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# Retinal Isomer Ratio in Dark-Adapted Purple Membrane and Bacteriorhodopsin Monomers†

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ABSTRACT: On the basis of data obtained by spectroscopic analysis and chromatography of retinal extracts, a consensus has been adopted that dark-adapted purple membrane (pm) contains 13-*cis*- and *all-trans*-retinal in equal amounts, whereas the light-adapted membrane contains *all-trans*-retinal only. We have developed an improved extraction technique which extracts up to 70% of the retinal in pm within 4 min. In the extracts from dark-adapted pm at room temperature, we consistently find 66–67% 13-*cis*-retinal and 33–34% *all-trans*-retinal, and more than 98.5% all-trans isomer in light-adapted samples. The spectrum obtained by reconstitution of bacterioopsin with 13-*cis*-retinal at 2 °C (to minimize isomerization) shows an absorbance maximum at 554 nm and agrees well with the spectrum for the 13-cis component calculated from the dark-adapted and light-adapted bR spectra with our extraction data. The ratio of 13-cis:all-trans isomer in dark-adapted pm is 2:1 and nearly constant between 0 and 38 °C but begins to decrease distinctly above 40 °C, and more rapidly near 70 °C, reaching 0.75 at 90 °C. The van't Hoff plot of the isomer ratio shows a nonlinear temperature dependence above 40 °C, indicating a more complex system than a simple thermal 13-cis/all-trans isomer equilibrium. We attribute the broadening, absorbance decrease, and blue shift of the visible absorption band with increasing temperature to the appearance of at least one and possibly two or three new chromophores which contain, mainly or exclusively, the all-trans isomer. The 2:1 ratio of the isomers and its small temperature dependence between 0 and 40 °C suggest that it could be determined by the trimeric association of bR in the planar protein lattice of pm and that the lowest free energy conformation in the lattice is a trimer which contains one all-trans and two 13-cis isomers. However, the lattice remains intact up to ~75 °C, and trimers of bR exist at still higher temperatures. Moreover, monomeric bR solubilized with Triton X-100 shows a similar isomer ratio near room temperature and slightly higher 13-cis isomer content (e.g., 71% 13-cis and 29% all-trans at 4 °C) at low temperature, and also a nonlinear but steeper temperature dependence in the van't Hoff plot. We argue that the lattice stabilizes the protein in a conformation which slightly favors the 13-cis chromophore but restricts its structure from assuming the conformation still more favorable to 13-*cis*-retinal which it attains in the monomer.

Bacteriorhodopsin (bR),[1] the protein in the purple membrane (pm) of *Halobacterium halobium*, functions as a light-driven proton pump [for review, see Stoeckenius et al. (1979), Birge (1981), and Stoeckenius and Bogomolni (1982)]. The bR molecules are arranged within the membrane in a hexagonal lattice, and structural analysis by electron microscopy suggests a structure where the polypeptide chain forms seven helix segments spanning the membrane. Like visual pigments, bR contains one retinylidene chromophore (Oesterhelt & Stoeckenius, 1971) bound to Lys-216 as a protonated Schiff base (Lewis et al., 1974; Katre et al., 1981; Bayley et al., 1981; Lemke & Oesterhelt, 1981; Mullen et al., 1981). The chromophore has a main absorption band near 560 nm; the more than 100-nm red-shift compared to a protonated Schiff base of retinal in solution is explained by additional noncovalent protein–retinal interactions (Blatz et al., 1972; Spudich et al., 1986; Lugtenburg et al., 1986). After exposure to light, the absorption maximum is found at 568 nm, and this light-adapted bR ($bR^{LA}$) contains >98% *all-trans*-retinal. In the dark, the absorption maximum shifts

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[1] Abbreviations: pm, purple membrane; bR, bacteriorhodopsin; $bR^{LA}$, light-adapted bacteriorhodopsin; $bR^{DA}$, dark-adapted bacteriorhodopsin.

