Color Regulation in the Archaebacterial Phototaxis Receptor Phoborhodopsin (Sensory Rhodopsin II)†

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ABSTRACT: Phoborhodopsin, a repellent phototaxis receptor in *Halobacterium halobium*, exhibits vibrational fine structure, a feature that has not been identified for any other rhodopsin pigment at physiological temperatures. This conclusion follows from analysis of the absorption properties of the pigment in *H. halobium* membranes containing native retinal and an array of retinal analogues. The absorption spectrum of the native pigment has a maximum at 487 nm with a pronounced shoulder at 460 nm; however, the bandwidth is that expected for a single retinylidene species. Gaussian band-shape simulation with a spacing corresponding to the vibrational frequencies of polyene stretching modes reproduces the structured absorption spectra of native pigment as well as of analogue phoborhodopsin. Absorption shifts produced by a series of dihydroretinal and other retinal analogues strongly indicate that the dominant factor regulating the color of the pigment is planarization of the retinal ring with respect to the polyene chain.

Retinal (vitamin A aldehyde) is widely used as the chromophore of photosensory pigments from microorganisms (Spudich & Bogomolni, 1984; Foster et al., 1984) to mammals (Applebury & Hargrave, 1986). Retinylidene chromoproteins (rhodopsins) are commonly found as intrinsic membrane proteins composed of seven α-helices which surround the chromophore (Applebury & Hargrave, 1986). Phenomenologically, a key feature responsible for the wide distribution of retinal in nature is its ability to undergo alteration of its absorption properties. The chromophore retinylidene imino group (Figure 1), via protonation and further interaction with an apoprotein, regulates its absorption maximum over a wide range [345–610 nm (Schwemer & Langer, 1982)], giving organisms the ability to detect color (Spudich & Bogomolni, 1984; Nathans, 1987).

To attain maximal sensitivity, organisms tune the absorption maxima (λ_{max}) of their photoreceptors to the wavelength at which the radiation energy incident to their habitat is maximal (Lythgoe, 1984; Yoshizawa & Kandori, 1989). The spectrum of solar radiation passed through our atmosphere is maximal near 490 nm, and our sensitive dim-light visual system uses the scoptic visual pigment rhodopsin (λ_{max} = 498 nm) in rod outer segments. Phototropism, photomovement, and other photosensitive functions in plants, algae, and fungi are controlled by "blue-light photoreceptors" which, though most of them are yet unidentified, show action maxima at 440–500 nm (Ninnemann, 1980; Senger, 1981, 1987). The archaebacterial species *Halobacterium halobium* uses the retinylidene phototaxis receptor phoborhodopsin [pR (Takahashi et al., 1985b; Tomioka et al., 1986); λ_{max} = 487 nm, this study; also called sensory rhodopsin II (Spudich, E. N., et al., 1986) or P480 (Marwan & Oesterhelt, 1987)] to avoid solar radiation, potentially damaging during aerobic growth.

The evolutionary relationship between archaebacterial rhodopsins and our visual pigments is not known, and it might be difficult to clarify because of the large phylogenic distance. A possible hypothesis is that a progenitor photopigment gene first evolved in unicellular organisms (Martin et al., 1986), which may or may not have been ancestors of archaebacterial species. Though no apparent amino acid sequence homology was observed between bacteriorhodopsin (bR) and bovine rhodopsin (Ovchinnikov, 1982), it is natural from the structural similarity to assume a common ancestor for both archaebacterial rhodopsins and visual pigments (Doolittle, 1989). In any case it is worthwhile to compare functionally important molecular architecture between rhodopsins from different origins. Recently, several groups have analyzed the molecular mechanisms of color regulation of visual pigments (Loppnow et al., 1989; Koutalos et al., 1989; Sakmar et al., 1989) and archaebacterial pigments [bacteriorhodopsin, halorhodopsin, and sensory rhodopsin I (Spudich, J. L., et al., 1986; Lanyi et al., 1988; Fodor et al., 1989)]. It is especially interesting to compare the mechanism in pR with that of visual pigments, because the λ_{max} of pR is close to that of rhodopsin in rod outer segments [e.g., 500 and 475 nm for bovine and octopus rhodopsins, respectively (Koutalos et al., 1989)]. To this end, we have studied pR absorption properties by incorporating a variety of retinal analogues into apomembrane suspensions prepared from a pR-rich mutant (Flx3b) lacking apoproteins of any other bacterial rhodopsins (Takahashi et al., 1988; Spudich et al., 1989). Differences in the mechanisms of color regulation in pR from that of visual pigments explain well the structured band shapes of the absorption and action spectra of pR (Takahashi et al., 1987), unusual among known retinylidene proteins.

Materials and Methods

Chemicals. all-trans-Retinal was purchased from Sigma Chemical Co. (St. Louis, MO) and 3,4-dehydroretinal from Eastman Kodak. Dihydroretinal analogues were synthesized

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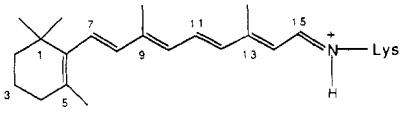


FIGURE 1: Structure of the protonated retinylidene chromophore, covalently linked via a Schiff base to a lysine residue of the protein.

as described in Arnaboldi et al. (1979), "C-18 ketone" (β-ionylid-3-en-2-one) as in Towner et al. (1981), naphthylretinal as in Iwasa et al. (1984), *ring*-demethylretinal analogues as in Courtin et al. (1987), and acyclic retinal analogues as in Jayathirtha Rao et al. (1987). Retinal and retinal analogues were purified by high-performance liquid chromatography (10 × 250 mm μ-Porosil column) eluted with 8% ethyl acetate in hexane at a flow rate of 3.0 mL/min. Solvent was removed with an argon stream and the retinal or analogue dissolved in absolute ethanol before addition to membrane preparations.

