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Interaction of Aromatic Retinal Analogues with Apopurple Membranes of *Halobacterium halobium*†

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ABSTRACT: Absorption spectral properties of aromatic analogues of retinal with apopurple membrane of *Halobacterium halobium* were studied. The spectra of the all-trans forms were composed of two or more absorption bands. During incubation at 20 °C, an absorption band above 500 nm increased in intensity gradually at the expense of an absorption band in the shorter wavelength region with no isomerization of the chromophore. The longer wavelength species was shown to be the protonated form of the shorter wavelength species by changing the pH of the medium. Upon irradiation with blue light, the bandwidth of the spectrum became smaller with isomerization of the chromophore to its 13-cis form. Irreversible binding of protons on the membrane occurred during this process. The rate of the increase in the longer wavelength absorption band was especially low in the reaction with the all-trans form of retinal analogues having a bulky substituent at the para or meta positions of the phenyl ring. In contrast, the 13-cis isomer of aromatic retinal analogues gave a single absorption peak. The extent of the spectral shift upon binding to apopurple membranes was compared over a series of aromatic retinals, and the results were explained in terms of steric interactions of the chromophore with the protein.

Bacteriorhodopsin (bR)[1] in the purple membrane of *Halobacterium halobium* transports protons unidirectionally across the membrane by using light energy absorbed by its chromophore (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1982). Conversion of light energy into chemical energy is most probably carried out in the vicinity of the retinylidene chromophore. In order to investigate the structure of the retinal binding site, experiments with a series of chemically modified chromophores (Nakanishi et al., 1980) have been done along with chemical modification of the protein itself

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[1] Abbreviations: bR, bacteriorhodopsin; λ_{max}, the wavelength of maximum absorption; HPLC, high-performance liquid chromatography.

0006-2960/84/0423-2507$01.50/0

(Lemke et al., 1982; Maeda et al., 1982).

Retinal consists of two parts, a polyene side chain and a trimethylcyclohexene ring. Replacement of the ring with aromatic derivatives retained the proton pumping activity but in reduced extents (Marcus et al., 1977; Bayley et al., 1981). Motto et al. (1980) have observed that the maximum absorption (λ_{max})[1] of the phenyl analogue of bR in the membrane is shifted to a much shorter wavelength than that of the parent purple membrane. They suggested that the spectral properties dictated by an electrical charge close to the ring of the chromophore (Nakanishi et al., 1980) was perturbed, owing to the presence of the aromatic ring. The λ_{max} value for the phenyl-bR reported by Motto et al. (1980), 480 nm, however, was somewhat smaller than the 510-nm value by Bayley et al. (1981). Huang et al. (1982) subsequently described the use of a carbenoid photoaffinity label attached to the aromatic ring for identifying the amino acid residues around the ring. This analogue having a bulky substituent at the meta position showed the λ_{max} at 470 nm upon binding with apopurple membrane. An analogue pigment with a highly substituted aromatic retinal, [4-methoxy-2,3,6-trimethylphenyl]retinal, gave the λ_{max} at 480 nm (Marcus et al., 1977). In contrast, Ahktar et al. (1982) have observed a larger extent of the red shift with naphthyl-bR. These results indicate that further studies are required in order to reveal the spectral properties due to the interactions of aromatic retinal in the retinal binding site of apopurple membrane.

Several analogues of aromatic retinal were previously used for the purpose of probing the structure of the retinal binding site of bovine opsin (Matsumoto et al., 1980). In the present paper, these analogues were recombined with bacterioopsin in the membrane. The spectral properties of these artificial pigments, which differ significantly from those of bR, were explained in terms of steric factors in the interaction of the chromophore with bacterioopsin.

Materials and Methods

Retinals. Figure 1 shows the structures of the all-trans form of the aromatic retinal used in this study with their trivial names. Procedures for their synthesis were described (Matsumoto et al., 1980). The synthetic mixtures containing both the all-trans and 13-cis isomers were separated by high-performance liquid chromatography (HPLC).[1] Butylamine derivatives were synthesized as described by Blatz et al. (1972): A small amount of the retinal analogue was dissolved in 0.05 mL of anhydrous butylamine and the solution was kept at 0 °C for 3 h under N_2. The excess butylamine was removed by evaporation under a stream of N_2. The Schiff base thus obtained was dissolved in methanol and protonated by HCl (Nakanishi et al., 1980).

Apopurple Membranes. Purple membranes were prepared from the cell lysate of *Halobacterium halobium* R_1M_1 (donated by Prof. D. Oesterhelt) by the standard method (Oesterhelt & Stoeckenius, 1974).