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slowly back to 558 nm, and for this dark-adapted state ($bR^{DA}$), roughly equimolar amounts of 13-*cis*- and *all-trans*-retinal have been reported repeatedly but with rather large variations between individual measurements (Oesterhelt et al., 1973; Dencher et al., 1976; Pettei et al., 1977; Maeda et al., 1977; Tsuda et al., 1980). Nevertheless, it has become the dogma that dark-adapted pm contains equal amounts of the 13-cis and all-trans isomers. Both the 13-cis and the all-trans components undergo cyclic photoreactions, during which a fraction of the 13-cis isomer containing bR is converted to all-trans isomer containing bR (Lozier et al., 1975; Dencher et al., 1976).

Without a catalyst, retinal isomers in solution do not thermally isomerize to a significant extent for days at room temperature; at thermal equilibrium, established at higher temperature or in the presence of a catalyst, they contain 60% all-trans, 20% 13-cis, and 16% 9-cis isomers (Sperling et al., 1977). The fast equilibration and ratio of the isomers in bR indicate that the protein functions as an isomerase and stabilizes the 13-cis isomer.

After optimizing extraction conditions for speed and yield, we have reinvestigated the isomer composition in the bR chromophore and confirmed that $bR^{LA}$ extracts contain virtually only *all-trans*-retinal at room temperature. However, we have consistently found a ratio of $[bR^{13\text{-cis}}]/[bR^{trans}] = 2$ in $bR^{DA}$. This observation creates some difficulties, because the 1:1 isomer ratio found earlier in extracts has been confirmed by absorption spectroscopy (Fischer & Oesterhelt, 1979; Maeda et al., 1977) and—under the assumption that excitation of the 13-cis chromophore does not produce an M intermediate and proton translocation—by flash spectroscopy and ion transport measurements (Dencher et al., 1976; Ohno et al., 1977a,b; Fahr & Bamberg, 1982). Furthermore, the absorption spectrum of the 13-cis chromophore (Dencher et al., 1976) and other parameters, e.g., the photoelectric signal (Trissl & Gaertner, 1987), have been calculated by subtracting 50% of the $bR^{LA}$ signal from $bR^{DA}$ records. Therefore, thorough reexamination of these observations and conclusions becomes necessary. Here we only consider environmental effects on the chromophore ratio. Preliminary results have been published (Scherrer et al., 1987).

## Materials and Methods

*Materials.* Purple membrane from *H. halobium* strains ET-1001 and R1 was isolated as described previously (Oesterhelt & Stoeckenius, 1974). The pm preparations were either used immediately, stored at 4 °C and used within 1 week, or stored as collected from the density gradient in 40% sucrose 0.2% $NaN_3$ solution for times up to several months at −80 °C.

A purple membrane suspension containing 60–80 μM bR in 20 mM MES, pH 6.0, 10 mM $MgCl_2$, and 100 mM NaCl was dark adapted by incubation for 3 h at 37 °C followed by 2 h at room temperature (22 °C). The $MgCl_2$ was added to minimize formation of the blue form of pm at high temperature (Chang et al., 1986).

For the temperature dependence study, additional incubation times were required. The dark-adaptation rates for pm follow first-order kinetics at any tested temperature between 20 and 55 °C; only the 60 °C dark-adaptation rate was fitted better with two exponentials. However, at this temperature, the rate becomes too rapid for a reliable measurement with our setup. The Arrhenius plot shows a linear dependence of the logarithm of the rate constant vs inverse temperature between 20 and 50 °C and yields an activation energy of 115 kJ/mol. It is obvious that long times are required for complete dark adaptation and that the often used "overnight in the refrigerator" protocol is insufficient. We, therefore, incubated our samples for 18 h at 10 °C, 3 days at 4 °C, and 1 week at 1 °C to ensure complete dark adaptation at each temperature. Higher temperatures required much shorter incubation times; above 40 °C, we used 1 h or less.

*Extraction of Retinal.* All operations were carried out in dim red light. A total of 100 μL of pm suspension was mixed rapidly with 250 μL of ice-cold ethanol on a vortex mixer. For extractions at and above 40 °C, the ethanol was precooled in methanol/dry ice. After 2 min on ice with intermittent vortexing, 250 μL of ice-cold hexane was added and the sample mixed continuously for 2 min. The emulsion was centrifuged in a Beckman microfuge for 60 s, and the retinal isomers in the hexane phase (upper) were immediately separated by HPLC (Spectra-Physics, SP-8700). We used two Zorbax-Sil columns (4.6 × 250 mm each; Du Pont) in series with 8% diethyl ether in hexane as solvent at a flow rate of 1.5 mL/min and monitored the isomers at 365 nm with a Kratos Spectroflow 783 connected to an integrator (Hewlett-Packard 3392). The peaks were identified and quantitated with recrystallized *all-trans*- and 13-*cis*-retinal for calibration.