Strains and Culture Conditions. Strains Flx3b and Flx5R are bR^-hR^- mutants (Spudich & Spudich, 1982a). Flx3b is $sR\text{-}I^-sR\text{-}II(pR)^+$. Flx5R is retinal deficient and produces no detectable amount of pR apoprotein (Spudich, E. N., et al., 1986). Flx3b and retinal-regenerated Flx5R contain relatively large amounts of pR (Takahashi et al., 1989) and sR-I, respectively. JW10, a retinal-deficient mutant that generates bR as its predominant pigment upon retinal addition, was provided by R. A. Bogomolni (University of California, San Francisco). Flx5R and JW10 were grown to early stationary phase as described (Spudich & Spudich, 1982b). Since the cellular content of pR is maximal in exponential phase (Tomioka et al., 1986), cultures of Flx3b were grown under extensive aeration and harvested at 2×10^8 cells/mL after cooling (4 °C). Retinal is undetectable in Flx3b under these conditions, and 1–2 mM nicotine was added to further ensure retinal-deficient Flx3b cells.

Preparation of Apomembranes. Cells from 4 L of culture were pelleted by centrifugation (10000*g*, 15 min, 4 °C), washed, repelleted, and resuspended with 20 mL of ice-cold basal salts. The suspension was rapidly frozen with liquid nitrogen. DNase (Sigma Chemical Co.) was added at 1–2 mg/10 mL to the slowly thawing cell suspension and the mixture stirred for 2 h at room temperature. The membrane fraction was collected by centrifugation (100000*g*, 2 h, 4 °C) and washed with 4 M NaCl by repeated centrifugations (100000*g*, 30 min, 4 °C) until the supernatant became clear. The final pellet was resuspended in 10 mL of 4 M NaCl–25 mM HEPES, pH 7.0.

Absorption Spectroscopy. Spectra were recorded on an SLM-Aminco DW2000 spectrophotometer (SLM Instruments, Inc., Urbana, IL). Native membrane suspensions (3–10 mg of protein/mL) were degassed under vacuum for 15 min prior to transfer to a pair of cuvettes (10-mm path length). The cuvettes were sealed with Parafilm and equilibrated for at least 1 h in the spectrophotometer sample housing, which was strictly maintained at 25 °C. A baseline spectrum was recorded of membrane suspension in both reference and sample cuvettes, and spectra were recorded at various times after retinal or analogue addition.

Results and Discussion

Vibrational Fine Structure

The absorption spectra obtained during regeneration of pR with *all-trans*-retinal (Figure 2) show a shoulder at 460 nm *in addition* to the main peak at 487 nm, though the isosbestic point at ~414 nm indicates formation of a single species. The pR spectrum is not like that of other retinylidene proteins, which exhibit simple bell shapes with half-bandwidths of

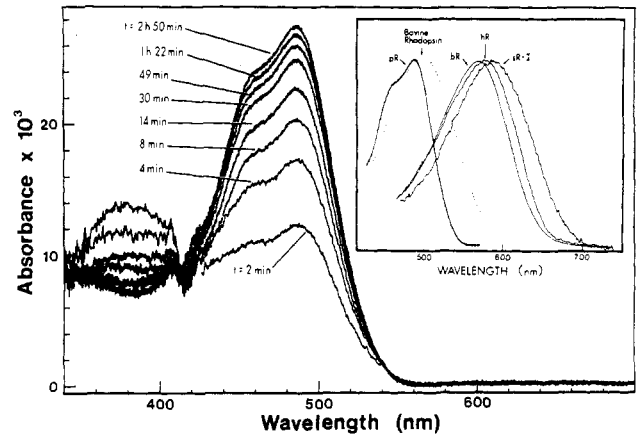


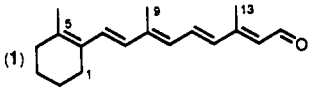
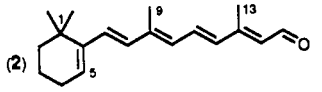
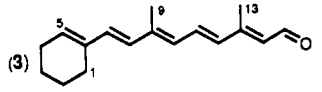
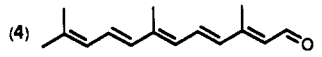
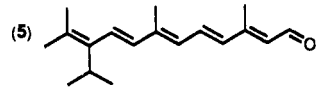
FIGURE 2: Formation of pR from *all-trans*-retinal and the apoprotein. To a 2.7-mL suspension of the membrane fraction containing 9 mg of protein/mL prepared from Flx3b cells grown in the presence of 2 mM nicotine (apomembrane preparation) was added 1.4 nmol of *all-trans*-retinal at $t = 0$ (pH 7, 25 °C). At the times indicated, absorption spectra were recorded by scanning at 0.5 nm/s (2 nm/s for the first two spectra) on an SLM-Aminco DW2000 spectrophotometer. The reference cuvette contained the same apomembrane preparation without retinal. Path length, 1 cm. The small peak at 408 nm and the dip at 417 nm are due to the perturbed spectrum of cytochromes abundant in the membrane preparation. Inset: Absorption spectra of bovine rhodopsin from Koutalos et al. (1989) and halobacterial rhodopsins bacteriorhodopsin (bR), halorhodopsin (hR), and sensory rhodopsin I (sR-I) from Spudich and Bogomolni (1983).

