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Effect of lipid–protein interaction on the color of bacteriorhodopsin

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Detergent solubilization and subsequent delipidation of bacteriorhodopsin (bR) results in the formation of a new species absorbing maximally at 480 nm (bR_{480}). Upon lowering the pH, its absorption shifts to 540 nm (bR_{540}). The p*K* of this equilibrium is 2.6, with the higher pH favoring bR_{480} (Baribeau, J. and Boucher, F. (1987) Biochim. Biophysica Acta, 890, 275–278). Resonance Raman spectroscopy shows that bR_{480}, like the native bR, contains a protonated Schiff base (PSB) linkage between the chromophore and the protein. However, the Schiff base vibrational frequency in bR_{480}, and its shift upon deuteration, are quite different from these in the native bR, suggesting changes in the Schiff base environment upon delipidation. Infrared absorption and circular-dichroism (CD) spectral studies do not show any net change in the protein secondary structure upon formation of bR_{480}. It is shown that deprotonation of the Schiff base is not the only mechanism of producing hypsochromic shift in the absorption maximum of bR-derived pigments, subtle changes in the protein tertiary structure, affecting the Schiff base environment of the chromophore, may play an equally significant role in the color regulation of bR-derived pigments.

Introduction

Bacteriorhodopsin, the only protein present in the plasma membrane of the extreme halophile *Halobacterium halobium* contains a retinal molecule, covalently linked to Lys-216. The Schiff-base linkage between retinal (chromophore) and the protein is protonated in the native state. The pigment has an absorption maximum at 560 nm in its dark-adapted state. Upon absorption of light, bR undergoes a photocycle with the eventual extrusion of protons from the cell interior against their concentration gradient. The free energy associated with this electrochemical gradient is utilized by the cell to support endergonic metabolic processes like the synthesis of ATP and the transport of nutrients into the cell interior.

In the purple membrane, bR molecules exist as trimers laterally dispersed in a two-dimensional hexagonal lattice [2]. Almost 80% of the lipids constituting the membrane are acidic [3]. Solubilization of the membrane in detergents results in a collapse of the trimeric structure and the bR molecules are rendered essentially monomeric [4]. Depending on the nature of detergent, this structural change produces hypsochromic shifts of 10–20 nm in the absorption maximum of the pigment. Almost complete removal of intrinsic lipids and extraneous detergents from bR results in the formation of a new species, bR_{480} ($\lambda_{max} = 480$ nm), in a pH-dependent equilibrium with a higher-wavelength-absorbing species, namely bR_{540} ($\lambda_{max} = 540$ nm). The apparent p*K* for this acid-base titration depends on the detergent used and has a value of 2.6 for the lipid- and detergent-free bR, with lower pH yielding the bR_{540} form [1].

The main factors governing the spectroscopic properties of retinal pigments are generally believed to be the protonation state of the Schiff base linkage between the chromophore and the protein and the spatial organization of the charged residues in the protein matrix surrounding the chromophore [5]. Deprotonation of the Schiff-base linkage in bR at high pH (p*K* = 13.5) results in the reversible formation of bR_{460}, a pigment absorb-

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Abbreviations: bR, bacteriorhodopsin, PSB, protonated Schiff base; CD, circular dichroism; FTIR spectroscopy, Fourier transform infrared spectroscopy.

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ing at approx. 460 nm [6]. Similarity between the absorption maximum of the high-pH-induced bR_{460} and bR_{480} prepared by delipidation of bR led to the suggestion that bR_{480} contained an unprotonated Schiff-base linkage, although this species was shown to have a photocycle similar to bR [7]. According to our current understanding of energy transduction, such photochemistry is thought to require a protonated Schiff base [8]. Although the pH-dependent equilibrium between bR_{480} and bR_{540} in the detergent- and lipid-free bR system is analogous to the equilibrium between native bR and bR_{460}, as judged by the absorption maxima of the species involved, there is, however, a large difference in the pK values of the two equilibria – 2.6 for the former and 13.5 for the latter. Thus, we undertook this study to characterize this hypsochromic absorbing species.

