Picosecond Absorption Studies on Rhodopsin and Isorhodopsin in Detergent and Native Membrane[†]

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ABSTRACT: Picosecond transient absorption spectra of rhodopsin and isorhodopsin were measured at room temperature with a double-beam laser spectrophotometer after excitation at 355 nm. Photolysis studies were performed on rhodopsin solubilized in two different detergents (digitonin and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The resulting rhodopsin/bathorhodopsin absorption difference spectra were measured at times from 35 ps to 250 ns following photoexcitation. Rhodopsin and isorhodopsin in native disk membrane were studied after suspension in 75% glycerol. Isorhodopsin was prepared by photoisomerizing rhodopsin in disk membrane at 77 K. Transient spectra obtained from the visual pigments in native membrane were of a quality approaching that obtained from detergent-solubilized rhodopsin. The batho intermediate derived from isorhodopsin was spectrally the same as that generated by rhodopsin photolysis and was produced with a quantum yield higher than had been predicted on the basis of other studies.

The visual pigment rhodopsin has been extensively studied for many years, yet the mechanism for visual transduction remains poorly understood. Studies on the photochemistry of the early intermediates of the visual sequence have been limited by available technology. While low-temperature spectroscopy allowed the intermediates of the rhodopsin bleaching sequence to be identified and characterized, it was the development of picosecond lasers in the 1970's which facilitated studies on the early events in vision at physiological temperatures (Peters & Leontis, 1982; Hilinski & Rentzepis, 1983). Investigations with both low-temperature and picosecond techniques, however, have utilized rhodopsin predominantly in detergent-solubilized form. Rhodopsin can be purified from virtually all contaminating protein and lipid (Fong et al., 1982), depending on the detergent and technique used, but not without consequences. Rhodopsin bleaching kinetics (Applebury et al., 1974; Stewart et al., 1977), thermal stability (DeGrip, 1982), absorption and circular dichroism spectra (Ebrey & Yoshizawa, 1973; Waddell et al., 1976; Suzuki et al., 1977), and bleaching quantum yield and photoproducts (Waddell et al., 1976; Sasaki et al., 1980) all vary with detergent solubilization. The kinetics of the early bleaching intermediates (in contrast to the later intermediates) have not been found to be sensitive to detergents (Busch et al., 1972; Applebury & Rentzepis, 1982; Horwitz et al., 1983). However, it has been recognized that although membrane vesicle samples of rhodopsin are more difficult to study, they should give results more relevant to events which occur under physiological conditions than do detergent extracts (Lewis et al., 1981).

Uncertainty still exists about the early intermediates in the visual sequence, in particular whether rhodopsin (containing 11-*cis*-retinal) and isorhodopsin (9-*cis*-retinal) both isomerize to a transoid form to yield the same bathorhodopsin intermediate upon photolysis. For this reason, we have used picosecond absorption spectroscopy to examine the photochemistry of rhodopsin and isorhodopsin. Techniques are presented for studying the pigments in native membrane in such a way that the quality of data obtained approaches that obtained from detergent-solubilized protein. Special attention was given

membrane were studied after suspension in 75% glycerol. Isorhodopsin was prepared by photoisomerizing rhodopsin in disk membrane at 77 K. Transient spectra obtained from the visual pigments in native membrane were of a quality approaching that obtained from detergent-solubilized rhodopsin. The batho intermediate derived from isorhodopsin was spectrally the same as that generated by rhodopsin photolysis and was produced with a quantum yield higher than had been predicted on the basis of other studies.

to maximizing signal to noise ratios and minimizing potential artifacts due to multiple absorption of photons and photoproduct absorption. The data are discussed in terms of current models for the early events in vision.

Materials and Methods

Visual Pigment Samples. Bovine rod outer segments were prepared from frozen retinas (Hormel) as described (Hong et al., 1982). Disk membranes were prepared from the rod outer segments (Smith et al., 1975) and were either extracted with detergents or suspended with glycerol and "low-salt" buffer [1 mM tris(hydroxymethyl)aminomethane (Tris),[1] 0.4 mM MgCl$_2$, and 0.1 mM EDTA, pH 7.0 (Lewis et al., 1981)] to achieve a glycerol concentration of 75%. Detergent extractions with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Calbiochem) were preceded by first lyophilizing the disk membranes and then partially delipidating them with petroleum ether. Extractions with digitonin (Merck) were performed with both fresh and lyophilized/delipidated membranes. Detergent and buffer concentrations used are indicated in the figure legends. All sample absorbance measurements were made at room temperature with a Perkin-Elmer Lambda 3 spectrophotometer using 1.0-cm path-length cells.

