorhodopsin
META I	metarhodopsin I
META II	metarhodopsin II.

EXPERIMENTAL

Synthesis of dihydro-retinal chromophore

Cis-5,6-diH-9-*cis*-retinal was prepared from β-ionone by hydrogenation over 5% $Pd/CaCO_3$ poisoned with lead in benzene to give *cis*-5,6-dihydro-β-ionone (Spudich et al., 1986). Condensation with the sodium salt of $(EtO)_2POCH_2CN$, reduction with diisobutylaluminum hydride (DIBAH), and separation of isomers gave the *cis* isomer of the 5,6-dihydro-C_{15} aldehyde. From this, *cis*-5,6-diH-9-*cis* retinal (I) was formed by standard methods (Spudich et al., 1986).

Regeneration/nanosecond photolysis

Bovine rod outer segments (ROS) were prepared from frozen retinas as previously described (Lewis et al., 1984). Freshly prepared, pH 7.0 hydroxylamine stock was added to produce a final concentration of 20 mM, and the ROS were bleached for 2 min using a 25-W bulb within 2 in. Hydroxylamine was removed by three washes which consisted of sedimentation of the ROS, pouring off the supernatant, and resuspending in pH 7.0 buffer containing 10 mM Tris, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA. Immediately after the last wash, sufficient chromophore to produce a 50% excess was added from an ethanol stock solution. The mixture was then incubated in the dark at 37°C for 3 h. NADPH (0.1 mg/mg opsin) was then added and the mixture was incubated for 45 min to convert the remaining aldehyde chromophore to the alcohol form, using the endogenous ROS retinol dehydrogenase. Pigment yields were typically 70%.

Detergent suspensions were prepared in 2% octyl-β-D-glucopyranoside (octyl-glucoside, in pH 7.0 Tris buffer; Calbiochem-Behring Corp., La Jolla, CA) after washing the ROS three times with pH 7.0, 2 mM EDTA solution to remove the extrinsic membrane proteins. Detergent suspensions were frozen before use and usually developed an opsin precipitate when thawed. This was removed by centrifugation. The optical density of the solutions (1-cm path length) used in the nanosecond experiments was typically 0.6 at the λ_{max} of the pigment (461 nm in the case of *cis*-5,6-diH-ISORHO). Nanosecond photolysis measurements were performed as previously described (Einterz et al., 1987; Lewis et al., 1987) except that the actinic source was a Nd/YAG pumped dye laser which produced a 7-ns fwhm pulse of 477-nm light. Pulses with typical energies of 0.5 mJ were used to irradiate a sample area of 1 × 10 mm. A new sample was pumped into the irradiated portion of the cell after each pulse.

Correction for ground state bleaching

Ammonyx detergent was added to the octyl-glucoside–solubilized pigments on which photolysis measurements were performed to obtain a final solution containing 1% ammonyx. This ensured rapid total bleaching of the pigment when photolyzed by the laser pulse (as opposed to the slow decay of Meta II and formation of a Meta III intermediate). Single shot irradiation of the pigment then allowed measurement of the amount of ground state bleached by each laser pulse as follows. The spectral distribution of light transmitted through a fresh sample of unbleached pigment was measured using a single flashlamp pulse, the intensity of which caused <1% bleaching. The pigment was then irradiated with a single laser pulse. After several seconds another flashlamp pulse was used to probe the light transmitted through the bleached pigment. The difference spectrum, which we denote as the "bleaching spectrum," was then calculated as for the transient measurements. This process was repeated from 20 to 30 times, with a standard deviation of 5%.

The average of the bleaching spectra was used to calculate the amount of pigment bleached by a single laser pulse as follows. The bleaching spectrum was modeled as the difference between spectra representing the initial pigment and the stable end product in ammonyz after bleaching. The amplitude of the spectrum for the pigment used in this model thus represented the amount of pigment bleached by the laser. This pigment spectrum was then added back to the transient difference spectra to generate transient absorption spectra. The resulting spectra of intermediates did not change qualitatively even when the amount of pigment spectrum added back was changed by ±20%, which is significantly greater than our experimental uncertainty.