We report here our resonance Raman spectroscopic studies which provide direct evidence that bR_{480} contains a protonated Schiff base linkage between chromophore and protein, just as the native bR does. Moreover, there appear to be minimal chromophore–protein interactions in bR_{480} relative to the native bR, according to our Raman data. We do not observe any change in the secondary structure of the protein upon formation of bR_{480}, as judged by circular dichroism and infrared absorption spectroscopic studies. We discuss the possible mechanism responsible for the hypsochromic shift in the absorption maximum of bR_{480} relative to the native pigment.

Materials and Methods

H. halobium (strain S9, a generous shift from Dr. W. Stoeckenius, University of California, San Francisco) was grown as has been described earlier [7]. Delipidation of bR was carried out according to the earlier published method [9]. This method yields delipidated bR, at least, bR:phospholipids < 1 : 0.3 (see Ref. 7) in 0.25% deoxycholate. These samples were loaded on top of a 1×15 cm column of phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) pre-equilibrated with 0.25% deoxycholate in 10 mM Tris buffer (pH 8.0). The column was first washed with 20 vol. of 50 mM phosphate buffer (pH 7.0) and then with 3 vol. of the same buffer containing 0.2% octyl glucoside. Purified bR could then be eluted from the column in the presence of 0.5% (w/v) octyl glucoside in the same buffer. bR fractions were concentrated by ultrafiltration on Amicon PM 10 filters and dialyzed against water (30 ml sample/2 l of water for 36 h with water changed twice). Once dialyzed, bR can be further concentrated by sedimentation at $50000 \times g$ for 2 h. Pelleted bR can be resuspended in buffer or buffered detergent solutions.

At neutral pH, the sample has an absorption maximum of 480 nm. The effect of pH on the absorption maximum of purified bR and detergent-solubilized bR has been extensively discussed elsewhere [1].

Absorption spectra were measured in a Pye-Unicam SP8-100 spectrophotometer. This instrument has been modified to minimize the scattering artifacts due to turbid samples.

Resonance Raman measurements were made on these concentrated samples, suspended in minimum amount of H_2O or ^2H_2O at neutral pH/p^{2}H. Under these conditions, the sample is exclusively present in the bR_{480} form, as shown by absorption measurements on diluted parallel samples. This is further confirmed by the frequency of the in-phase C = C stretching vibration in the resonance Raman spectrum, which has been shown, for retinal pigments, to be linearly correlated with the absorption maximum [10]. Raman measurements on retinal pigments have also shown that, except for the intensities of various bands (S/N changes, but relative intensities do not), concentration has no observable effect on the frequency of the vibrational modes.

The 476.5 nm line from Ar^+ laser (Spectra Physics, Model 163) was used to excite Raman scattering from the sample at 80 K, in conditions that have been previously described [11]. The scattered signal was resolved using the Triplemate spectrograph (Spex Industries, Model 1877). The OMA detector in this apparatus consists of a Reticon detector and controller system (EG&G Princeton Applied Research, NJ; Model 1420-2/1218) which is controlled by an LSI-11 (Digital Equipment) computer system for data collection and storage, as has been described earlier [11]. All spectra were calibrated against the Raman lines of toluene. The spectral resolution under these conditions was approx. 8 cm^{-1}.

CD spectra were measured in a JASCO J-40 AS spectropolarimeter. The protein concentration was 0.6 μM and the cell path length 1 cm. Concentration of bR was obtained from the absorption spectrum using $\epsilon_{480} = 3.9 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

For infrared absorption measurements, a protein sample (a 4 μM solution) was layered on a CaF_2 window and allowed to dry overnight in dark at room temperature. Immediately prior to the measurements a stream of dry nitrogen was blown on top of the deposited film for about 5 min. The measurements were made with an IFS-85 Bruker FTIR spectrometer (Bruker Analytische Messtechnik, Karlsruhe, F.R.G.) operating at 2 cm^{-1} resolution.