*Reconstitution Experiments.* The pm was bleached with yellow light (Corning filter 3-71) from a 350-W arc lamp (General Electric Marc 300) in the presence of 500 mM hydroxylamine, pH 7.0 (Becher & Cassim, 1977), for 3 h and washed four times with distilled $H_2O$ by centrifugation. The apomembrane was resuspended in 50 mM HEPES, pH 7.0, containing 100 mM NaCl at ~10 μM bacterioopsin (bO) and reconstituted at 2 °C with a 1.2–1.4 molar excess of 13-*cis*- or *all-trans*-retinal dissolved in ethanol.

*Spectroscopy.* Absorption spectra were recorded with an Aminco DW-2a spectrophotometer connected to a Nicolet 1180 data acquisition system. The light–dark adaptation kinetics were followed by monitoring the absorbance change at 585 nm of a light-adapted pm suspension (11 μM bR in 10 mM MES, pH 6.0, 100 mM NaCl, 5 mM $MgCl_2$) in a Perkin-Elmer Lambda 4A spectrophotometer. The sample holders were thermostated with circulating water from a Haake A80 unit.

*pm Solubilization in Triton X-100.* Purple membrane (7 mg of bR/mL) in 20 mM sodium acetate buffer, pH 5.0, was incubated in 2% Triton X-100 (Pierce Chemicals, purified Triton X-100; Surfact-Amp X-100) in the dark for 18 h at room temperature under stirring. Then, the sample was centrifuged at 150000*g* for 2 h and the supernatant diluted with 20 mM MES, pH 6.0, 10 mM $MgCl_2$, 100 mM NaCl, and 0.4% Triton X-100 to give a 60 μM bR solution. Dark adaptation was carried out on either the concentrated or diluted samples for the same times as used with intact pm. Extraction conditions were the same as used for intact pm.

## Results

*Retinal Extraction.* Our technique consistently extracted between 55 and 70% of the retinal in the pm sample, and the HPLC analysis showed that, in the extracts from dark-adapted samples at room temperature, 66–67% was the 13-cis and 33–34% the all-trans isomer. In fully light-adapted samples, the ratio changed to >98.5% *all-trans*-retinal with only traces of the 13-cis isomer (Figure 1a,b). Increasing the concentration of NaCl and/or $MgCl_2$ up to 1 M did not affect the isomer ratio in dark-adapted or light-adapted pm; but membranes washed extensively with distilled water or suspended in phosphate buffer consistently showed lower amounts of the 13-cis isomer (58–62%) after dark adaptation. Millimolar concentrations of salt are required for reproducible results. The 13-cis to all-trans isomer ratio in dark-adapted membranes

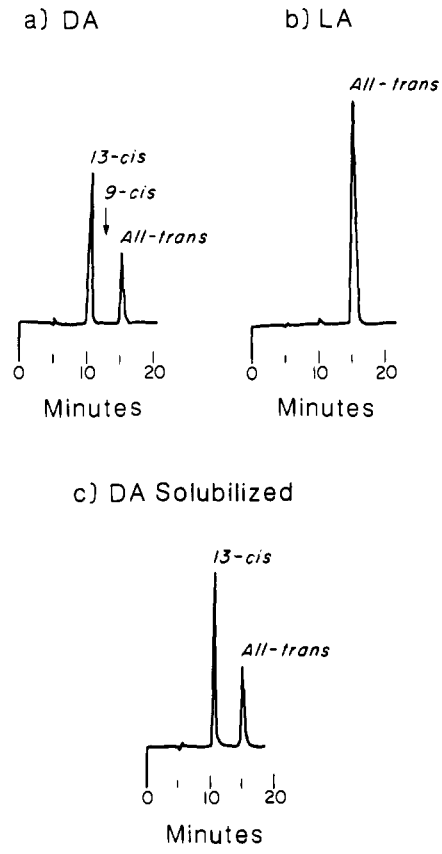


FIGURE 1: HPLC chromatograms of retinal extracts from dark-adapted (a) and light-adapted (b) purple membrane and from Triton-solubilized monomers (c).