Low temperature measurements

Cis-5,6-diH-ISORHO was mixed with glycerol resulting in a final glycerol concentration of 66%. For low temperature experiments, the sample was placed in a glass cryostat with quartz windows. Irradiation was carried out with a 100-W bulb and wavelengths were selected using glass cutoff filters. The difference spectra were measured using a Hewlett Packard Co. (Palo Alto, CA) 8450A diode array spectrophotometer. No photolysis was observed by the spectrophotometer monitoring source alone.

RESULTS

Fig. 2 *a* shows the transient difference spectra observed from 20 ns to 4 μs after laser excitation of *cis*-5,6-

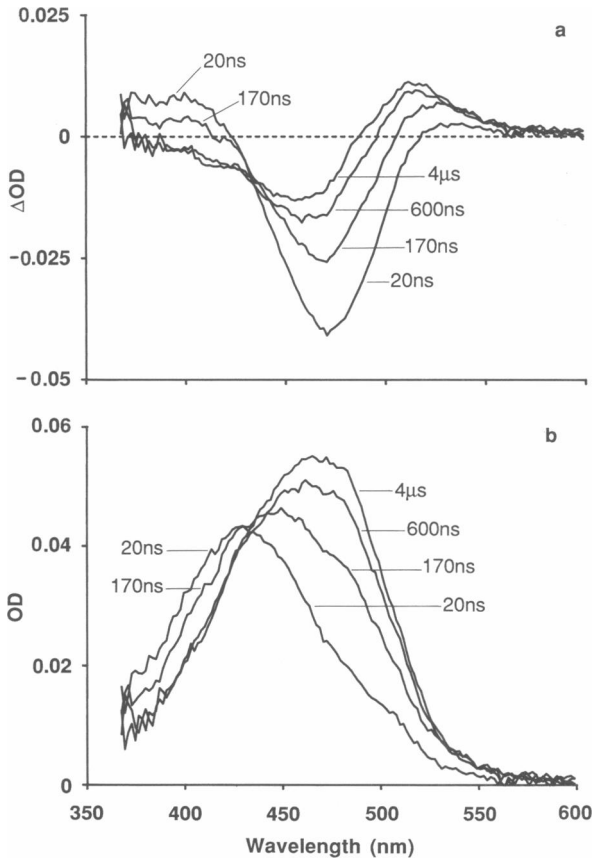


FIGURE 2. Transient spectra of detergent-solubilized 5,6-diH-ISORHO after 477-nm laser photolysis at room temperature. (*a*) Transient difference spectra from 20 ns to 4 μs after photolysis. Note that the predominant features in the earlier times correspond to a bleaching of the ground state and an increase in absorption to the blue of the parent pigment. (*b*) Transient absorption spectra calculated from the difference spectra in *a* according to the procedure outlined in the experimental section. In contrast to transient spectra observed after photolysis of RHO (see Fig. 3), the intermediate present 20 ns after photolysis of 5,6-diH-ISORHO is blue-shifted (λ_{max} 420 nm), rather than red-shifted, from the parent pigment (λ_{max} 461 nm).

diH-ISORHO. Fig. 3 *a* shows the spectral changes which occur after photolysis of RHO on an analogous time scale. In contrast to rhodopsin (Packer, 1982; Einterz et al., 1987) and isorhodopsin (Hug et al., 1988), no initial absorbance increase in the red is observed in the case of *cis*-5,6-diH-ISORHO. (Note that the small amount of positive ΔOD seen to the red of the maximum absorption of the pigment in the 20-ns data for *cis*-5,6-diH-ISORHO increases at longer times, and is thus attributed to a later intermediate, rather than a red-shifted BATHO intermediate.)

Figs. 4 and 5 present steady-state illumination experiments of *cis*-5,6-diH-ISORHO carried out in a glycerol-water mixture at low temperatures. After illumination at 93 K with 430-nm light, a bleaching was observed at 470 nm, accompanied by an absorbance increase at 405 nm corresponding to a blue-shifted photoproduct (Fig. 4 *a*). Subsequent illumination with 370-nm light (Fig. 4 *b*) reverses the effect, partially regenerating a red-shifted absorption band at 480 nm. The red-shifted band (relative to the original 470-nm absorption) is probably due to a mixture of 9-*cis* and 11-*cis* isomers. The initially blue-shifted band induced by 430-nm illumination at 93 K is stable up to 130 K. At about 130 K (Fig. 5), the above difference spectrum red shifts to a spectrum representing a 480-nm absorber. Similar results were obtained for illuminations carried out at 77 K.