Apomembranes were prepared from purple membranes essentially as described by Bayley et al. (1981): bleaching in 2 M hydroxylamine (pH 6.8) in the presence of light (>460 nm), washing by centrifugation for 30 min at 35000*g* after dilution in water, dialysis against 100 volumes of 5 mM phosphate buffer (pH 6.8) for 16 h and then three changes of 100 volumes of water for 24 h, lyophilization, and 5 times extraction of the retinal oxime in petroleum ether. The suspensions of apomembrane in 5 mM phosphate buffer (pH 6.8) were sonicated briefly and then mixed with an equal volume of glycerol for the spectrophotometric experiments in order to reduce light scattering and to avoid possible precipitation

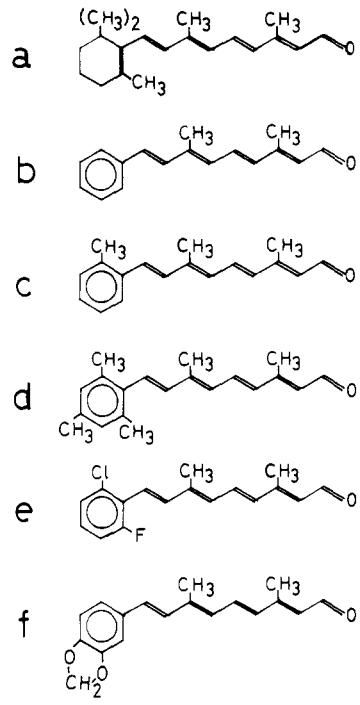

FIGURE 1: Structure of (a) retinal and (b–f) aromatic retinal analogues used: (b) [phenyl]retinal, (c) [*o*-tolyl]retinal, (d) [mesityl]retinal, (e) [2-chloro-6-fluorophenyl]retinal, and (f) [piperonyl]retinal. All the formulas are shown in the all-trans form.

during spectral measurements. The final pH measured was 6.6. The amount of bacterioopsin in the apomembranes was determined from the amount of bR formed by adding *all-trans*-retinal, using a molar extinction of 63 000, according to Oesterhelt & Hess (1973).

Reaction of Aromatic Retinal with Apomembranes. The retinal analogue was dissolved in ethanol and added to the apomembrane suspensions in 50% glycerol (about 10^{-5} M). The final concentration of ethanol was less than 1% of the total volume of the mixture, and the amount of the added retinal was 60–80% of the amount required for completely occupying the binding site in the apomembranes. Spectral measurements were carried out on a Hitachi recording spectrophotometer, type 124. Temperature was controlled by circulating water from the thermostated bath. Circulation of ice-cold water gave a temperature of 3 °C in the cuvette. Moisture on the surface of the cuvette was prevented by a stream of dry N_2. The spectra presented are those obtained after subtraction of the spectrum before addition of retinals.

Irradiation with blue light (400–530 nm) was done by a combination of a band filter and a cutoff filter as described previously (Maeda et al., 1981). A 1-kW halogen–tungsten lamp in a slide projector was used as a light source.

Analysis of Retinal Isomers. The method for the analysis of retinal isomers is based on the procedure developed by Pilkiewicz et al. (1976). Retinals were extracted from the membrane by mixing with an equal volume of dichloromethane in an ultradisperser, Ultraturrax of Ika Werke, at its maximum speed for 8 min. Details were described by Maeda et al. (1981).

Light-Induced pH Changes. One milliliter of the apomembrane suspension containing 1×10^{-8} mol of bacterioopsin was equilibrated at 20 °C in a water bath. The pH was adjusted to about 7.9 with a small amount of 0.01 N NaOH. The sample was kept under a constant flow of N_2 at 20 °C. The retinal in 1.5-fold molar excess of the amount required for the complete binding was added. The pH was measured after a 3-h incubation by a Horiba pH electrode, type 6128,

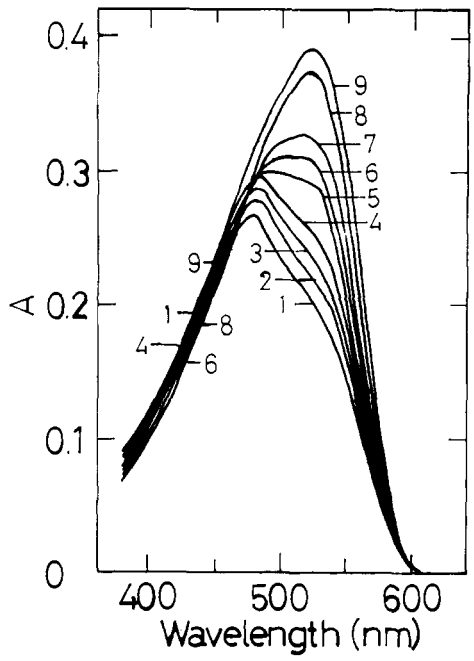

FIGURE 2: Changes in the absorption spectrum of *all-trans*-phenyl-bR. Apopurple membrane containing 1.4×10^{-5} M bacterioopsin, 0.4 mL, was incubated with *all-trans*-[phenyl]retinal (4 μL of the ethanolic solution, $A_{390nm} = 50$) at 3 °C. The absorption spectra were measured after 20 (curve 1), 40 (curve 2), 80 (curve 3), and 160 min (curve 4). The mixture was then incubated at 20 °C, and the spectra were measured after an additional 1 (curve 5), 2 (curve 6), 4 (curve 7), 16 (curve 8), and 40 h (curve 9). The measurements were always made after cooling to 3 °C.