Isorhodopsin in disk membrane was prepared photochemically at 77 K (Yoshizawa & Wald, 1964). Disk membranes suspended in 75% glycerol/low-salt buffer were immersed in liquid N$_2$ and irradiated for 3 h with orange light from an Oriel 250-W quartz–halogen lamp combined with a Hoya 0-56 filter. The 0-56 filter has the following transmittance values: λ ≤ 540 nm, 0%; λ = 560 nm, 28%; λ = 580 nm, 75%; λ ≥ 600 nm, 82%. Simultaneously, a control sample of disk membranes (the rhodopsin for comparative studies) was kept in liquid N$_2$ in the dark. The isorhodopsin/rhodopsin composition of the samples was assayed kinetically (Yoshizawa & Wald, 1964) after aliquots were solubilized in 2% Ammonyx-LO (Onyx Chemical), 0.2 M NH$_2$OH, and 60 mM phosphate, pH 6.5. The solubilized aliquots were irradiated with orange light as described above, and the decrease in absorbance at 495 nm was monitored for kinetic analysis.

Picosecond Spectroscopy. The picosecond double-beam absorption spectrometer has been described in detail elsewhere

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

(Simon & Peters, 1983). Rhodopsin samples were photoexcited at room temperature by a single 25-ps pulse at 355 nm generated by frequency tripling the output of a passively mode-locked Nd^{3+} YAG laser. The 355-nm excitation beam was focused onto the sample into a circular area of about 0.2-cm diameter. Two simultaneous 25-ps continuum interrogation pulses were focused onto the sample at a time arbitrarily delayed relative to the excitation pulse, the first continuum pulse (I) passing through the portion of the sample which had been excited by the 355-nm light and the second (I_0) passing through an unexcited region of the sample. The transient absorption difference spectra were calculated with

$$\Delta A = \log [I_0/I]_{\text{with excitation}} - \log [I_0/I]_{\text{without excitation}}$$

after corrections for vidicon dark current and extraneous light generated by laser operation. The latter included contributions from low-level fluorescence emission resulting from 355-nm excitation of detergent impurities in detergent-solubilized rhodopsin samples, and scattered light resulting from excitation of turbid disk membrane suspensions.

Detergent-solubilized rhodopsin samples (2.0 mL, 25 μM rhodopsin) were photolyzed in a 1.0-cm path-length cell equipped with a magnetic stirrer and stirred rapidly enough so that the sample was made essentially homogeneous between laser pulses. For disk membranes in 75% glycerol, a 1.0-cm path-length flow cell coupled with a small peristaltic pump was used. A fresh 6-mL sample of disk membranes in 75% glycerol (pigment concentration 25 μM) at room temperature was continuously pumped through the flow cell as the data for each transient absorption spectrum were collected. The flow rate was 9 mL/min, and the time required for a photolyzed volume of the sample to be displaced from the path of the photolysis beam was estimated to be 0.25 s. The variable repetition rate of the laser was such that the time between laser pulses hitting the sample was $0.1n$ s where $n = 1$ (minimum) to 10 (approximate maximum) and $n = 3$ was the average.

Results

Bovine rhodopsin in disk membrane was prepared with a yield of 0.5 mg of rhodopsin per retina and spectral ratios of $A_{280}/A_{498} \simeq 2.6$ and $A_{400}/A_{498} \simeq 0.27$ after detergent solubilization. CHAPS and digitonin extracts of disk membranes were used directly without further purification yet exhibited spectrally well-defined α, β, and protein bands. In the present study, rhodopsin is photolyzed at 355 nm, resulting in the eventual release of *trans*-retinal ($\lambda_{\text{max}} \simeq 380$ nm; Hubbard et al., 1971), a photoproduct also photoexcitable at 355 nm. It was thus necessary to limit laser bleaching of rhodopsin arbitrarily to <10% to minimize photoproduct excitation.