4000–5000 cm^{-1} at room temperature (Dartnall, 1953; Ebrey & Honig, 1977). For comparison, the absorption spectra of other bacterial rhodopsins (Spudich & Bogomolni, 1983) and bovine rhodopsin (Koutalos et al., 1989) are illustrated in the inset of Figure 2.

Polyenes similar to retinal in the length of their double bond systems frequently show a progression of vibronic transitions, and a spacing of 1450 cm^{-1} is characteristic (Stern & Timmons, 1970). However, such vibrational fine structure has not been detected for retinal in solution nor for any retinylidene protein in its native state. Consequently, a broad unstructured spectrum has become an expected feature of absorption and action spectra from retinylidene pigments. A significant finding from the present study is that the unusual band shape of pR is due to vibrational fine structure. This result implies that band-shape analysis based on vibrational spectroscopy rather than the conventional nomogram, which assumes the broad unstructured absorption, becomes more appropriate to test for the involvement of retinylidene proteins by action spectroscopy.

Evidence for Structured Absorption of pR. The narrow 3500-cm^{-1} bandwidth of the absorption generated after retinal addition together with the isosbestic point argues that the double peak derives from a single pigment rather than from multiple species (Figure 2). Furthermore, heterogeneity in the configuration of the chromophore is unlikely because no detectable retinal isomerization occurred during the period of reconstitution in Figure 2 with *all-trans*-retinal as judged by extraction of the chromophore. Heterogeneity of the apoprotein or perturbation of the spectrum by other membrane-bound pigments is improbable because preparations from different strains differing in pigment content or in the amount of carotenoids consistently show two peaks at 460 and 487 nm with an absorption ratio similar to that in Figure 2. Furthermore, similar double peaks are observable in the blue-green region of the flash-induced absorption difference spectrum and the phototactic action spectrum (Takahashi et al., 1987), which strongly support that both peaks belong to pR.

Table I: Absorption Maxima and Opsin Shifts of *ring*-Demethylretinal and Acyclic Retinal Analogues in pR

chromophore[a]	absorption max (nm) aldehyde	absorption max (nm) PSB^+	λ_{max} in pR	opsin shift (cm^{-1})
(1) 	404	476	480	180
(2) 	390	458	475	780
(3) 	394	465	468	140
(4) 	400	465	474	410
(5) 	382	445	485	1850

[a] **1**, 1,1-didemethylretinal; **2**, 5-demethylretinal; **3**, 1,1,5-tridemethylretinal; **4**, 3,7,11-trimethyldodeca-2,4,6,8,10-pentaenal; **5**, 3,7,11-trimethyl-10-isopropyldodeca-2,4,6,8,10-pentaenal.