connected to a Radiometer M26 pH meter. The changes in pH were recorded on a Hitachi QPD_{34} recorder with an attached amplifier made by Tarou Oguruso of Kyoto University. The sample was irradiated with blue light (400–530 nm). Light intensity measured by a thermopile (Kipp & Zonen, type CA1) at the place of the surface of the sample tube was 4×10^4 ergs·cm^{-2}·s^{-1}. The pH change was calibrated by adding a known amount of HCl.

Results

Absorption Spectrum of all-trans-Phenyl-bR. Upon addition of *all-trans*-[phenyl]retinal (Figure 1b) to apomembrane suspensions at 0 °C, a complex spectrum was produced with a peak around 480 nm and two shoulders around 440 and 520 nm (curve 1 in Figure 2). The intensities increased gradually during subsequent incubation for 160 min at 3 °C (curves 2–4 in Figure 2). In order to accelerate the reaction, the temperature was increased to 20 °C. During incubation at 20 °C, the spectrum shifted to longer wavelengths with an increase of the absorption around 520 nm at the expense of the absorbance below 480 nm. The spectra presented in Figure 2 (curves 5–9) were measured at 3 °C after the additional incubation at 20 °C. The absorption band around 520 nm increased slightly upon cooling of the sample to 3 °C but reverted when the temperature was returned to 20 °C. These spectra seemed to be composed of two absorption bands having λ_{max} around 480 and 520 nm, respectively. Prolonged incubation for 16 (curve 8 in Figure 2) and 40 h (curve 9 in Figure 2) resulted in large increases of the absorbance around 520 nm, while leaving the shoulder around 480 nm.

These spectral shapes are somewhat different from the smoothly shaped spectra presented by Bayley et al. (1981). We could not obtain a complete conversion to the state of a single absorption band by duplicating their experimental conditions (incubation at pH 8.0 in the absence of glycerol) or by conducting the reaction at 20 °C without intermittent cooling. It is probable that the reaction with [phenyl]retinal, which requires a long duration in our experiments, is somewhat dependent on the apomembrane preparation used. With respect to the time dependence of the initial absorbance changes, our preparation gave the same results as those presented by Bayley et al. (1981) upon mixing with either *all-trans*-retinal or *all-trans*-[phenyl]retinal. The spectral changes after 5 min, however, were not described by the previous authors.

Two different values of λ_{max} have previously been obtained for phenyl-bR. A value of 510 nm reported by Bayley et al. (1981) is close to that of our longer wavelength value. The 480-nm value given by Motto et al. (1980), which was obtained after incubation for 1 h, is rather similar to the λ_{max} of the prevailing species of phenyl-bR at the early stage of incubation at 3 °C. Coexistence of these two species has not been reported previously.

During the course of regeneration of purple membrane at low temperatures, Schreckenbach et al. (1977) detected an intermediate with two maxima at 430 and 460 nm. This 430/460 nm complex, which converted directly to the final purple membrane, seems to correspond to the precursor species that was formed initially in our experiments.

pH-Dependent Spectral Changes in all-trans-Phenyl-bR. At strong alkaline pH, the spectrum of purple membrane shifts to shorter wavelengths with an accompanying deprotonation from the Schiff base (Druckmann et al., 1982). Thus, a species absorbing around 480 nm is expected to be produced from *all-trans*-phenyl-bR at alkaline pH.

The pH-dependent spectral changes in *all-trans*-phenyl-bR that was prepared by incubation for 3 h at 20 °C were examined at 3 °C by increasing pH with the successive addition of small volumes of NaOH solution (Figure 3). A drop of absorption around 520 nm was accompanied by an increase of the absorption band around 470 nm. The midpoint for the conversion was about pH 10. Neutralization of the sample at pH 12.0 by HCl did not completely regenerate the original spectrum if the measurement was made after an 80-min incubation at 3 °C. The original spectrum was regained after a 60-min incubation at 20 °C (not shown in figure). Thus, the species produced by the deprotonation at alkaline pH is very similar to the precursor species observed during incubation at neutral pH.

A similar alkali-induced conversion with a midpoint at pH 8.1 was described by Bayley et al. (1981) for phenyl-bR in 0.1 M NaCl. The presently observed conversion at pH 10 in the absence of NaCl was not seen by Bayley et al. (1981), since their experiments were carried out at pH below 9.5.