Refractive index matching disk membranes with 75% glycerol proved advantageous for the photochemical preparation of isorhodopsin, since the suspension conveniently formed a glass at liquid N_2 temperature. Kinetic analysis of the isorhodopsin generated indicated the isorhodopsin content to be >95%. Maeda et al. (1978) have shown that photolysis (λ > 530 nm) of rhodopsin in rod outer segments/67% glycerol at liquid N_2 temperature produces only the 9-*cis*-retinal isomer of rhodopsin, as judged by high-pressure liquid chromatography. Figure 1 shows the absorbance spectra of the rhodopsin and isorhodopsin samples in disk membrane suspended in 75% glycerol/low-salt buffer. The spectra were affected by turbidity but nonetheless show the isorhodopsin α band to be blue shifted by ~11 nm and to be higher in molar absorptivity by ~10% relative to rhodopsin, as expected (Hubbard et al., 1971). Solubilization of membrane aliquots in 2% Ammonyx-LO/60 mM phosphate, pH 7.0, allowed confirmation of
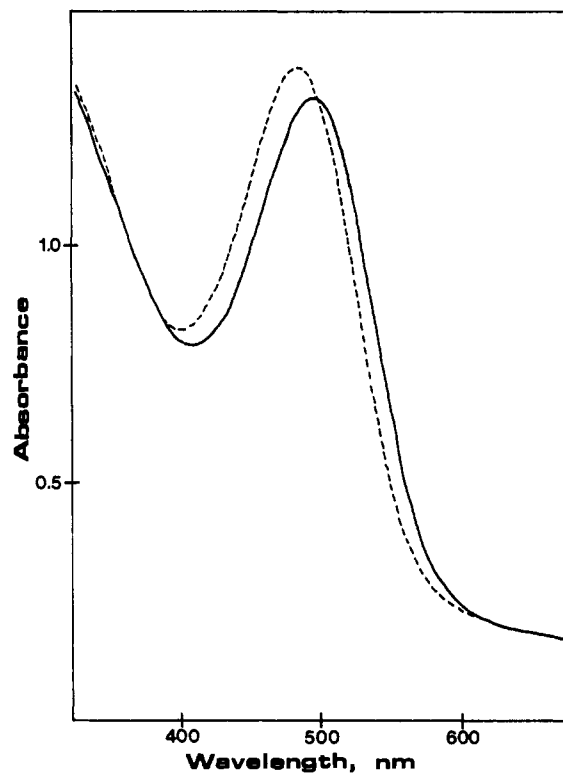

FIGURE 1: Absorbance spectra of rhodopsin and isorhodopsin disk membranes suspended in 75% glycerol/low-salt buffer: (—) rhodopsin; (- - -) isorhodopsin.
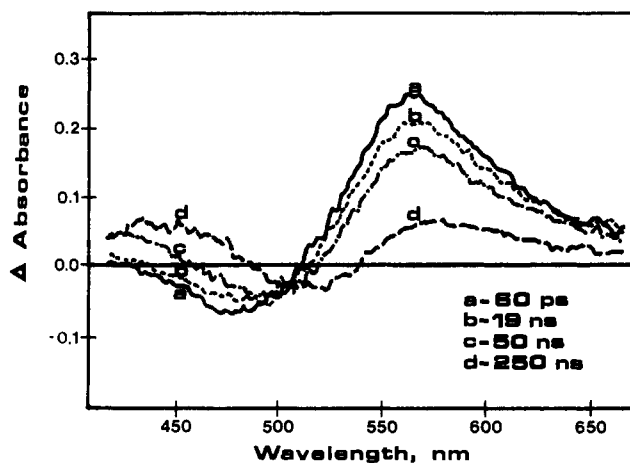

FIGURE 2: Transient absorption difference spectra of rhodopsin solubilized in 6 mM CHAPS/15 mM phosphate, pH 7.0, following lyophilization and partial delipidation of disk membranes. Sample absorbances were A = 1.0 at 498 nm (1.0-cm path length). The following times after photolysis at 355 nm are shown: (a) 60 ps; (b) 19 ns; (c) 50 ns; (d) 250 ns.

the spectral identity of the pigments by minimizing the effects of light scattering.

Transient Difference Spectra of Detergent-Solubilized Rhodopsin. Picosecond and nanosecond transient absorption spectra for CHAPS- and digitonin-solubilized rhodopsin are shown in Figures 2 and 3. The form of the picosecond difference spectra, including the ratio of the bathorhodopsin absorbance to rhodopsin bleach ($|\Delta A_{\text{batho}}/\Delta A_{\text{rho}}|)^2$ and the