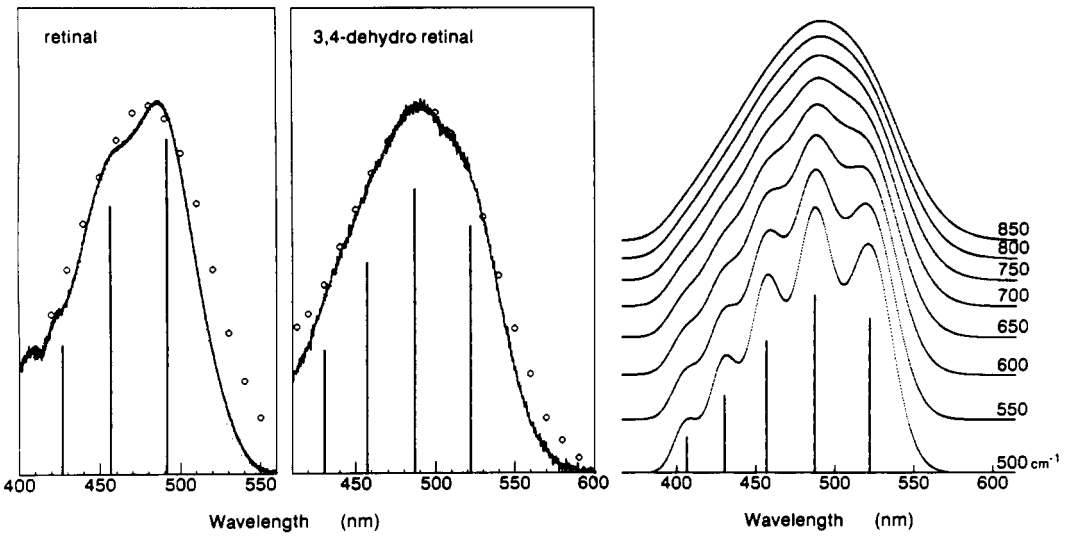


FIGURE 3: Band-shape analysis of pR and the analogue pR formed from 3,4-dehydroretinal. Right: Variation of the band envelope with changes in intrinsic band widths of vibronic components. Band widths (half-width at $1/e$ of maximum absorption) of the Gaussian components used are as indicated in cm^{-1}. Spacing and intensities are those for the 3,4-dehydroanalogue of pR (middle panel). Equal band width is assumed for all the components that form a band envelope. Left and middle panels: Comparison of the absorption spectra to predicted curves. The solid lines represent observed spectra (25 °C), circles show the expected absorption from the conventional nomogram for retinylidene proteins (Ebrey & Honig, 1977), and the dotted lines are calculated as the sum of Gaussian components having a standard deviation of 740 cm^{-1} for both pigments and spacing of 1550 and 1360 cm^{-1} for pR and the analogue pigment, respectively. Bars indicate the position and the intensity of each Gaussian component. Note the dotted line in the middle panel is nearly completely obscured by the empirical data because of the closeness of the fit. The overlap of the dotted lines with the observed spectra shows that both pigments fit the sum of Gaussian components with equal spacing and band width.

Retinal analogues with reduced lengths of their conjugated double bond systems exhibit simple single-peaked spectra when incorporated into the pR apoprotein (see below). Analogues with the same number of conjugated double bonds as native retinal (Table I) invariably exhibit shoulders with a consistent separation of $\sim$1500 cm^{-1} (data not shown). The pigment formed from 3,4-dehydroretinal (Figure 3, middle panel) shows the typical three-peaked spectrum usually observed in long-conjugated homopolyenes (Bentley & Kirby, 1972).

The three-peaked spectrum is also observed when C-18 ketone, which lacks two olefinic carbons from the aldehyde end of retinal, is bound to the apoprotein of pR (Figure 4). Free C-18 ketone exhibits a single broad absorption peak in solution.

Band-Shape Analysis of pR. The measured spectra of both native pR and its analogue formed from 3,4-dehydroretinal are compared with band envelopes calculated on the basis of vibronic transitions (Figure 3). Vibrational frequencies of polyene stretching modes are sufficient to reproduce the observed spectra. We have estimated the effect of Gaussian inhomogeneous broadening (Warshel & Karplus, 1974; Hemley & Kohler, 1977; Myers et al., 1983) on the calculated overall band shapes (Figure 3, right panel). Even a slight change in intrinsic bandwidth around the Gaussian standard deviation of 750 cm^{-1} is sufficient to cause the total loss of fine structure. The spectra of pR and of its 3,4-dehydro analogue fit well to the calculated envelopes with equal standard deviations of 740 cm^{-1} for each component (Figure 3, left panel). Note that both pigments fit the sum of Gaussian components with equal spacing and bandwidth. The parameters for band-shape analysis were arbitrarily chosen to obtain the best fit, in the absence of detailed information to allow calculation of Franck–Condon deltas and vibrational frequencies.

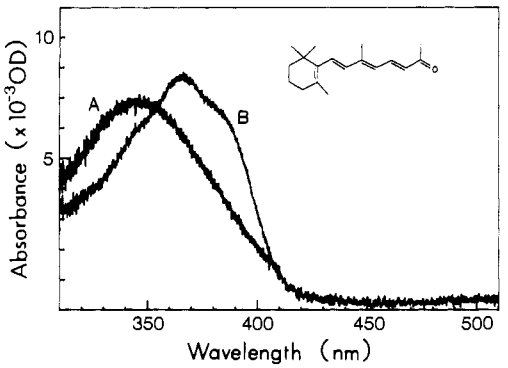


FIGURE 4: Binding of C-18 ketone to the apoprotein of pR in the membrane suspension. Spectrum B was recorded 30 min after addition of an equimolar concentration of the analogue (structural formula shown) to the apoprotein in the membrane suspension. Spectrum A is the difference between the spectrum with a 3-fold excess of C-18 ketone and the spectrum with a 2-fold excess, representing the absorption of the free chromophore. Temperature, 25 °C.

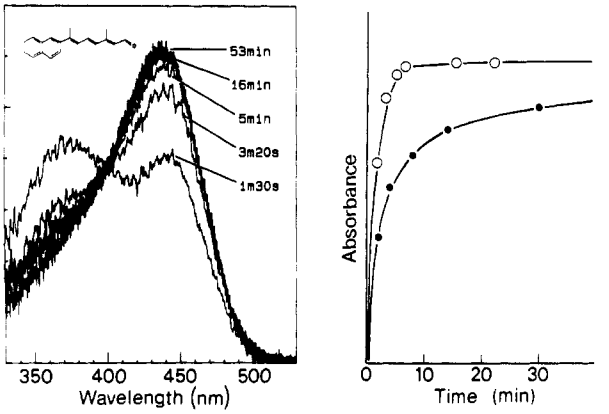


FIGURE 5: Formation of pR analogue from β-naphthylretinal in apomembrane suspension. Left: Absorption spectra recorded at the times indicated after addition of the retinal analogue. Each division on the ordinate equals an absorbance of 1×10^{-3}. Right: Time course of the reaction with the analogue (○) and natural retinal (●).