The spectrum was unchanged over a wide range of pH from 6.6 to 8.1 (curves 1–3 in Figure 3). Further decreases of the pH to 4 also left the spectrum unchanged (data not shown). These results indicate that the species of *all-trans*-phenyl-bR with the absorption band around 480 nm could not be converted to the 520-nm species by simply increasing the proton concentration.

Effect of Blue Light on all-trans-Phenyl-bR. In contrast to the case of purple membrane, the retinals extracted from the *all-trans*-phenyl-bR produced after a 3-h incubation at 20 °C (solid line in Figure 4) remained unisomerized in the darkness, as shown by the HPLC pattern in the upper part of the inset in Figure 4. When the *all-trans*-phenyl-bR thus produced was irradiated with blue light at 0 °C (in ice-cold water), a single absorption peak around 505 nm appeared with accompanying decreases of the absorbance on both the shorter and the longer wavelength sides (dashed line in Figure 4). In

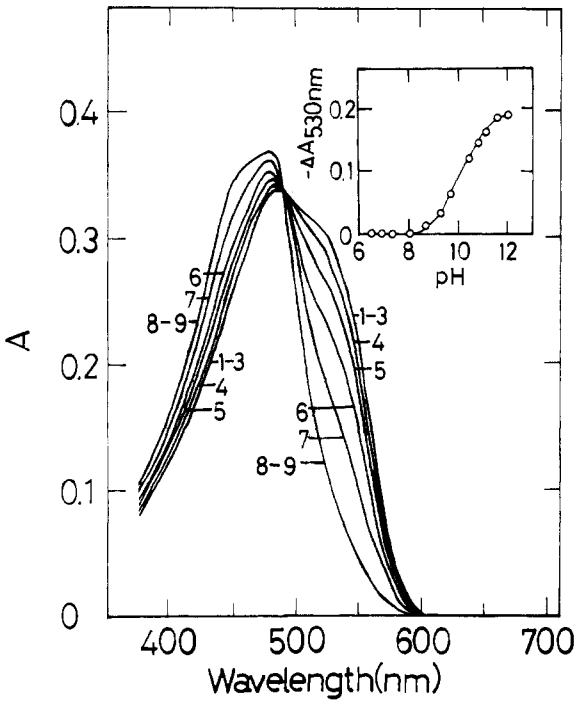

FIGURE 3: pH-dependent changes in the absorption spectra of the *all-trans*-phenyl-bR that was prepared by incubation of apomembranes (2.5 mL containing 1.2×10^{-5} M bacterioopsin) with *all-trans*-[phenyl]retinal (20 μL, A_{390nm} = 54) for 3 h at 20 °C. The sample was cooled to 3 °C in order to suppress further spectral changes during the titration. Small amounts of NaOH were added successively, and the pH was determined before measurements of the absorption spectrum. Curves 1–9 were the spectra at pH 6.6, pH 7.4, pH 8.1, pH 8.7, pH 9.3, pH 9.8, pH 10.5, pH 11.6, and pH 12.0, respectively. In the figures inserted, the decrease of extinction at 530 nm from the value at pH 6.6 is plotted against pH. Points not shown in the spectral curves are included.

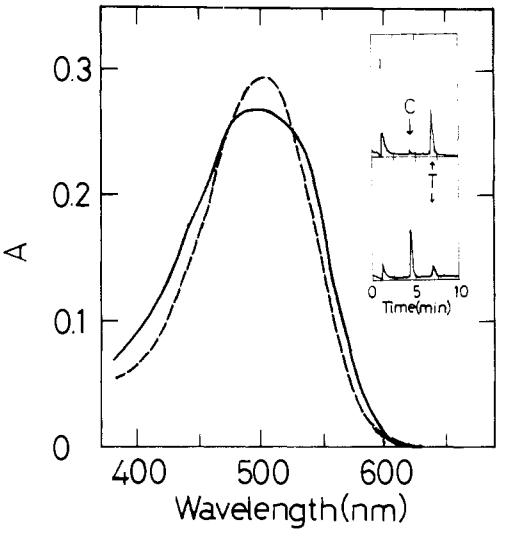

FIGURE 4: Blue light induced conversion of the *all-trans*-phenyl-bR. Apopurple membrane suspensions containing 1.0×10^{-5} M bacterioopsin, 1.0 mL, were mixed with *all-trans*-[phenyl]retinal (5 μL of the ethanolic solution, A_{390nm} = 70) at 3 °C. After 80 min, the temperature was increased to 20 °C. The spectrum after 3 h was recorded at 3 °C (solid line). The sample was divided into two parts. The one was irradiated with blue light for 10 min in ice-cold water, followed by the spectral measurement at 3 °C (dashed line). Consecutive 10-min irradiation did not result in further spectral changes. Insets show the HPLC patterns of the retinals extracted from both the unirradiated sample for incubation for 3 h at 20 °C (upper part) and the irradiated sample (lower part). The vertical scale in the inset represents an absorbance of 0.001 at 360 nm, and "C" and "T" show 13-cis and all-trans isomers.

other words, blue light induced a red shift of the absorption band in the shorter wavelength region along with a blue shift of the other band in the longer wavelength region. The chromophore of the photostationary state products was largely isomerized to the 13-cis form, as shown in the lower part of the inset in Figure 4. A similar spectral change was obtained by irradiating with light at 530 nm.