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[2] $|\Delta A_{\text{batho}}/\Delta A_{\text{rho}}|$ is the absolute value of the ratio of the maximum positive absorbance arising from bathorhodopsin production (ΔA_{batho}) to the maximum negative absorbance arising from rhodopsin bleaching (ΔA_{rho}). In Figure 2a, for example, ΔA_{batho} is measured at 565 nm while ΔA_{rho} is measured at 475 nm.
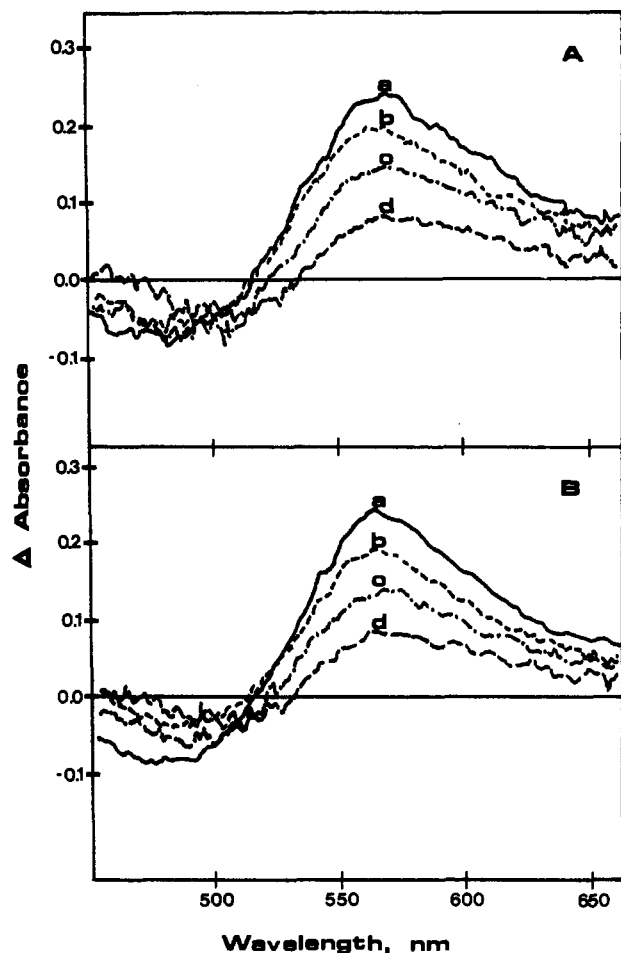
FIGURE 3: Transient absorption difference spectra of rhodopsin solubilized in 2% digitonin/67 mM phosphate, pH 7.0. Sample absorbances were $A = 1.0$ at 498 nm (1.0-cm path length). (A) Rhodopsin solubilized from fresh disk membranes. (B) Rhodopsin solubilized from lyophilized and partially delipidated disk membranes. The following times after photolysis at 355 nm are shown: (a) 35 ps; (b) 19 ns; (c) 50 ns; (d) 250 ns.

Table I: Spectral Characteristics of Rhodopsin and Isorhodopsin Photolyzed with 355-nm, 25-ps Pulses at Room Temperature

form of sample	$\left\|\dfrac{\Delta A_{batho}}{\Delta A_{rho}}\right\|$ [a]	isosbestic point (nm)[a]
rhodopsin solubilized in CHAPS from delipidated disk membranes	3.6 ± 0.4	510 ± 3
rhodopsin solubilized in digitonin from delipidated disk membranes	2.9 ± 0.2	515 ± 3
from untreated disk membranes	3.1 ± 0.2	515 ± 3
rhodopsin in disk membranes	2.1 ± 0.8	530 ± 5
isorhodopsin in disk membranes	2.2 ± 0.6 [b]	518 ± 3

[a] Transient absorption difference spectra taken 35–60 ps following photolysis. [b] Represents $|\Delta A_{batho}/\Delta A_{iso}|$.

isosbestic point, was found to vary according to how fast the sample was stirred during photolysis. Low stirring rates resulted in lower $|\Delta A_{batho}/\Delta A_{rho}|$ ratios, red-shifted isosbestic points, and, in the case of little to no stirring, the appearance of a positive absorbance with $\lambda_{max} \simeq 450$ nm in addition to the negative absorbance at ~ 500 nm and a positive absorbance at ~ 570 nm. The rate of stirring was set to a point such that any further increase in stirring resulted in no further changes in the difference spectrum.