Mechanism of Wavelength Regulation

Conformation of the Retinal in pR. It is generally accepted that the major causes of spectral broadening in retinal and related compounds in solution are a distribution of vibronic activity among a large number of excited-state vibrational modes and/or a ground-state conformational heterogeneity, both of which arise from a distorted cis conformation around the C_6–C_7 single bond (Warshel & Karplus, 1974; Birge et al., 1982; Myers et al., 1983, 1986; Loppnow & Mathies, 1988) (Figure 1). Therefore, the development of fine structure of retinal-related compounds at the binding site can be used as an indication of a forced coplanar conformation around the C_6–C_7 bond, which was first proposed to be coplanar (Schreckenbach et al., 1977) in the archaebacterial light-driven proton pump bR and later shown to be coplanar 6-s-trans in bR by ^{13}C NMR (Harbison et al., 1985) and by analogue binding (van der Steen et al., 1986). Because this conformation implies better conjugation between the double bonds in the ring and the polyene chain, one expects a spectral shift to longer wavelengths (van der Steen et al., 1986). Both the fine structure and associated red-shift in the C-18 ketone bound to the apoprotein of pR (Figure 4) suggest that retinal in pR exhibits ring/chain coplanarity at the binding site. A planar conformation is expected to be 6-s-trans because steric hindrance argues against a planar 6-s-cis geometry.

Unlike bovine opsin (Matsumoto & Yoshizawa, 1975; Towner et al., 1981), pR apoprotein does not appreciably bind β-ionone. The rate of pR formation with *all-trans*-retinal is not influenced even in the presence of 500-fold molar excess of β-ionone to the apoprotein, indicating different geometry around the retinal binding pocket between the two rhodopsins (data not shown). Strong support for the ring/chain coplanarity in pR is the incorporation of "naphthylretinal" (3,7-dimethyl-2,4,6-heptatrienal) in which the bond corresponding to the C_6–C_7 bond in retinal is already fixed to form a planar conformation (Figure 5). The naphthylretinal pigment generates much faster than the native pR, as was reported in pigment formation from the apoprotein of bR and this analogue (Akhtar et al., 1982).

Opsin Shifts. In all known rhodopsins, retinal is linked via a protonated Schiff base to the ε-amino group of a lysine residue of the apoprotein (Lewis et al., 1973; Oseroff & Callender, 1974; Pande et al., 1987; Loppnow et al., 1989). The "opsin shift" is defined as the difference in the absorption maxima between a retinylidene pigment and the corresponding protonated Schiff base model compound (PSB^+) in standard conditions (i.e., in methanol in the presence of Cl^-). The value of the opsin shift depends on the conformational difference of the analogue in the pigment and in solution, as well as on other contributions of the protein to the absorption properties of the chromophore, and can be used as a measure of the specific protein–chromophore interaction that regulates the absorption wavelength (Honig et al., 1979; Nakanishi et al., 1980; Motto et al., 1980).

Theoretical calculations indicate that ring/polyene chain coplanarization would account for 800–1500 cm^{-1} of opsin shift (Honig et al., 1976; Kakitani et al., 1985). Since the distorted C_6–C_7 cis conformation of retinal in solution and in the crystal (Honig et al., 1971; Simmons et al., 1981) results from a steric constraint due to the C_1 and C_5 methyl groups and the C_8 proton, analogue chromophores without C_1 or C_5 methyl groups are likely to have a C_5=C_6-C_7=C_8 planar conformation in their PSB^+ in solution. Therefore, such analogues are expected to exhibit less opsin shift in pR than natural retinal, which produces 1950 cm^{-1}. Table I shows that several *ring*-demethylretinal and acyclic retinal analogues except **5**, which has the same steric hindrance as natural retinal, showed relatively small opsin shifts, thus providing further evidence for a coplanar conformation at the binding site of pR. The calculated value of opsin shifts in Table I may have errors of ±50 cm^{-1} due to the accuracy of measurement of the absorption peak, and altered ring conformations in the pigments and in solution may contribute to the measured opsin shifts produced by these ring-modified analogues.

Electrostatic Perturbation between the Chromophore and Its Protein Environment. A negative charge or a negative end of an electric dipole near the retinylidene chromophore strongly stabilizes a resonance structure with a carbonium ion character, which causes a substantial spectral shift to longer wavelength (Honig et al., 1976; Schulten et al., 1980; Kakitani et al., 1985). Following previous studies (Honig et al., 1979; Nakanishi et al., 1980; Spudich, J. L., et al., 1986; Lanyi et al., 1988; Koutalos et al., 1989), we measured the contribution of this factor to pR by means of a series of analogues having incrementally reduced lengths of their conjugated double bond system (Figures 6 and 7). In Figure 7, the opsin shifts in pR are compared with the values obtained previously in archaebacterial (Spudich, J. L., et al., 1986; Lanyi et al., 1988), vertebrate (Koutalos et al., 1989), and invertebrate (Koutalos et al., 1989) rhodopsins. The profile of the opsin shifts in pR is significantly different from those of both other archaebacterial and eucaryotic rhodopsins.