A blue shift with the concomitant isomerization to the 13-cis form is observed during the dark adaptation in purple membrane (Stoeckenius et al., 1979) or during the light-dependent reaction of α-retinal complexed with apomembrane (Towner et al., 1980). Thus, the blue light dependent red shift may not be a consequence of the isomerization of the chromophore.

Figure 5 shows that irradiation with blue light induced changes in the pH of the membrane suspensions at 20 °C. Under these conditions, the spectra measured before and after the irradiation were similar to those obtained at 3 °C (see Figure 4), except for lower absorbances over a range of wavelengths above 480 nm. This is due to the slow reaction at higher pH (pH 7.9) and the decrease of the intensity around 520 nm caused by high temperature (20 °C). When the blue light was turned on, the membrane suspensions containing the *all-trans*-[phenyl]retinal bound protons from the medium (Figure 5a). The proton uptake terminated in about 2 min, when the spectral changes were almost completed. All the protons remained bound even after cessation of irradiation. No further binding of protons was observed after subsequent reirradiation with blue light. Such a light-dependent fixation of protons to the membranes did not occur with 13-*cis*-phenyl-bR (Figure 5b), or without added retinals (Figure 5c). Fixation of protons to the membranes has been reported by

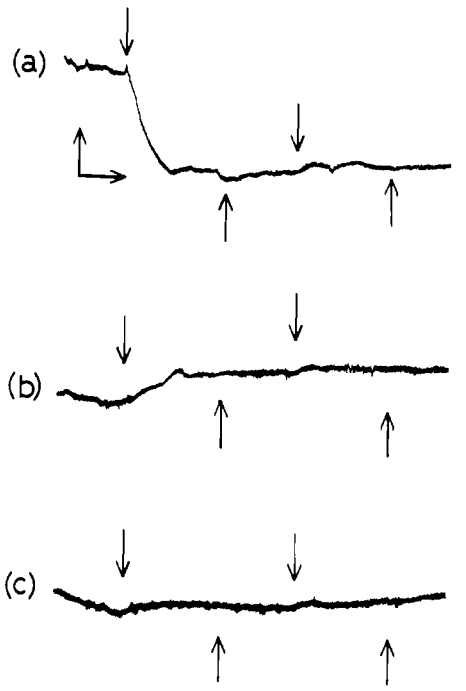

FIGURE 5: Light-dependent pH changes with the membrane constructed (a) with *all-trans*-[phenyl]retinal, (b) with 13-*cis*-[phenyl]retinal, and (c) without added retinals. The initial pH value was 7.9. Arrows pointed downward represent the time for the start of irradiation with blue light (400–530 nm), and those pointed upward represent the time for cessation of irradiation. The vertical bar represents a scale of pH changes brought about by the addition of 2×10^{-9} mol of HCl, and the horizontal bar shows a scale for 2 min. pH values increased downward. Details were explained under Materials and Methods.

Fischer & Oesterhelt (1980) during a light-dependent process in the formation of purple membrane from 9-*cis*-retinal with apopurple membranes.

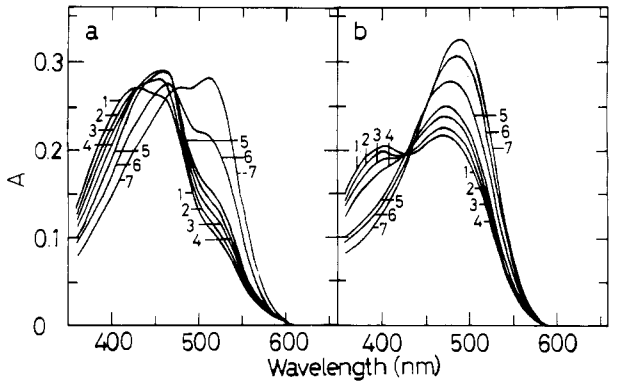

FIGURE 6: Changes in the absorption spectra during the course for binding of (a) *all-trans*-[2-chloro-6-fluorophenyl]retinal and (b) 13-*cis*-[2-chloro-6-fluorophenyl]retinal. To 0.4 mL of apomembrane suspensions (1×10^{-5} M) was added an ethanolic solution of isomers (3 μL, A_{370nm} = 73 and 61 for all-trans and 13-cis isomers, respectively) at 3 °C. The sample was first incubated at 3 °C. Curves 1–5 show the spectra at 20, 40, 80, 160, and 320 min, respectively. The temperature was then increased to 20 °C, and the spectra were recorded at 3 °C after 30 min (curve 6) and 16 h (curve 7).