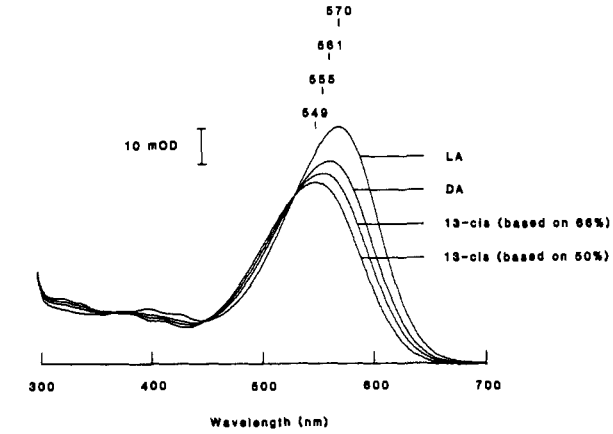


FIGURE 2: Absorbance spectra of light-adapted pm (LA) and dark-adapted pm (DA) at 2 °C and calculated spectra for 13-cis bR assuming an all-trans bR absorbance contribution of 50% and 33%, respectively. The $bR^{13\text{-cis}}$ spectra are normalized for equal bR concentration.

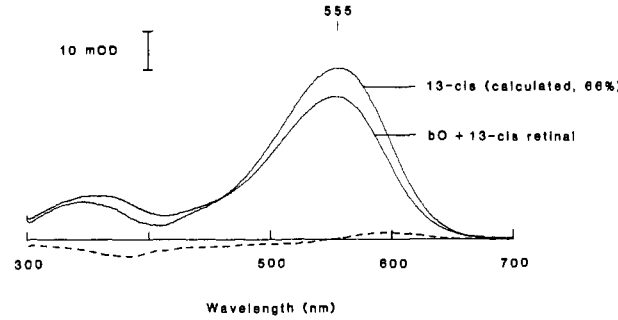


FIGURE 3: Comparison of the 13-cis bR spectrum calculated as in Figure 2 assuming 33.3% *all-trans*-, 66.6% 13-*cis*-retinal for $bR^{DA}$ and a 13-cis bR spectrum obtained by reconstitution of bacterioopsin with 13-*cis*-retinal 20 min after retinal addition. The dashed (--) line indicates the difference spectrum after normalization at 555 nm.

remained constant when the amount extracted was decreased from 68% to 43% by decreasing extraction time for each extraction step from 150 to 30 s [see Figure 2 in Scherrer et al. (1987)]. Extraction times longer than 5 min or extraction at room temperature resulted in lower ratios without significant increases in the extracted amount.

Since the trimer structure of the lattice could affect the isomer ratio, we also extracted monomeric bR. In Triton X-100 solubilized, dark-adapted monomers (Casadio et al., 1980; Reynolds & Stoeckenius, 1977; Dencher & Heyn, 1978) at and below room temperature, we consistently found a slightly higher 13-cis isomer content (71% at 4 °C) than in intact pm (Figure 1c).

*Reconstitution of the Chromophore with 13-cis- and all-trans-Retinal.* Knowing the amount of *all-trans*-retinal in dark-adapted pm and the $bR^{all\text{-}trans}$ spectrum ($bR^{LA}$), the spectrum for $bR^{13\text{-cis}}$ can be calculated by subtracting the $bR^{all\text{-}trans}$ contribution from the $bR^{DA}$ spectrum. Assuming an equimolar 13-cis to all-trans ratio, Dencher et al. obtained a spectrum with an absorbance maximum at 548 nm for $bR^{13\text{-cis}}$ at 20 °C (Dencher et al., 1976). Using 66–67% 13-cis isomer in $bR^{DA}$, we calculate a $bR^{13\text{-cis}}$ spectrum with an absorption maximum at 555 ± 1 nm at 2 °C (Figure 2).