Spectra of the photolysis intermediates at room temperature were obtained from the corresponding difference spectra of Figs. 2 *a* and 3 *a* by correcting for the contribution of the bleached pigment according to the procedure outlined in the experimental section. The spec-

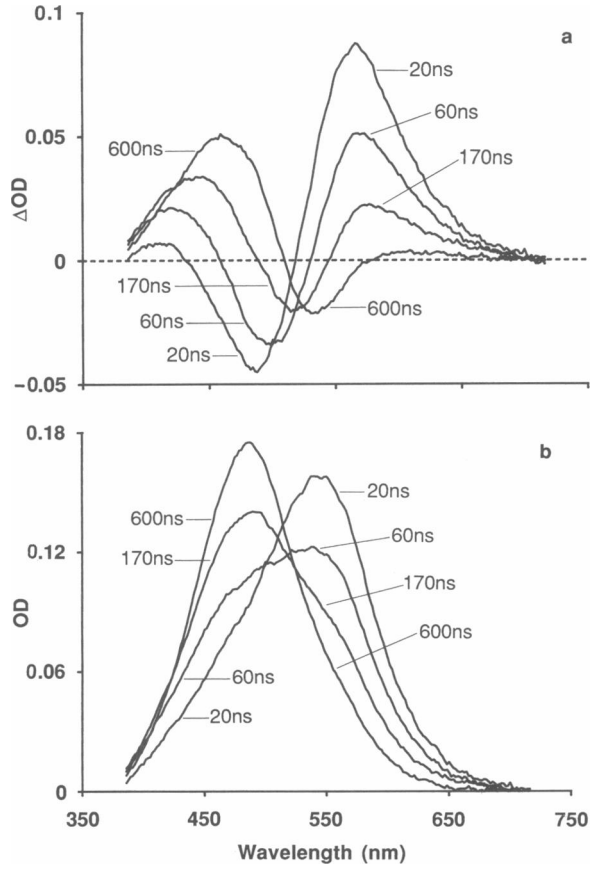


FIGURE 3. Transient spectra of detergent-solubilized RHO after 477-nm laser photolysis at room temperature. (*a*) Transient difference spectra from 20 to 600 ns after photolysis. Note that the predominant features in the earlier times correspond to a bleaching of the parent pigment and an increased absorption to the red of the parent pigment. The shifting isosbestic is due to the decay of two spectrally distinct BATHO products. (*b*) Transient absorption spectra calculated from the difference spectra in *a*. As expected, the intermediates seen at 20 ns, corresponding to the BATHO stage, are red-shifted from the parent pigment, and decay over several hundred nanoseconds to an absorption near 490 nm, corresponding to LUMI. It should be noted that the sectra observed after photolysis of ISORHO are qualitatively very similar to those shown here for RHO (Hug et al., 1988).

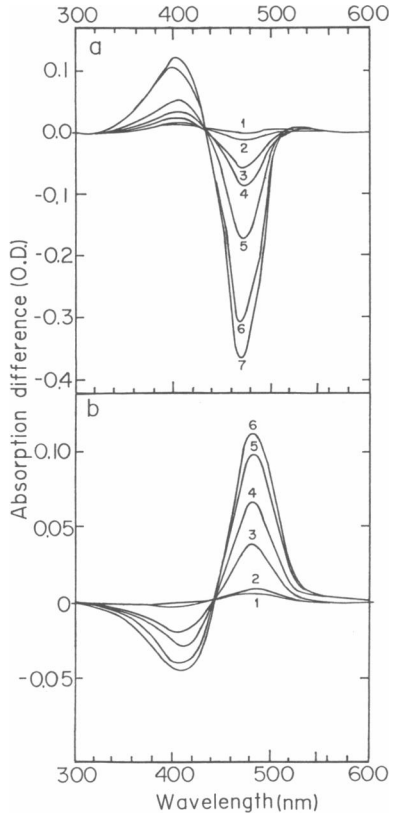


FIGURE 4 (*a*) Difference spectra after 430-nm illumination of *cis*-5,6-diH-ISORHO at 93 K. Curves 1–7 represent illumination for 2, 6, 16, 46, 80, 106, and 166 s, respectively. (*b*) Difference spectra after 370-nm illumination at 93 K of a sample that was previously irradiated with 430 nm (Fig. 4 *a*). Curves 1–6 represent illumination for 2, 12, 42, 102, 122, and 462 s, respectively.