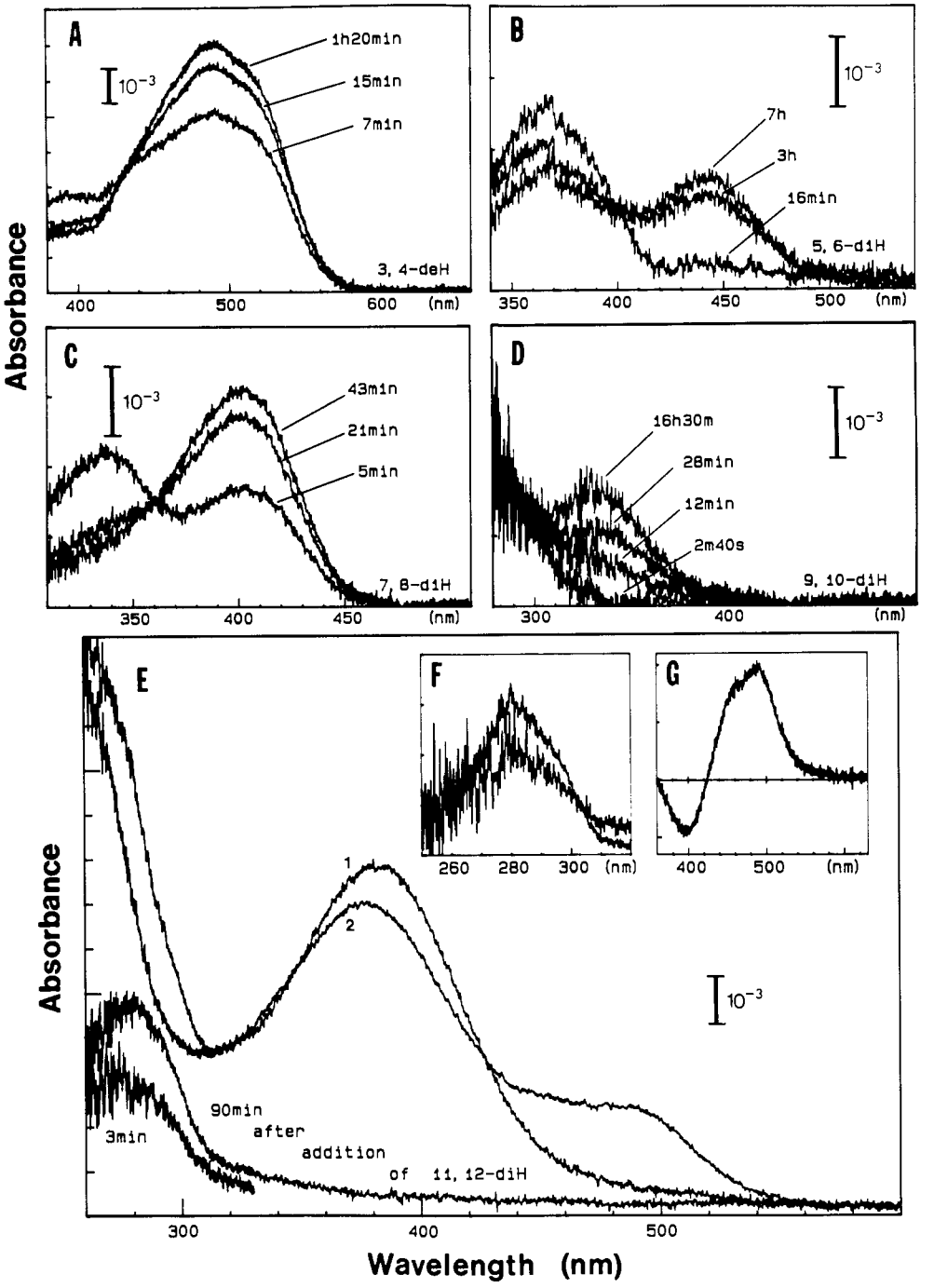


FIGURE 6: Formation of pR analogues from dihydroretinal and dehydroretinal analogues in apomembrane suspension. Absorption spectra were recorded during the incubation with the analogues *all-trans*-3,4-dehydro- (A), 5,6-dihydro- (B), 7,8-dihydro- (C), and 9,10-dihydroretinal (D). In (E), subsaturating amounts of 11,12-dihydroretinal were added to the apomembrane, spectra were recorded at 90 and 3 min after addition (bottom two spectra), and their difference was plotted as the bottom spectrum in (F). A similar result [top spectrum in (F)] is obtained as a difference spectrum after further addition of native retinal to the sample [spectrum 1 minus spectrum 2 in (E)]. Spectrum 1 was recorded immediately after addition of native retinal and spectrum 2 after overnight incubation at room temperature. The difference spectrum (2 minus 1) at longer wavelengths (G) demonstrates formation of pR as the result of chromophore exchange.

In the vertebrate and invertebrate rhodopsins, the largest opsin shifts (for 11,12-dihydroretinal, $n = 2$) have been explained by electrostatic perturbation at the C_{13} position (Honig et al., 1979; Smith et al., 1987; Koutalos et al., 1989). The relatively large opsin shifts in the other three archaebacterial pigments [bR; halorhodopsin (hR), a light-driven Cl^- pump; sensory rhodopsin I (sR-I), another phototaxis receptor] for native retinal ($n = 6$) have been attributed partly to a similar electrostatic effect near the C_5 position in the cyclohexene ring (Nakanishi, et al., 1980; Spudich, J. L., et al., 1986). On this basis, the results of incorporation of the same series of dihydroretinal analogues into pR leads to the conclusion that there is no electrostatic perturbation to either the ring or the polyene chain of the chromophore retinal. Note that the homoannular 3,4-dehydroretinal cannot be considered as a simple linear extension of the double bond system of native retinal, and the decrease in opsin shifts from $n = 6$ to $n = 7$ in all the pigments is likely to be due to a distorted ring conformation at the binding pocket. The independence of the opsin shift from the length (two to five double bonds) of the conjugated system (Figure 7) strongly implies no detectable local electrostatic effects around the pR chromophore.