To verify the extraction data, we reconstituted bleached pm with a 1.2–1.4 molar excess of 13-*cis*- and *all-trans*-retinal, respectively. To minimize isomerization during the reconstitution, the samples were kept at 2 °C. Similar to the results obtained earlier by Schreckenbach et al. (1977), we observed that reconstitution within 1–2 min generated intermediates with absorption maxima at 420–430 nm with *all-trans*-retinal and 400–410 nm with 13-*cis*-retinal, followed by conversion to $bR^{all\text{-}trans}$ and $bR^{13\text{-cis}}$ chromophores with absorbance maxima at 570 ± 1 and 554 ± 1 nm, respectively. The $bR^{13\text{-cis}}$ chromophore formed significantly faster; about half of the 13-cis pigment was reconstituted within 3 min while the all-trans pigment required 15 min. The $bR^{13\text{-cis}}$ spectrum obtained 30 min after 13-*cis*-retinal addition agrees well with the calculated spectrum obtained if a 2:1 13-cis to all-trans isomer ratio is assumed for dark-adapted bR (Figure 3). Extracts of the 30-min-reconstituted sample showed less than 1% isomerization from 13-*cis*- to *all-trans*-retinal.

*Temperature Dependence of the Isomer Ratio and Spectral Changes.* As one would expect, the isomer ratio in dark-adapted pm is temperature-dependent. Increasing the temperature from 0 to 90 °C reversibly changes the 13-cis to all-trans ratio from 2:1 to 0.75:1 (Figure 4). The ratio changes by less than 1% between 0 and 38 °C but begins to decrease more rapidly above 40 °C. Plotting the logarithm of the equilibrium constant $K = [bR^{trans}]:[bR^{cis}]$ vs the inverse temperature yields a nearly horizontal straight line between 0 and 40 °C with rapidly increasing upward curvature at higher temperatures (Figure 5). The isomer ratio for monomeric $bR^{DA}$ in Triton also shows a nonlinear van't Hoff plot but with no straight portion and a much stronger temperature dependence, so that above 25 °C, the 13-cis:all-trans isomer ratio is lower than that in pm. The monomers can only be tested over a more limited temperature range because irreversible changes already appear at 50 °C. The small pH changes which accompany the temperature change are less than 0.4 unit between 2 and 80 °C and, at least for pm, have no effect on the isomer composition, as tested by pH adjustments.

The absorbance spectrum of bR also shows a considerable temperature dependence (Jackson & Sturtevant, 1978;

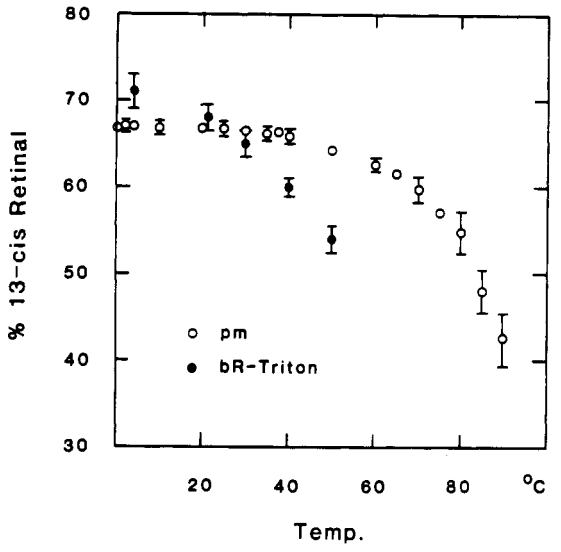


FIGURE 4: Percent 13-*cis*-retinal in extracts from dark-adapted purple membrane and Triton-solubilized bR as a function of temperature.