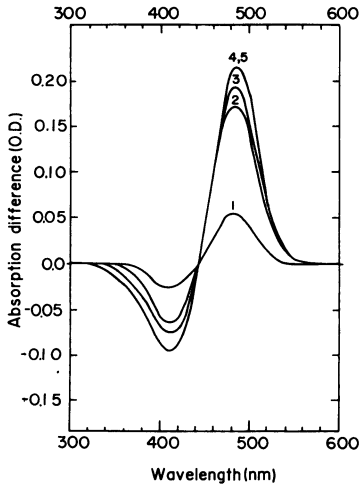


FIGURE 5 Difference spectra of *cis*-5,6-diH-ISORHO that was irradiated first at 93 K with 430 nm and then warmed to 113 K and subsequently to 133 K. Curve 1 is the difference spectrum between 113 K and 133 K, following the warming process. Curves 2–5 are the difference spectra between 113 K and the spectrum at 133 K after 5, 8, 13, and 18 min, respectively, at this temperature.

tra of the various intermediates obtained after photolysis of *cis*-5,6-diH-ISORHO and RHO are shown in Figs. 2 *b* and 3 *b*.

It is evident from Figs. 2 and 3 that in the case of *cis*-5,6-diH-ISORHO, the transient absorption observed 20 ns after photolysis (which in both RHO and ISORHO corresponds to the BATHO intermediate) is markedly different from that of BATHO. In contrast to RHO, the transient observed 20 ns after photolysis of *cis*-5,6-diH-ISORHO, which we denote as BSI, is blue-shifted rather than red-shifted, relative to its parent pigment. Identical conclusions may be derived from the low temperature experiments of Fig. 4. Namely, the light-induced change in absorption at 93 K associated with the red-shifted BATHO intermediate in RHO (Packer, 1982) is replaced by a difference spectrum which is qualitatively similar to that of the blue-shifted intermediate observed at room temperature.

DISCUSSION

Identification of the photointermediates of 5,6-diH-ISORHO and correlations with those of RHO

When attempting to characterize BSI in terms of the photointermediates of the native rhodopsin or isorhodopsin pigments, kinetic differences (i.e., decay times and thermal stability) as well as spectroscopic differences should be examined. Given these considerations, we propose below that BSI is a decay product of 5,6-diH-BATHO which is formed via changes in the retinal conformation. BSI decays to 5,6-diH-lumirhodopsin via a process which, like the decay of BATHO in the native pigment (and unlike the decay of 5,6-diH-BATHO), is rate-limited by protein conformational changes.

We first note that BSI decays over a time scale of several hundred nanoseconds, which is only slightly longer than that of BATHO in the native pigment. It is relevant to interject that it has been shown that transient absorption spectra of RHO and ISORHO are consistent with the decay of two spectrally distinct, red-shifted BATHO products (Einterz et al., 1987; Hug et al., 1988). While there is clear evidence in the transient spectra of 5,6-diH-ISORHO presented here that more than one blue-shifted species is decaying on the submicrosecond time scale, our signal-to-noise ratio precludes quantitative resolution of the kinetics or spectra of these species. We thus feel it would be premature to interpret BSI in terms of its spectral components. Instead, for the purposes of this discussion we consider only a single decay on the nanosecond timescale, with a lifetime of 200 ns (the closest single exponential fit to the kinetic decay at 470 nm). Of the intermediates identified after photolysis of rhodopsin, this lifetime compares most closely with those of the BATHO products, which have lifetimes of 36 and 170 ns.

Given the similar lifetimes of BATHO from RHO and of BSI from *cis*-5,6-diH-ISORHO, it is tempting to identify BSI as a transient comparable to BATHO, but with a blue-shifted, rather than red-shifted, absorption. Alternatively, BSI may constitute an additional step between a red-shifted *cis*-5,6-diH-BATHO intermediate and a *cis*-5,6-diH-LUMI intermediate, which is unresolved after photolysis of the native pigment. Since we see no red absorption 20 ns after photolysis of *cis*-5,6-diH-ISORHO, this would imply that any red-shifted *cis*-5,6-diH-BATHO intermediate formed is characterized by a room-temperature lifetime of less than 10 ns (the time resolution of our apparatus). Moreover, it would also imply that *cis*-5,6-diH-BATHO is thermally unstable at 77 K, whereas BATHO is stable up to 133 K (Packer, 1982; Shichida, 1986).