A summary of spectral data from Figures 2 and 3 appears in Table I. Within experimental error, the photochemical behavior of rhodopsin solubilized in digitonin was the same regardless of membrane treatment (lyophilization and partial delipidation or no treatment). This suggests that no serious
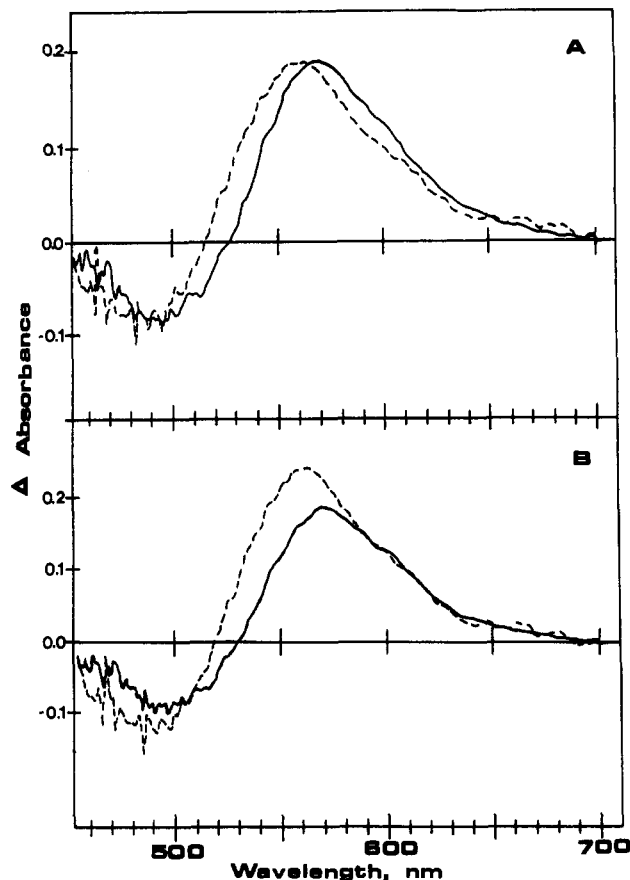

FIGURE 4: Transient absorption difference spectra of rhodopsin (—) and isorhodopsin (---) in disk membrane/75% glycerol at 40 ps following photolysis ($\lambda_{ex} = 355$ nm). The samples were the same as those shown in Figure 1. (A) Transient spectra resulting from identical treatment of rhodopsin and isorhodopsin samples. (B) Same as (A), but with the isorhodopsin spectrum expanded by a factor of 1.3 after both spectra were normalized to zero absorbance at 700 nm.

artifacts were introduced into the behavior of rhodopsin solubilized in CHAPS due to the necessity of lyophilizing and partially delipidating the disk membranes to facilitate extraction with CHAPS. The transient absorption spectra shown in Figures 2 and 3 do not provide enough data for a definitive kinetic analysis of the decay of bathorhodopsin, but do provide a qualitative picture of the visual sequence intermediates from 35 ps to 250 ns following absorption of a photon.

Transient Difference Spectra of Rhodopsin and Isorhodopsin in Disk Membranes. In order to obtain transient absorption difference spectra of disk membranes in 75% glycerol, a flow system was used to minimize the possibility of multiple absorption processes by a given rhodopsin molecule. The peristaltic pump was run at the maximum flow rate which still resulted in the smooth flow of 75% glycerol through the flow cell. As shown in Figure 4A, the form of the difference spectrum for rhodopsin in disk membrane was slightly different from that of the detergent-solubilized rhodopsin spectra (Figures 2 and 3). Specifically, for rhodopsin in disk membrane, the $|\Delta A_{batho}/\Delta A_{rho}|$ ratio was lower, and the isosbestic point was red shifted (Table I). In analogy to the artifacts observed for the stirred system with low stir rates, these features are most likely due to the difficulties in rapidly flowing the sample. However, within the constraints of the experimental setup, the transient spectra were highly reproducible, and thus a comparison of the bathorhodopsins from rhodopsin and isorhodopsin was attempted.

Comparison of Rhodopsin and Isorhodopsin. The rhodopsin and isorhodopsin samples were handled identically

during the laser photolysis studies and had the same initial absorbance at 355 nm. The isosbestic points between the pigment bleach and the resulting batho intermediate at 40 ps following photolysis (Figure 4A) were 530 ± 5 nm for rhodopsin and 518 ± 3 nm for isorhodopsin, a difference which agrees well with the 11-nm difference in the absorption maxima of rhodopsin and isorhodopsin (498 and 487 nm, respectively). Laser-induced bleaching of rhodopsin and isorhodopsin was determined spectrophotometrically after aliquots of the disk membranes were solubilized with 2% Ammonyx-LO/60 mM phosphate, pH 7.0. For the samples used to obtain the transient spectra in Figure 4A, the isorhodopsin samples were bleached 5.5% ± 0.6% by 100 laser pulses at 355 nm while the rhodopsin samples were bleached 8.3% ± 0.9% by 100 pulses. The values reported have been corrected for changes in the average energy of the pulses and represent the average of five determinations each. Hence, under identical conditions, isorhodopsin is bleached less than rhodopsin by a factor of $\phi_{iso}/\phi_{rho} = 0.66 \pm 0.15$, where ϕ_{iso} and ϕ_{rho} represent overall bleaching quantum yields. If the batho intermediates resulting from rhodopsin (batho$_{rho}$) and isorhodopsin (batho$_{iso}$) photolysis were the same, one would expect the magnitude of the batho$_{iso}$ absorbance to be ~ 0.66 times that of the batho$_{rho}$ absorbance. This conjecture can be tested by evaluating the difference spectrum in Figure 4A at 600 nm, a wavelength where bathorhodopsin absorbs but rhodopsin and isorhodopsin do not. The spectrum shows $\Delta A_{batho_{iso}}/\Delta A_{batho_{rho}} = 0.79 \pm 0.04$; i.e., the batho$_{iso}$ absorbance is approximately equal to what would be predicted on the basis of the total bleach values. The same values were found after laser photolysis of rhodopsin and isorhodopsin solubilized in digitonin.