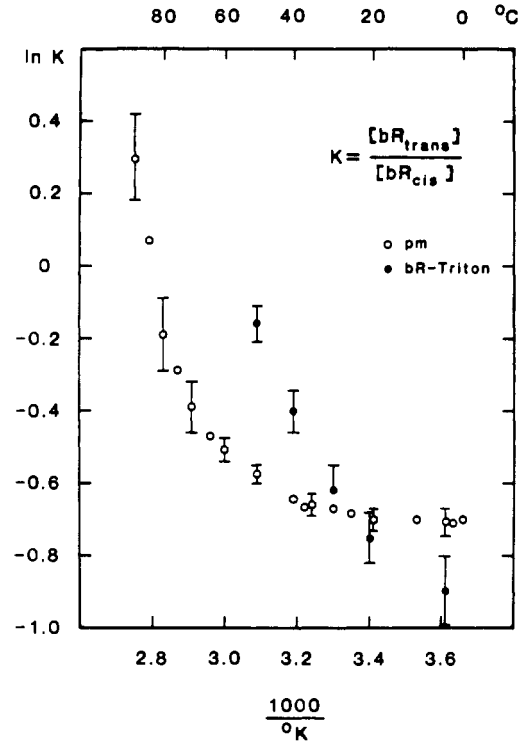


FIGURE 5: Temperature dependence of the all-trans to 13-cis isomer ratio plotted as the logarithm of the equilibrium constant versus inverse temperature (van't Hoff plot). Data from Figure 4.

Brouillette et al., 1987). Under our conditions with increasing temperature, the visible absorbance band of dark-adapted pm shifts to shorter wavelengths and decreases with a simultaneous decrease in 280-nm absorbance. At the same time, the spectrum broadens somewhat asymmetrically from 110-nm half-width at 2 °C to 139 nm at 79 °C with nearly isosbestic cross overs at 525 and 625 nm for temperatures up to 70 °C on the short-wavelength side and up to 80 °C on the long-wavelength side (Figure 6a). The shift and decrease of the absorbance appear almost linear between 0 and 60 °C but become more pronounced at higher temperatures, similar to the accelerated change in the isomer composition. The absorbance changes of the spectrum are reversible up to 80 °C. When the sample is kept above this temperature for more than a few minutes, the 560-nm absorbance does not fully recover

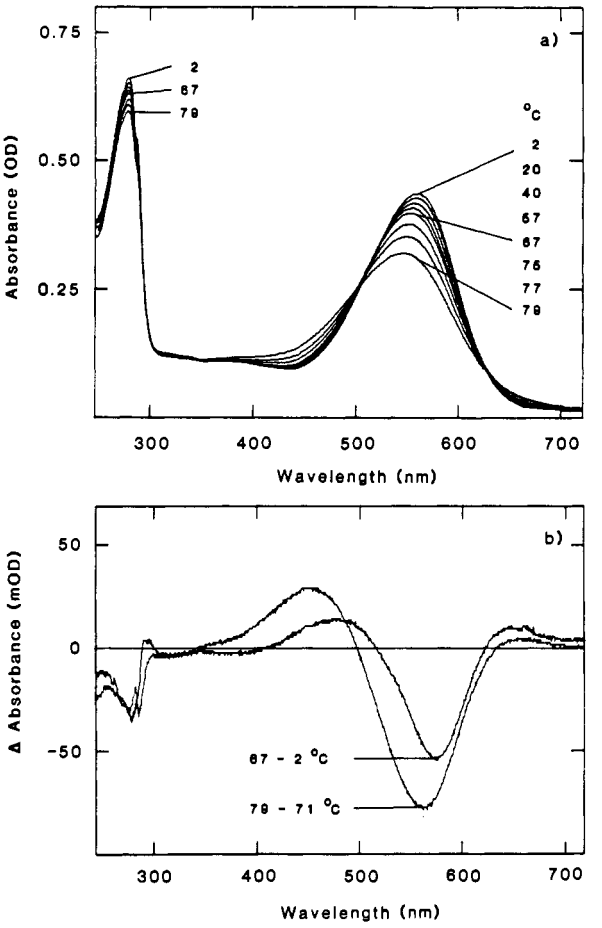


FIGURE 6: (a) Absorbance spectra of dark-adapted pm at various temperatures. The pm was resuspended in 100 mM NaCl–50 mM HEPES, pH 7.0, and $MgCl_2$ was omitted to prevent pm aggregation at higher temperature. For clarity, only selected temperatures are shown. (b) Difference spectra for 67–2 °C and 79–71 °C.

and the 9-cis isomer appears in the extract, indicating denaturation of the protein.