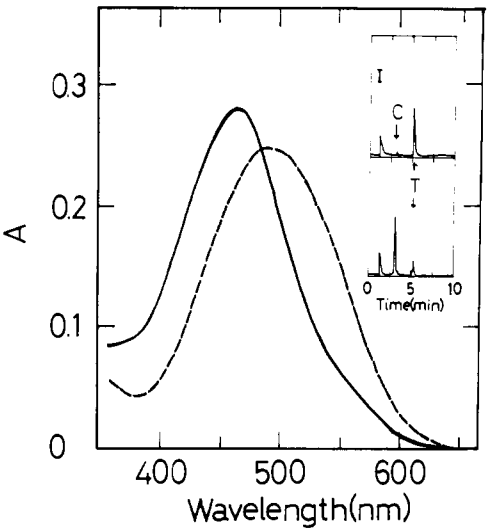

FIGURE 7: Absorption spectra of mesityl-bR. To 0.8 mL of suspensions of apopurple membranes (1.0×10^{-5} M) was added on ethanolic solution of *all-trans*-[mesityl]retinal (2 μL, A_{370nm} = 84). The mixture was incubated for 3 h at 20 °C in the dark, followed by spectral measurements at 3 °C (solid line). The sample was divided into two parts. The one was irradiated with blue light for 20 min at 0 °C, and the spectrum was measured at 3 °C (dashed line). The other was kept in the darkness at 3 °C. The inset shows the HPLC patterns of the retinals extracted from the unirradiated sample (upper part) and from the irradiated sample (lower part). The vertical bar in the insets represents a scale for absorbance of 0.001 at 360 nm, and "C" and "T" show 13-cis and all-trans isomers.

It is well-known that irradiation of purple membrane sheets releases protons [see a review of Stoeckenius & Bogomolni (1982)]. As is evident from Figure 5, irradiation with blue light did not release protons from the membrane of phenyl-bR. Since irradiation with blue light induced a large extent of isomerization of the all-trans chromophore to the 13-cis form, only a minor fraction should be capable of the proton pumping activity that is inherent in the all-trans isomer of purple membranes. Although phenyl-bR pumps protons across the membrane, the extent is much less than that with purple membranes (Bayley et al., 1981).

Light dependent fixation of protons most probably occurs through the binding of protons by the shorter wavelength species of all-*trans* phenyl bR, with an accompanying isomerization of the chromophore to the 13-cis form, which has a λ_{max} between those of the two all-trans species of phenyl bR. Whether the site responsible for the proton uptake in this case is the same or not with the site for the deprotonation during the titration (see Fig. 3) is presently unknown.

Comparison of the Spectral Shapes of Various Aromatic bR's. We also studied the spectral properties of the chromophore constructed with other aromatic retinal analogues shown in Figure 1. Figure 6a shows the reaction of *all-trans*-[2-chloro-6-fluorophenyl]retinal (Figure 1e) with apomembranes. The absorption band around 460 nm was increased with an isobestic point around 430 nm during incubation at 3 °C (curves 1–4 in Figure 6a). On further incubation at 20 °C, the species with λ_{max} around 460 nm was converted into a species absorbing around 510 nm (curves 5–7 in Figure 6a). Two absorption bands remained even after a 16-h incubation at 20 °C (curve 7 in Figure 6a). Such a pocess is quite similar to that observed with *all-trans*-phenyl-bR (Figure 2), and it is likely that the 460-nm species with a shoulder around 430 nm corresponds to the 430/460 nm complex in the case of purple membrane (Schreckenbach et al., 1977). A similar broad spectral shape was obtained with *all-trans*-[*o*-tolyl]retinal (Figure 1c) (the spectrum is not shown). Thus, two absorption bands were observed also when either a halogen or methyl substituent was present at the ortho position to the polyene side chain in all-*trans* aromatic retinal. As shown for *all-trans*-phenyl-bR (Figures 4 and 5), isomerization to the 13-cis form and irreversible binding of protons also occurred upon irradiation of these artifical pigments (not shown in figures).