Figure 4B shows the same difference spectra as in 4A but with the isorhodopsin spectrum expanded by a factor of 1.3 (the reciprocal of 0.79) after both spectra were normalized to zero absorbance at 700 nm. Qualitatively, there is very good fit between the difference spectra in the region $\lambda \geq 590$ nm where only bathorhodopsin absorbs appreciably. The difference in isosbestic points remains ~ 12 nm. In addition, the λ_{max} values of the bleaches of rhodopsin and isorhodopsin correspond well to their actual λ_{max} values (498 and 487 nm, respectively), and the magnitude of the isorhodopsin bleach is larger than that of the rhodopsin bleach, consistent with the higher extinction coefficient of isorhodopsin (cf. Figure 1; Hubbard et al., 1971). These features are consistent with, but do not necessarily prove, that batho$_{iso}$ is spectrally identical with batho$_{rho}$.

No attempt was made to correct the transient difference spectra in Figure 4 for parent pigment absorbance to calculate the net absorbance spectra of the batho intermediates. Light is scattered equally in both the sample (I) and reference (I_0) beams of the transient spectrometer in obtaining a transient difference spectrum of a turbid sample. Sample turbidity shows its effects predominantly as decreased signal to noise ratios in the spectra. In contrast, the parent pigment absorbance spectra in Figure 1 were obtained by using a conventional double-beam spectrophotometer with the sample (I) beam passing through the turbid sample and the reference (I_0) beam passing through water. The turbidity of the sample is evident throughout the spectra in Figure 1 but particularly at $\lambda \geq 600$ nm. All apparent absorbance in this region is due to scattered light. It would thus be erroneous to correct the transient spectra in Figure 4 by using the spectra in Figure 1 to compute the batho intermediate absorbance spectra. In addition, the signal to noise ratios of the reported transient spectra would not allow a definitive determination of the batho

intermediate absorbance maxima (to within a few nanometers) even if suitable parent pigment spectra could be obtained. With these restrictions on the potential methods for comparing the batho intermediate spectra, we conclude that no differences exist between batho$_{iso}$ and batho$_{rho}$ and therefore rhodopsin and isorhodopsin isomerize to the same bathorhodopsin intermediate.

Discussion

The work presented herein involves several novel approaches to the study of rhodopsin by picosecond absorption spectroscopy. These approaches include the photoexcitation of bovine rhodopsin in the β-band spectral region using UV light, which differs from previous picosecond studies using α-band photoexcitation with green light (Busch et al., 1972; Applebury et al., 1974; Peters et al., 1977; Monger et al., 1979; Spalink et al., 1983). Rhodopsin and isorhodopsin were studied successfully in native disk membranes, thus obviating the need for detergents and eliminating the potential artifacts resulting from detergent solubilization. Detergents were used, however, for comparison with native membrane studies. Although essentially all picosecond absorption work on rhodopsin thus far has been performed on Ammonyx-LO-solubilized rhodopsin (Busch et al., 1972; Applebury et al., 1974; Peters et al., 1977; Monger et al., 1979; Spalink et al., 1983), Ammonyx-LO was not chosen for the present work because of its relative harshness as a detergent (Fong et al., 1982; DeGrip, 1982). Instead, the classic detergent digitonin, used for many years in low-temperature rhodopsin studies, and the recently synthesized detergent CHAPS (Hjelmeland, 1980) were used. CHAPS is a very mild detergent and the only one known in which rhodopsin exhibits the same circular dichroism spectrum as it does in native membrane (Kropf, 1982).