The absorbance changes are obviously complex, and in the opposite direction of what one would expect from the increase in all-trans isomer. Up to 67 °C the blue shift and absorbance decrease at 560 nm are relatively small and accompanied by small absorbance increases around 480 and 650 nm. At higher temperatures, the 650-nm increase remains small, but the 480-nm increase grows more rapidly and blue shifts, reaching 450 nm at 90 °C, with a corresponding decrease and blue shift of the 560-nm band (Figure 6b). The absorbance increase between 500 and 450 nm clearly involves two steps, the second becoming dominant at temperatures above 70 °C. The isosbestic point for the first step is near 525 nm and could be attributed to a species with an estimated absorption maximum near 520 nm. The corresponding parameters for the second species are an isosbestic point at 505 nm and absorption maximum at 460–480 nm.

## Discussion

Our results for the isomer ratio in dark-adapted pm clearly conflict with earlier extraction data (Pettei et al., 1977; Maeda et al., 1977; Tsuda et al., 1980; Fischer & Oesterhelt, 1979). The differences may be attributable, at least in part, to the more carefully controlled membrane preparation and faster extraction technique we used, and also to the integrator calibrated with 13-*cis*- and *all-trans*-retinal standards and the greatly improved HPLC columns and equipment developed over the last few years. Data reported in the literature for

comparable conditions vary between 45 and 59% 13-cis isomer and were obtained by more elaborate methods, most of which gave lower yields and/or larger variations. Our data were reproducible within 1% up to 60 °C, and the temperature effect was fully reversible.

The high 13-cis to all-trans isomer ratio we observe cannot be the result of isomerization during the extraction and/or chromatography since the amount of 13-*cis*-retinal found in light-adapted pm is <1.5% and can mostly be accounted for by a small degree of dark adaptation between illumination and the beginning of the extraction procedure. It is unlikely that 13-cis isomer is formed during unfolding of the protein only in $bR^{DA}$ and not in $bR^{LA}$, and a preferential 13-cis extraction can be ruled out since reducing the extraction times changes the yield from 68% to 44%, but not the isomer ratio.

In deionized blue membrane, only 52–53% 13-cis isomer is found after dark adaptation (Mathew et al., 1986). Therefore, the lower 13-cis to all-trans isomer ratio of pm washed extensively in distilled water can be explained by partial formation of that species. Similarly, removal of divalent cations by phosphate buffer may cause lower ratios since addition of excess NaCl or $MgCl_2$ to phosphate buffer restores the 2:1 isomer ratio (our unpublished results). Note that in experiments where the isomer ratio was measured by flash photolysis (Dencher et al., 1976) 25 mM phosphate buffer without added salt was used.

While we are confident that our results are more reliable than any reported so far; extraction data, in principle, cannot determine the isomer distribution in the intact protein conclusively. Resonance Raman, FTIR, and NMR spectroscopy can give information on the isomer ratio, and inspection of the best published spectra generally shows larger amounts of 13-*cis*-retinal than *all-trans*-retinal in dark-adapted pm, but quantitation is difficult. A reinvestigation by resonance Raman spectroscopy yielded 58 ± 4% 13-cis isomer (S. Fodor, J. Ames, and R. Mathies, personal communication), and NMR spectra gave a very similar value (De Groot et al., 1988). It is, however, not possible to exactly reproduce the conditions of our extraction experiments in the samples for vibrational and NMR spectroscopy, and we have seen that the ratio is quite sensitive to sample-handling conditions. We, therefore, consider the good agreement of the 13-cis bR absorption spectrum calculated with the 2:1 isomer ratio and the spectrum obtained after reconstitution of bO with 13-*cis*-retinal as the stronger argument for validity of the extraction data. Similar bR spectra obtained by reconstitution with 13-cis-retinal and showing an absorption maximum well above 550 nm can be found in the literature, e.g., Schreckenbach and Oesterhelt (1977) and Iwasa et al. (1981).