The reaction with 13-*cis*-[2-chloro-6-fluorophenyl]retinal is shown in Figure 6b. In contrast to the case of all-trans chromophore, absorption bands that appeared in the shorter wavelength region (curves 1–4 in Figure 6b) were converted into a single absorption band after incubation at 20 °C (curves 6–7 in Figure 6b). The λ_{max} in the spectrum after a 16-h incubation at 20 °C (curve 7 in Figure 6b) appeared around 490 nm. This is between the two shoulders obtained with *all-trans*-(2-chloro-6-fluorophenyl)-bR, which were located around 470 and 510 nm (curve 7 in Figure 6a). About 95% of the chromophore retinal remained in the 13-cis form. The 13-cis forms of [phenyl]retinal and [*o*-tolyl]retinal also gave similar smooth spectral shapes, whose λ_{max} values were invariably located between the two shoulders obtained with the corresponding all-trans chromophores. Expectedly, the spectrum of 13-*cis*-phenyl-bR (not shown) quite resembled that of the irradiated products of the all-trans chromophore (curve 3 in Figure 2), and no striking spectral changes occurred upon irradiation with blue light. In this respect, it is consistent that no protons were taken up during irradiation of 13-*cis*-phenyl-bR (Figure 5b).

all-trans-[Mesityl]retinal (Figure 1d) showed an apparently different spectral shape. The increases of the absorption intensity in the longer wavelength region proceeded very slowly. As a result, the spectrum showed a main absorption peak around 460 nm in addition to an absorption tail over a range of wavelengths greater than 500 nm (Figure 7). The spectrum remained unchanged even after a 16-h incubation at 20 °C. The chromophoric retinals extracted after 3 h were composed almost completely of *all-trans*-[mesityl]retinal (the upper part of the inset in Figure 7). Upon irradiation of this product with blue light, the absorption maximum shifted to the red, and the chromophore largely isomerized to the 13-cis form (the lower part of the inset in Figure 7). These facts are consistent with the findings on phenyl-bR, if one assumes that the tail absorption of the mesityl-bR before irradiation is due to the absorption band corresponding to that of *all-trans*-phenyl-bR

Table I: Spectral Properties of All-Trans Aromatic Retinal Complexes

	[phenyl]-retinal	[*o*-tolyl]-retinal	[mesityl]-retinal	[2-chloro-6-fluorophenyl]-retinal
(a) λ_{max} (nm) of the absolute spectrum of the protonated Schiff base in acidic methanol	452	453	442	434
(b) λ_{max} (nm) of the difference spectrum of (3 h at 20 °C) minus (80 min at 3 °C)[a]	530	540	535	520
(a) − (b) (cm^{-1})	3250	3560	3930	3810

[a] See the text for details.

in the longer wavelength region. *all-trans*-[Piperonyl]retinal (Figure 1f), which has bulkier substituents at para and meta positions of the ring, also showed a spectral shape similar to that of *all-trans*-mesityl-bR (not shown).

These results indicate that the complete conversion to the species having the absorption band in the longer wavelength region was restricted generally in the all-trans aromatic bR, and this sort of restriction was especially severe in the case of the retinal analogue having a bulky substituent at the para or meta positions. The isomerization of the chromophore to the 13-cis form could relieve the chromophore from the restriction by the decrease of the length from the Schiff base nitrogen to the ring. A similar concept that the allowable longitudinal length of retinal is restricted in the binding site has previously been proposed by Matsumoto & Yoshizawa (1978) for explaining the ability of various isomers to bind with bovine opsin.

Comparison of the Absorption Bands in the Longer Wavelength Region. The λ_{max} values of the all-trans aromatic bR's could not be determined precisely because of their broad absorption spectra. As stated above, the absorption band in the longer wavelength region became greater after the increase of temperature for incubation from 3 to 20 °C. The difference between the spectra after and before warming to 20 °C must reflect the formation of the final red-shifted all-trans product. The difference spectra obtained in such ways with *all-trans*-(2-chloro-6-fluorophenyl)-bR (curve 6 or 7 minus either of curves 1–5 in Figure 6a) exhibited a λ_{max} value of about 520 nm (±2 nm). Table I shows the λ_{max} values of the difference spectra obtained by subtracting the spectrum after 80 min at 3 °C from that after subsequent incubation for 3 h at 20 °C for each all-trans aromatic retinal.

In order to normalize the extent of the spectral red shift on the different retinal analogues bound to the apomembranes, Nakanishi et al. (1980) have proposed a term of "opsin shift", the difference in wavenumber (cm^{-1}) between the λ_{max} value after binding to the apomembrane and that of the protonated Schiff base in solution. Table I also includes the λ_{max} values of the retinylidene–butylamine complex in acidic methanol (protonated Schiff base). The extent of the spectral shift was compared for different analogues of aromatic retinal from the value of the difference between the λ_{max} of the protonated Schiff base and that of the difference spectrum obtained as described above. These values (±70 cm^{-1} from the expected error mentioned above) increased in the order of [phenyl]-retinal < [*o*-tolyl]retinal < [2-chloro-6-fluorophenyl]retinal < [mesityl]retinal.

A similar comparison was made by using the wavelengths of the longer wavelength shoulders in the spectra having similar spectral shapes. These values were 520 nm for phenyl-bR (Figure 2), 530 nm for *o*-tolyl-bR (not shown), and 510 nm for (2-chloro-6-fluorophenyl)-bR (Figure 6a). Respective values of opsin shift were then calculated to be 2890, 3210, and 3430 cm^{-1}. The corresponding value for mesityl-bR could not be determined. These two lines of evidence both indicate that the spectral shift is smallest with unsubstituted phenyl-bR and is increased by the introduction of methyl or halogen substituents on the ring.