In photoexciting rhodopsin with 355-nm UV light, it was assumed that the resulting photochemistry would be the same as that initiated by 532-nm visible light. An earlier study by Bensasson et al. (1977) compared nanosecond flash photolysis of detergent-solubilized rhodopsin using 530-nm light to that using 353-nm light. No significant differences between the resulting transients were observed. A major advantage of 355-nm photolysis is that the measured transient absorption spectra are complete in the visible region. In contrast, because of experimental design, with 532-nm photolysis it is not possible to get spectral data in the region 520–540 nm (Spalink et al., 1983). This region is very important for analyzing data involving rhodopsin/bathorhodopsin/isorhodopsin relationships. The major disadvantages of 355-nm photolysis are that all rhodopsin bleaching intermediates and photoproducts absorb at 355 nm and that impurities in the detergents used absorb 355-nm light, resulting in low-level fluorescence. To minimize the former, rhodopsin samples were not used after photolysis had depleted the rhodopsin content by 10%, and all spectra were corrected for emission and/or scattering.

Absorption difference spectra of detergent-solubilized rhodopsin taken at times <100 ps following photolysis showed $|\Delta A_{batho}/\Delta A_{rho}|$ ratios of 3 to 1 (Table I), thus agreeing with low-temperature (Peters et al., 1977) and room-temperature (Spalink et al., 1983) spectra obtained from 532-nm photolysis of Ammonyx-LO-solubilized rhodopsin. Ratios less than 3 to 1 have been associated with photon saturation effects (Spalink et al., 1983). Although an analysis of photon saturation effects was not undertaken in the present study, a calculation of the probability of more than one photon being absorbed by a given rhodopsin molecule indicated that multiple absorption of photons was not likely. In our experiments, the energy of the 355-nm pulses incident on the rhodopsin samples

was 0.2 ± 0.1 mJ, giving a fluence of 6 ± 3 mJ/cm² or, in terms of photons, (1.1 ± 0.6) × 10¹⁶ photons/cm². The absorption cross section[3] of rhodopsin at 355 nm, assuming ϵ_{355} = 0.3ϵ_{498} (ϵ_{498} = 4.06 × 10⁴ L mol⁻¹ cm⁻¹; Hubbard et al., 1971), is 4.6 × 10⁻¹⁷ cm²/rhodopsin molecule (Braude, 1950). The product of the fluence and the absorption cross section yields the value 0.5 ± 0.3 photon/rhodopsin molecule, indicating a relatively low probability for multiple absorption events.

Our choice of CHAPS for solubilizing rhodopsin was based on the relative newness of the detergent (Hjelmeland, 1980) and the properties exhibited by rhodopsin when solubilized in CHAPS (Kropf, 1982). The mildness of CHAPS became evident when extractions of fresh disk membranes with the detergent proved too inefficient for practical use. It was thus necessary to lyophilize the disk membranes and partially delipidate them with petroleum ether to facilitate CHAPS extraction. This treatment, while seeming harsh, was used by Kropf (1982) in circular dichroism studies, showing that CHAPS-solubilized rhodopsin more closely resembles rhodopsin in native membrane than does rhodopsin solubilized in any other detergent. Problems did arise when working with CHAPS, however. Buffering CHAPS with phosphate resulted in the formation of some precipitate, possibly indicating incompatibility between CHAPS and phosphate, and rhodopsin solubilized in CHAPS/phosphate buffer partially sedimented over a period of days when stored at 4 °C. Attempts at separating rhodopsin photoproducts from intact pigment after partial photolysis of a CHAPS-solubilized rhodopsin sample were unsuccessful. In this respect, Ammonyx-LO solubilization and hydroxylapatite purification of rhodopsin (Applebury et al., 1974) are preferable to techniques involving CHAPS.

Picosecond absorption spectra of rhodopsin in disk membranes were very similar to those of detergent-solubilized rhodopsin. The technique of index-matching rod outer segment fragments with high glycerol concentrations for flash photolysis studies is not new (Pratt et al., 1964). Of several methods for reducing light scattering by turbid membrane suspensions, index matching appears to be the mildest (Lewis et al., 1981). In our hands, suspensions of disk membranes in 75% glycerol were quite workable for picosecond studies, the major disadvantage being that large quantities of disk membranes (one-sixth of a preparation from 100 retinas) were required per spectrum. On the other hand, extensive biochemical preparation and purification were neither required nor desired, as our objective was to study rhodopsin in an environment as minimally perturbed as possible.