In the purple membrane, bR trimers form a rigid hexagonal lattice, and the 13-cis to all-trans isomer ratio of 2:1 could be explained by the assumption that the lowest free energy conformation of a trimer contains one all-trans and two 13-cis isomers. However, trimers persist at temperatures above 80 °C when the lattice has already dissociated (Jackson & Sturtevant, 1978; Brouillette et al., 1987; Hiraki et al., 1981), and one would not expect that the symmetric interactions in the lattice would promote a difference in the monomers forming the trimer. Also, to our knowledge, no lattice transition has ever been reported near 40 °C. Tsuda and Ebrey (1980) have reported an increase of the 13-cis to all-trans isomer ratio of pm from 1:1 at ambient temperature and pressure to 3:1 at 2.5 kbar and 30 °C. Their absolute values may need correction in view of our results, but the increase of the 13-cis component beyond 67% is probably real. Together with the increase to >70% 13-cis isomer in Triton-solublizied bR at 2 °C which we observed, the results indicate that the 13-cis chromophore represents the lowest free energy state of the monomer and suggest that the lattice restricts the required conformational change of the protein, e.g., by restricting the minimal volume it can occupy. According to this alternative view, the 2:1 ratio would be determined not by the trimer but would represent the averaged conformation of the individual monomers in the lattice.

The strong curvature of the van't Hoff plot cannot be due to isomerization in the extract because known amounts of 13-*cis*- or *all-trans*-retinal added to the ethanol just prior to extraction of 80 °C samples are recovered unchanged and the monomer preparation shows a very similar curvature, even below room temperature. Independently, the spectral changes also indicate that more complex processes take place in the intact protein, which could explain the curved van't Hoff plot. The absorption decrease and blue shift are the opposite of what one would expect from an increase of the all-trans chromophore and too large for a simple temperature effect. Apparently, we are not just dealing with a simple equilibrium between two conformational states but are generating new states with different protein and chromophore conformations. The 650-nm absorbance increase can be attributed partially to broadening of the spectrum and to the formation of blue membrane ($bR_{605}$), which has been shown to be favored by higher temperature (Chang et al., 1986). Its formation is apparently not completely inhibited by the salt concentration and pH used here. As expected, its contribution becomes larger when the salt concentration is decreased (data not shown). Absorbance increases on the short-wavelength side, which are favored by high temperature and/or pH, have been described before and dubbed the 500-nm chromophore ($bR_{500}$) and the 460-nm chromophore or 460/490-nm complex (Oesterhelt et al., 1973; Schreckenbach et al., 1977; Fischer & Oesterhelt, 1979). The chromophore absorbing at 460–480 nm has been repeatedly described (Lozier et al., 1976; Scherrer & Stoeckenius, 1984). The monomeric state, or a disturbed lattice, apparently promotes it, and it is not surprising, therefore, that this species increases rapidly above 70 °C in pm and at lower temperature in Triton-solubilized bR. It is also generated by organic solvents or detergents and has been investigated more thoroughly under these conditions (Baribeau & Boucher, 1985, 1987; Henry et al., 1988; Nishimura et al., 1985). It contains only the all-trans isomer (Pande et al., 1986) (and R. Callender, personal communication). Of the other two species suspected to be present, dark-adapted blue membrane has a lower 13-cis:all-trans isomer ratio than $bR^{DA}$ (Mathew et al., 1986), and the isomer composition of the more elusive 500-nm-absorbing species is not known. The coexistence of some or all of these species could easily account for the strong curvatures of the van't Hoff plot, but a rigorous quantitative treatment at present is hardly warranted.

As pointed out in the introduction, the observed isomer ratio conflicts with reports that dark-adapted pm shows 50% of the pumping efficiency and amount of M intermediate seen in the same preparation after light adaptation. If correct, these observations necessarily lead to the conclusion that the 13-cis component in dark-adapted pm pumps protons and produces M with ~25% of the efficiency of the all-trans component, given equal quantum yields for photocycling. This conclusion conflicts with the generally accepted view that excitation of 13-cis bR does not lead to M formation and proton translocation. However, during one photocycle, 10–15% of the 13-cis component in dark-adapted pm may be converted to $bR^{LA}$

(Takeda et al., 1986) (and our unpublished results). If the cross over into the light-adapted photocycle should occur before the L intermediate, this fraction should form an M intermediate and pump protons. The effect would be sufficient to account for the observed amount of M intermediate and pumping efficiency in dark-adapted pm, given the error limits in these results. We have begun experiments to test this hypothesis.

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**Registry No.** 13-*cis*-Retinal, 472-86-6; *all-trans*-retinal, 116-31-4.

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