Evidence favoring the alternative of an additional BSI intermediate can be obtained from the steady-state photolysis experiments of *trans*-5,6-diH-ISORHO carried out by Yoshizawa et al. (1984) at 4 K. As summarized in Fig. 1, irradiation at 4 K forms a thermally stable red-shifted intermediate (λ_{max} 508 nm), which was proposed to be *trans*-5,6-diH-PHOTO (i.e., identical to the picosecond precursor of BATHO at room temperature [Yoshizawa et al., 1984; Shichida, 1986]). Upon warming to 31 K this

species transforms to a blue-shifted intermediate (λ_{max} 414 nm), proposed to be analogous to hypsorhodopsin (HYPSO) (see Shichida, 1986, for a discussion of the HYPSO intermediate). Upon warming to 187 K, 223 K, and 253 K, species interpreted as *trans*-5,6-diH-BATHO (488 nm), *trans*-5,6-diH-LUMI (473 nm), and *trans*-5,6-diH-META I (340 nm) were observed. No stage attributed to *trans*-5,6-diH-META II was assigned. Although not shown here, the transient absorption of detergent-solubilized *cis*-5,6-diH-ISORHO was also monitored from 5 μs to 2 ms after laser photolysis at room temperature. The results were qualitatively similar to those observed for RHO on analogous time scales. Both exhibited a decay of the absorption observed after 5 μs to an intermediate blue-shifted by ~6,000 cm^{-1} (see Fig. 1). It is evident that the basic photochemical phenomena observed at low temperatures for *trans*-5,6-diH-ISORHO are in keeping with those observed by us for *cis*-5,6-diH-ISORHO at both low and room temperatures.

We wish to interpret our observations, as well as those of Yoshizawa et al., by suggesting that both *cis*- and *trans*-5,6-diH-ISORHO exhibit analogous photolysis intermediates. However, taking advantage of information obtained since the original measurements of Yoshizawa et al., we propose assignments for the various photointermediates which differ from their assignments. As shown in Fig. 1, we identify the first (508 nm) low-temperature intermediate as 5,6-diH-BATHO (rather than 5,6-diH-PHOTO), and we propose that the second (414 nm) intermediate represents our BSI species (rather than 5,6-diH-HYPSO). The subsequent phototransients are identified as the analogs of LUMI, META I and Meta II (rather than BATHO, LUMI and META I, as suggested earlier [Yoshizawa et al., 1984]). Namely, these are identified as 5,6-diH-LUMI (488 nm), 5,6-diH-META I (473 nm), and 5,6-diH-META II (340 nm), respectively. According to this interpretation the analogies between the native pigment and 5,6-diH-ISORHO are drawn on the basis of the spectral similarities between the early red-shifted (BATHO) species and the later blue-shifted (META II) species. This implies that 5,6-diH-BATHO is highly destabilized with respect to BATHO, rather than stabilized as previously suggested.

Several major arguments favor the present interpretation over the previous one:

(*a*) Later experiments by Yoshizawa and co-workers (see Shichida, 1986, and Fig. 1) have shown that HYPSO is a photoproduct of PHOTO, while the 414-nm intermediate is a thermal product of its 508-nm precursor. Moreover, the 414-nm species is stable up to 113 K, while HYPSO in bovine rhodopsin decays at 22 K (Yoshizawa et al., 1984).

(*b*) The markedly blue shifted 340-nm transient is more likely to represent a deprotonated Schiff base such as in META II (λ_{max} 380 nm) than a protonated species such as META I (λ_{max} 478 nm).