Never was a positive absorbance detected in the blue spectral region (400–500 nm) at early times (<40 ps following photoexcitation) with a fresh rhodopsin sample under stir/flow conditions which minimized the potential for photoproduct excitation. Thus, within the resolution of our experiment, no hypsorhodopsin was detected. A positive absorbance with λ_{max} ≃ 450 nm was detected when partially photolyzed samples were used or when a fresh sample was not stirred or flowed rapidly between laser pulses. The blue-absorbing artifact may be attributed to some form of *all-trans*-retinal. When *all-trans*-retinal dissolved in ethanol is photoexcited at 355 nm, a transient species with λ_{max} ≃ 450 nm is observed at times <35 ps following photolysis. It is still not clear whether

hypsorhodopsin is a genuine intermediate in the bovine visual sequence or an artifact. Cooper (1983) has suggested that hypsorhodopsin is not a discrete photoproduct of bovine rhodopsin but rather a low-temperature artifact resulting from the photoselection of different conformational substates of the protein chromophore in the frozen matrix.

Our comparison of rhodopsin and isorhodopsin picosecond photochemistry is limited by the interpretation of broad, unstructured electronic spectra in terms of isosbestic points and the magnitudes of parent pigment bleaches and apparent batho intermediate absorbances. We are nonetheless led to conclude that batho$_{iso}$ is the same as batho$_{rho}$, both in spectral shape and in molar absorptivity. Our conclusion agrees with the low-temperature, nonphotostationary state work of Mao et al. (1980) comparing digitonin-solubilized rhodopsin with digitonin-solubilized isorhodopsin prepared photochemically. The resulting batho intermediates were found to be identical both in λ_{max} and in molar absorptivity. [Digitonin-solubilized isorhodopsin prepared by direct chromophore substitution, on the other hand, yielded a batho intermediate with a 15% higher molar absorptivity (Mao et al., 1980).] It should be noted that in our studies, isorhodopsin was prepared photochemically while the protein was still imbedded in native membrane, potentially the most gentle way of performing this synthesis. Our conclusion that the batho intermediates are the same agrees as well with recent nanosecond flash photolysis (Horwitz et al., 1983) and picosecond resonance Raman (Hayward et al., 1981) studies and further supports the hypothesis that cis–trans isomerization is the primary event in vision.

Other workers have found differences in the batho intermediates from rhodopsin and isorhodopsin. Spalink et al. (1983), using picosecond spectroscopy, found the absorbance maximum of batho$_{iso}$ to be shifted ~10 nm to the blue of the maximum of batho$_{rho}$. Studies by Waddell et al. (1984) at 77 K showed the same hypsochromic shift to be 4–5 nm. In both of these studies, isorhodopsin was prepared by regenerating opsin with 9-*cis*-retinal. It is possible that removal of the chromophore from rhodopsin induces some irreversible change in the protein conformation which is later manifested as altered bleaching properties in the regenerated pigment.

Our study of isorhodopsin led to the additional observation that the overall bleaching quantum yield of isorhodopsin is about the same as the apparent quantum yield for the formation of batho$_{iso}$ as measured by picosecond absorption spectroscopy. The overall bleaching ratio observed here (ϕ_{iso}/ϕ_{rho} = 0.66 ± 0.15) is significantly higher than previously published (Hurley et al., 1977; Suzuki & Callender, 1981) overall bleaching ratios of ϕ_{iso}/ϕ_{rho} = 0.3 (room temperature) and ϕ_{iso}/ϕ_{rho} = 0.1–0.2 (77 K; wavelength dependent). Spalink et al. (1983), using picosecond pulses at 532 nm, and Horwitz et al. (1983), using nanosecond pulses at 457 nm, also found that significantly more batho$_{iso}$ was observed by transient absorption spectroscopy than would have been predicted on the basis of known quantum yield data. In addition, Horwitz et al. (1983) found significantly different transient spectra for rhodopsin and isorhodopsin on the nanosecond and microsecond time scales.

A coherent picture of isorhodopsin photochemistry thus remains to be formulated. Differences in the photon saturation properties of rhodopsin and isorhodopsin (Spalink et al., 1983), as well as the uncertainties in the quantum yield for isomerization of the two pigments, make even carefully controlled studies difficult to evaluate. Our work has shown, however, that rhodopsin and isorhodopsin may be studied facilely while still in native membrane. Hence, future studies may be per-

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[3] The absorption cross section of a chromophore, σ, is defined by the relationship ϵ = (2.62 × 10²⁰)σ, where ϵ is the extinction coefficient in units of liters per mole per centimeter and σ has units of centimeters squared (Braude, 1950).

formed under conditions which minimally perturb the opsin/retinal interactions responsible for the visual bleaching sequence.

Acknowledgments

Registry No. CHAPS, 75621-03-3; digitonin, 11024-24-1.

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