[Naphthyl]retinal, having a bulkier group around the ring, showed a larger extent of the shift, 3690 cm^{-1} (Ahktar et al., 1982), upon binding with apomembranes. Nevertheless, all of these values of opsin shift are smaller than the 4870-cm^{-1} value with the natural retinal (Motto et al., 1980), whose size of the ring is greater than that of the aromatic ring. All these results support the view that the presence of the bulky groups, especially those close to the polyene side chain, seems to be important in order to develop the interactions of the chromophore in the retinal binding site.

Discussion

The absorption spectra of all-trans aromatic retinals changed gradually after the binding of the retinals to apopurple membranes. All the all-trans aromatic bR showed two absorption bands. These may arise from two species, which differ in protonation state. The site for the protonation is presently unknown. The Schiff base is a possible site for the protonation, since the Schiff base in the alkaline form of purple membrane, which is similar to the shorter wavelength species of aromatic bR, has been shown to be in an unprotonated state by resonance Raman spectroscopy (Druckmann et al., 1981). Similar experirments with resonance Raman spectroscopy as well as with relevant chemical modifications are required for the aromatic bR's.

The absorption band in the shorter wavelength region was larger in the spectrum of analogues having substituents at the para of meta positions in the ring. Bulky substituents at these positions may cause steric hindrance on the chromophore in the retinal binding site. The low λ_{max} values previously observed with the aromatic analogue of bR with substituents at these positions, (4-methoxy-2,3,6-trimethylphenyl)-bR (Marcus et al., 1977) and (*m*-diazirinophenyl)-bR (Huang et al., 1982), might result from the absorption bands that correspond to those in the shorter wavelength region. On the other hand, the featureless spectrum with the 13-cis chromophore could be due to a decrease of the steric hindrance by isomerization to the 13-cis form, whose length from the Schiff base nitrogen to the ring is smaller than that of the all-trans form. The absorption band of the all-trans aromatic bR in the shorter wavelength region thus could be related to steric hindrance in the retinal binding site.

The value of the opsin shift [defined by Nakanishi et al. (1980)] of the absorption band in the longer wavelength region increased with the size of the ring, dependently on the number of the substituents at the ortho position in the ring. Therefore, steric interactions in the vicinity of the side chain seem to be important in determining the spectral red shift of the longer wavelength species. The largest opsin shift obtained in the present paper was 70% of that with natural retinal. A larger value of 75% has been reported for naphthyl-bR (Ahktar et al., 1982), in which the aromatic ring extends to the side chain.

Such an incomplete shift with aromatic bR could be due to a lack of contact of the chromophore with the protein. But a possibility that a distinct electronic property of the aromatic ring may also affect the spectrum cannot be eliminated completely.

In spite of these notions, the least substituted *all-trans*-[phenyl]retinal still gave two absorption bands even after prolonged incubation. The two bands may be due to the presence of both the protonated species and the unprotonated one. These two species could be present even at neutral pH in a situation where a subtle change in the steric interaction with the chromophore is accompanied by a geometrical alteration of the charged residues interacting with the Schiff base proton. The presence of two tautomeric chromophores was postulated by Fischer & Oesterhelt (1980) for purple membrane at alkaline pH. In this connection, it is interesting that purple membranes allow the presence of both all-trans and 13-cis chromophores in the dark. Another possibility, that a single species has two maxima, has not been eliminated completely. Further studies, however, will have to be done in order to examine this point.

As has been proposed by Nakanishi et al. (1980), the electrical interactions between the chromophore and bacterioopsin are critical in determining the spectral properties of bR. We think that the electrical interactions are perturbed by a steric incompatibility between the chromophore and bacterioopsin in the case of aromatic bR. The present paper gives some specification of the steric factor.

Similar absorption bands in the shorter wavelength region have been observed with purple membrane at strong alkaline pH (Druckmann et al., 1982) and with the nitrated membrane at moderately alkaline pH (Lemke & Oesterhelt, 1981). All-trans aromatic bR shows such an absorption band at neutral pH. In this respect, the results presented on aromatic retinal analogues will be valuable for the analysis of the unprotonated state of purple membrane, if used in parallel with the sample with the natural chromophore. The results also give some guide for experiments with aromatic bR with various substituents in the ring.

Acknowledgments

We are indebted to Marlene Denny for her technical assistance.

Registry No. [Phenyl]retinal, 75001-15-9; [*o*-tolyl]retinal, 75001-16-0; [mesityl]retinal, 75001-17-1; [2-chloro-6-fluorophenyl]retinal, 75001-18-2.

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