(*c*) Identifying the 340-nm species as 5,6-diH-META II (assuming that BSI is a new phototransient which cannot be correlated to an intermediate in the native pigment) would suggest that the low temperature 488- and 473-nm phototransients of *trans*-5,6-diH-ISORHO should be analogous to LUMI and META I, respectively. (Accordingly, we identify the 130 K transient of *cis*-5,6-diH-ISORHO as *cis*-5,6-diH-LUMI; see also legend to Fig. 1.) This would be in keeping not only with the respective low-temperature thermal stabilities and with the room temperature decay rates, but also with the corresponding absorption maxima (see Fig. 1). This interpretation is consistent with the idea that thermal stabilities of the early intermediates may be perturbed more by changes in the chromophore (which lead to bleaching) than later intermediates, which are more directly affected by protein conformational changes.

Furthermore, the suggestion that in 5,6-diH-ISORHO the PHOTO intermediate is stable at 4 K (Yoshizawa et al., 1984), is only in keeping with the observation of a psec precursor of BATHO (analogous to PHOTO) at this temperature (Peters et al., 1977) if one assumes a large stabilization of PHOTO in 5,6-diH-ISORHO. While not necessarily implausible, this point will only be clarified by psec studies of 5,6-diH-ISORHO at temperatures as low as 4 K.

Implications for the primary event in visual pigment photolysis

The absorption maximum of ISORHO (λ_{max} 483 nm) is red shifted relative to a protonated Schiff base of 9-*cis* retinal in ethanol (λ_{max} 440 nm) (Derguini and Nakanishi, 1986). The corresponding frequency difference of 2,100 cm^{-1}, denoted the "opsin shift," has been attributed to the effect of a nonconjugated protein charge in the vicinity of C_{12}—C_{14} (Honig et al., 1979*a,b*). *Cis*-5,6-diH-ISORHO (λ_{max} 461 nm) exhibits a similar opsin shift (2,000 cm-1) with respect to the corresponding *cis*-5,6-dihydro-protonated Schiff base in ethanol (λ_{max} 425 nm). This suggests that the dihydro chromophore binding interaction with the protein is similar to that in native ISORHO. Thus, the observation of a reduced barrier for the 5,6-diH-BATHO decay and the accompanying appearance of the new BSI intermediate should be discussed in terms of different chromophore–protein interactions which occur after pigment photolysis.

It is now well accepted that the stage of bathorhodopsin represents an isomerized all-*trans* chromophore (Akita et

al., 1980; Mao et al., 1981; Fukada et al., 1984; Hubbard and Kropf, 1958; Rosenfeld et al., 1977; Eyring et al., 1980, 1982). However, the resonance Raman spectrum of bathorhodopsin shows unusually intense hydrogen out-of-plane wagging vibrations in the 800–920 cm^{-1} region, which indicate that the chromophore is conformationally distorted (Eyring et al., 1980, 1982). It is possible that such a distortion may account for a substantial part of the energy storage in BATHO (Palings et al., 1987; Birge and Hubbard, 1980; Warshel and Barboy, 1982). FTIR data indicate that the transition from BATHO to LUMI involves a relaxation of the above strain via conformational changes in both the retinal and in the protein (DeGrip et al., 1987; Rothschild and DeGrip, 1986). We suggest that saturation of the 5,6 double bond affects the retinal–protein interactions at the BATHO stage by changing the ring–chain and/or the ring–5 methyl conformation. Consequently, the initially formed 5,6-diH-BATHO is kinetically destabilized and undergoes a fast change in the retinal conformation which results in the BSI intermediate. It is possible that in RHO the BSI intermediate is not observed either because this retinal conformation change is blocked, or occurs simultaneously with protein conformational changes which generate LUMI.

The drastic effect of merely saturating the 5,6 double bond is indicative of a tightly packed protein geometry in the vicinity of the β-ionone ring at the BATHO stage. A relatively tight protein geometry with respect to the β-ionone ring at this stage has also been suggested by low temperature studies of the back photoreactions of BATHO and LUMI (Shichida, 1986; Maeda et al., 1978, 1979). As indicated by the resonance Raman spectrum of BATHO, the primary 9-*cis* to all-*trans* isomerization in the native pigment induces a strain due to twisting around the C_{10}—C_{11}, C_{12}—C_{13}, and C_{14}—C_{15} bonds (Palings et al., 1987; Eyring et al., 1980, 1982). We suggest that this process also induces strain in the β-ionone ring region, which in the 5,6-dihydro pigment is in part rapidly relieved. This is due to the capability of the ring to readjust its geometry, giving rise to BSI. Such strain relief in BSI is supported by FTIR studies (Siebert et al., manuscript in preparation). Due to yet undetermined steric factors (see discussion below), such a readjustment takes place more slowly in the native pigment, occurring only at the stage of LUMI, when the ring–region strain, as well as the skeletal $C_{10} \ldots C_{15}$ strain (Palings et al., 1987; DeGrip et al., 1987; Rothschild and DeGrip, 1986) are both relieved by the protein rearrangement. It is interesting that, in contrast to rhodopsin, the K (BATHO) photointermediates in the photocycles of artificial bacteriorhodopsins are insensitive to modifications of the ring region (Ottolenghi and Sheves, 1987; Sheves et al., 1987). This could be evidence for a more loosely packed protein environment in the bacterial system.