Another determinant known to induce spectral red-shifts of retinylidene proteins is a weakened interaction between the

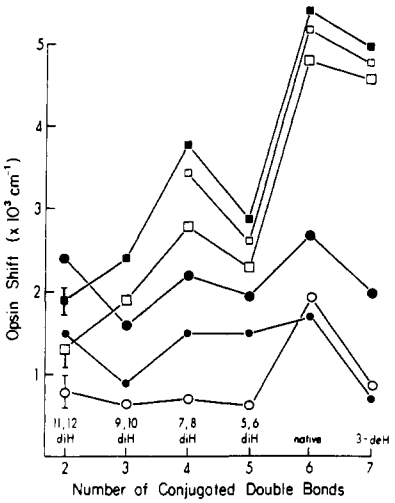


FIGURE 7: Opsin shifts in visual pigments and archaebacterial rhodopsins: (■) sensory rhodopsin I; (small squares) halorhodopsin; (large squares) bacteriorhodopsin; (●) bovine rhodopsin; (·) octopus rhodopsin; (○) phoborhodopsin. Opsin shifts of a series of retinal analogues with varied lengths of the conjugated double bond system from the Schiff base end are plotted vs the number of double bonds in the system. Data for bovine and octopus rhodopsins are taken from Koutalos et al. (1989), for hR from Lanyi et al. (1988), and for bR and sR-I from Spudich, J. L., et al. (1986) except for 11,12-dihydroretinal ($n = 2$) in both bR and sR-I and for 9,10-dihydroretinal ($n = 3$) in sR-I, which were determined in this study. Bars represent the uncertainty of the absorption maximum of the PSB^+ of 11,12-dihydroretinal due to the overlap of absorption between its two conjugated systems. λ_{max} values for protonated Schiff bases of *all-trans*-retinal (445 nm) and other analogues are from Spudich, J. L. et al. (1986).

Schiff base proton and its counterion (Blatz et al., 1972; Honig et al., 1976; Szeykowska et al., 1984; Kakitani et al., 1985; Lugtenburg et al., 1986; Smith et al., 1987; Mathies et al., 1987). Theoretically, an increased distance between the protonated nitrogen and its negatively charged counterion could regulate the absorption maximum from that of the corresponding PSB^+ in standard condition (445 nm for *all-trans*-retinal) up to 600 nm (Honig et al., 1976). From the flat part of the profile of the opsin shifts of pR in Figure 7, we can attribute only approximately 600 cm^{-1} to this factor. This value indicates that interactions at the Schiff base end of the chromophore contribute about 30% to the opsin shift of pR.

We extended the bR and sR-I measurements to include 11,12-dihydroretinal ($n = 2$). We observe a difference between the bR and sR-I opsin shifts, as expected from the earlier demonstration that the color difference between these two pigments is attributable to interactions at the Schiff base end (Fodor et al., 1989). Assuming that these smallest opsin shifts are attributable primarily to the weakened interaction between the Schiff base proton and its counterion, we conclude that *the small opsin shift in pR compared to those of other bacterial rhodopsins is due to both the lack of electrostatic perturbation of the chromophore and the relatively strong proton–counterion interaction at the Schiff base nitrogen.*

Why Is Vibrational Structure Evident in pR and Not in Other Well-Characterized Retinylidene Proteins?

The spectra of polyenes and related compounds frequently show vibrational fine structure of the ethylenic stretching mode because intensities of Franck–Condon active vibronic transitions in this mode predominate due to a reversal of bond alternation upon excitation (Warshel & Karplus, 1974). However, if the bandwidth of each vibronic transition is large due to inhomogeneous or homogeneous broadening in comparison with the spacing between the vibronic bands, such structured absorption will not be observed.

In visual pigments and in free retinal in solution (Christensen & Kohler, 1973), relatively large Franck–Condon active vibronic intensities of low-frequency torsional modes facilitate the loss of fine structure (Warshel & Karplus, 1974; Birge et al., 1982; Myers et al., 1986; Loppnow & Mathies, 1988). In visual pigments, this arises from a single bond distortion due to steric hindrance between the C_{13} methyl group and the C_{10} proton resulting from the $C_{11}=C_{12}$ cis configuration of the chromophore, in addition to the distortion around the C_6–C_7 bond (Smith et al., 1987). However, in light-adapted bR, retinal has been shown to have a C_6–C_7 planar configuration and an all-trans configuration at the binding site (Harbison et al., 1985), and therefore, these factors should contribute much less to broadening. The related pigments hR and sR-I (Baselt et al., 1989) and pR (this paper) evidently share this ring/chain coplanar conformation. Moreover, differences in polyene chain conformation of the chromophore between pR and the other bacterial rhodopsins are unlikely because we see a similar facility of binding of the dihydroretinal analogues, which differ in polyene chain conformation in solution, to pR and sR-I apoproteins. Why then is pR the only member of this family to exhibit vibrational fine structure? Three possible answers are as follows:

(i) A homogeneous broadening mechanism that causes a Gaussian line shape has been introduced to explain the observed diffuseness of the absorption spectrum of bR (Birge et al., 1982; Myers & Mathies, 1987). This is attributed to a barrierless excited-state potential surface, which might cause a rapid isomerization of a double bond. Recent kinetic hole-burning experiments have shown fast relaxation of the excited vibronic state and significant Gaussian homogeneous broadening in bR (Mathies et al., 1988; Pollard et al., 1989). Therefore, one possible explanation of the existence of fine structure in pR and its absence in bR is that this kinetic broadening is smaller in extent in pR than in bR.

(ii) A second possibility regards the relative one-photon intensity of the nearby forbidden $^1Ag^*$-like state, which has been studied with two-photon spectroscopy (Birge et al., 1985, 1986, 1990). The fairly large one-photon oscillator strength in bR may contribute to spectrum broadening (Birge et al., 1990). Accordingly, a low oscillator strength of the $^1Ag^*$-like state in pR could be responsible for the pR vibronic structure.

(iii) The above-mentioned two sources of broadening are not likely to be strongly temperature dependent, and appreciable resolution of structured absorption has been observed in the spectrum of bR at liquid nitrogen and helium temperatures (Iwasa et al., 1979; Balashov et al., 1989). These results suggest a third possibility, namely, that a larger electrostatic perturbation in bR, hR, and sR-I relative to that of pR, which has been suggested to be responsible for the greater red-shift of these pigments, leads to inhomogeneous broadening. Electrostatic perturbation at the ring end of the chromophore would permit thermal fluctuations, which cause a distribution of the distance between the chromophore ring and the charges or dipole fixed to the protein (El-Sayed et al., 1981). Weakened interactions between the Schiff base proton and its counterion may result in the destabilization of the geometry around the Schiff base end, thereby increasing the fluctuations of the distance between the counterion and the proton. It is also possible that the factors which weaken the interaction may in themselves provide a source of inhomogeneity of the interaction (site distribution effect). Several

ionizable groups have been proposed as candidates for the counterion of the Schiff base proton of bR (Braiman et al., 1988), implying a complex geometry around the Schiff base end. These inhomogeneous factors are evidently reduced in extent in pR compared to the other bacterial rhodopsins (Figure 7), which may account for its evident vibronic structure.

Bacterial Rhodopsins as a Family

Recent analysis of sR-I has shown a large degree of sequence homology between the photosensor and the two halobacterial ion pumps (Blanck et al., 1989). The sequence of pR is not known; however, the apparent molecular weight determined by [^{3}H]retinal labeling and SDS–polyacrylamide gel electrophoresis is 23 000 (Spudich, E. N., et al., 1986; Spudich et al., 1989), closer to the molecular weights of bR, hR, and sR-I (ca. 25 000), than to those of visual pigments (ca. 40 000). The chromophore structure and the wavelength regulation mechanism of pR further indicate that, despite its difference in absorption maximum, it is a member of the same family of proteins as bR, hR, and sR-I. Archaebacterial rhodopsins similar to each of the four halobacterial rhodopsins have been found in halophilic and haloalkaliphilic bacteria isolated from salt lakes in Australia and Africa, respectively (Bivin & Stoeckenius, 1986; Mukohata et al., 1989). One from an African species exhibits an absorption band shape similar to that of pR with a λ_{max} greater than that of pR by ~10 nm.

When *H. halobium* cells grow aerobically, they sythesize pR as their only retinylidene pigment, thereby avoiding sunlight. When they become anaerobic, biosynthesis of hR and bR is induced, enabling the cells to use light as an energy source. Also induced is the synthesis of sR-I, which mediates phototaxis into regions illuminated by orange light appropriate for photoactivation of bR and hR (Hildebrand & Dencher, 1975; Spudich & Bogomolni, 1984; Takahashi et al., 1985a), while production of pR is suppressed (Tomioka et al., 1986). Therefore, pR seems to be a primary photosensor which helps the cell avoid harmful irradiation during aerobiosis, as do most aerobic organisms living under intense sunlight without a photosynthetic system (Ninnemann, 1980; Senger, 1981). Our results show that the simple wavelength regulation mechanism of pR forms a basic part of the more complex mechanisms in the other three pigments. An attractive hypothesis is that pR arose first and the other three pigments later evolved as pR-related proteins acquired the ability to absorb longer wavelength light. Evolution of our color visual pigments may have occurred by an analogous process (Nathans, 1987).

The idea that pR is the progenitor of the other bacterial rhodopsins raises the possibility that pR may preserve a property of a "missing link" between the archaebacterial rhodopsins and the progenitor of the visual pigments. However, the differences in the mechanism of wavelength regulation in pR and in visual rhodopsin, a fundamental property of the active site of these molecules, argue that we should search elsewhere for vestiges of the missing link or that the two families originated independently. In either case, the convergence to the same phenotype via two distinct mechanisms reflects the great flexibility in color tuning inherent in the rhodopsin chromophore and can be interpreted in evolutionary terms as an example of homoplasy at the molecular level.

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