It is highly relevant to note that destabilization of BATHO, followed by generation of a blue-shifted intermediate between the BATHO and LUMI stages, has also been reported for the artificial pigment formed from bovine opsin and 9-*cis*-13-demethylretinal (13dm-ISORHO, λ_{max} 494 nm) (Shichida et al., 1981). Illumination of the pigment at 83 K forms 13dm-BATHO (λ_{max} 532 nm), which at 93 K converts to an intermediate which has been called 13dm-BL (BL, λ_{max} 475 nm), followed by the formation of 13dm-LUMI (λ_{max} 517 nm) at 133 K. Preliminary laser photolysis experiments on both 13dm-ISORHO and 13dm-RHO indicate that a blue-shifted intermediate, which corresponds to the low temperature BL intermediate, is also a precursor of the LUMI stage at room temperature (D. S. Kliger et al., manuscript in preparation).

The analogy with the BSI intermediate of 5,6-diH-ISORHO suggests that relieving the polyene strain at the BATHO stage, via a change in the retinal conformation, is also made possible by lack of the 13-methyl group. In other words, the changes in polyene conformation generating BSI and BL both involve a release of strain in the BATHO species, in either the ring or the C_{13} regions of the molecule, respectively. An early blue-shifted intermediate has also been observed in preliminary laser photolysis measurements in this lab on two other dihydro analogs, 4,5-dehydro-5,6-dihydro-ISORHO and 7,8-dihydro-ISORHO. However, no such blue-shifted product is observed after photolysis of 3,4-dehydro-ISORHO (which forms normal photolysis intermediates) (D. S. Kliger et al., manuscript in preparation). These results suggest that conformational stability near the ring may be largely determined by electronic or steric effects of conjugation between the ring and the chain regions of the chromophore.

The question obviously arises as to the mechanism accounting for the blue-shifted spectrum of either BL or BSI. No definite suggestion can be made at this time, since at present even the spectra of rhodopsin and isorhodopsin, and especially the basic red shift in BATHO, are not fully understood. Several factors, such as external protein charges (Derguini and Nakanishi, 1986; Honig et al., 1979; Birge et al., 1988), Schiff base-counterion and H-bond interactions (Sheves et al., 1985, 1987; Lugtenburg et al., 1986; Spudich et al., 1986), and single bond rotations (Shichida et al., 1981), may participate in determining the spectrum of the above blue-shifted intermediates.

We finally point out the similarity in the low temperature thermal stabilities of BATHO, BSI, and BL and in their decay rates at room temperature. If BATHO and

BSI (or BL) are characterized by different retinal conformations, it is plausible to suggest that the rate-determining step in their decay to the corresponding LUMI intermediates involves a conformation change in the protein rather than in the polyene. Namely, it is implied that in the visual process protein structural changes account for the decay of photointermediates at stages as early as BATHO. Thus, the only non-protein rate-determining step may involve the PHOTO (or PBATHO) to BATHO transformation.

Future work on both 5,6-diH-BSI and 13dm-BL could address questions such as the structure of the retinal in both species and its interactions with the protein environment. FTIR and resonance Raman spectroscopy would be instrumental in this respect. Also of interest will be measurements of the energy stored in both intermediates in comparison to the amount (36 Kcal/mol) stored in BATHO (Honig et al., 1979; Cooper 1979; Boucher and Leblanc, 1985; Schick et al., 1987). This could clarify the relationship between the red shift in BATHO and the mechanism of energy storage (Ottolenghi and Sheves, 1987; Palings et al., 1987).

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