Results

Resonance Raman spectra of bR_{480} suspended in water and in ^2H_2O are shown in Figs. 1a and 1b, respectively. The main ethylenic band resulting from the C = C stretch appears at 1558 ± 1 cm^{-1} in the two spectra. The slight shoulder at about 1530 cm^{-1},

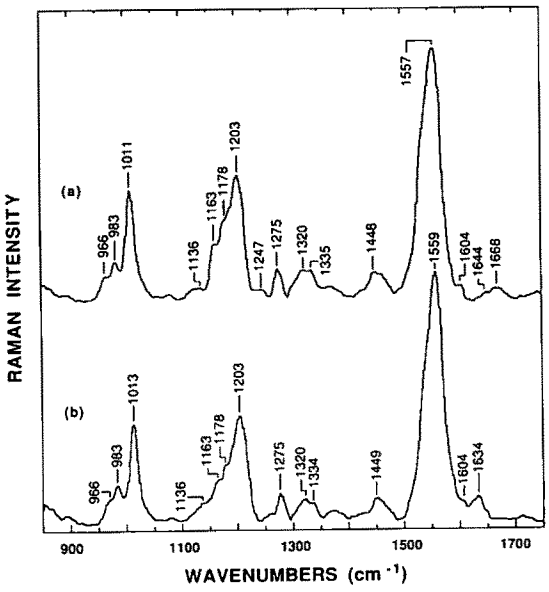

Fig. 1. Resonance Raman spectra of bR_{480} in (a) H_2O and (b) ^2H_2O at 88 K. The spectra were probed with approx. 5 mW of 476.5 nm light from an Ar^+ ion laser. The sample pH/p^{2}H was 6.

according to the observed inverse correlation between the ethylene stretching frequency and the absorption maximum in retinal pigments [10], would result from a species with absorption maximum of approx. 550 nm. It could either represent a small amount of photoproduct of bR_{480} or some unconverted bR_{540}, although the latter is less likely at this pH. In any event, the negligibly low intensity of the ethylenic mode due to this species clearly implies that the contribution of this pigment in other regions of the spectrum can be ignored.

The Schiff base vibrational frequency (C = N stretch) is sensitive to the protonation state of nitrogen and undergoes a shift upon deuteration if the Schiff base is protonated. Comparison of Figs. 1a and 1b shows that the Schiff-base vibrational band at approx. 1668 cm^{-1} in water solution shifts to 1634 cm^{-1} when the sample is in deuterium oxide, showing thereby that bR_{480} contains a protonated Schiff-base linkage between the chromophore and the protein. However, the frequency of Schiff-base mode in bR_{480} is rather high when compared to its frequency at approx. 1641 cm^{-1} in native bR [12]. Furthermore, the frequency shift of this mode to 1634 cm^{-1} is also quite different from that in native bR, where it shifts to approx. 1623 cm^{-1} upon deuteration. It should also be noted that corresponding values of this vibrational mode in the protonated and deuterated Schiff bases of retinal in solution are approx. 1663 and 1633 cm^{-1}, respectively [13]. Since the position as well as the shift upon deuteration of the Schiff-base vibrational frequency are independent indices of its environment [14–17], these data suggest a rather strong hydrogen-bonding interaction between the Schiff base and its counterion in bR_{480}, quite different from that in the native bR.

The 1100–1300 cm^{-1} region in the Raman spectrum (fingerprint region) of retinal pigments results mainly from the C–C stretching and the C–C–H bending motions of the chromophore [18] and is very sensitive to the isometric form of the chromophore as well as to the nature of chromophore–protein interactions. In this region the bR_{480} spectrum is distinctly different from the spectrum of the native bR in both the light- and dark-adapted forms [10,12]. Comparison with the spectra of model PSB in solution, however, suggests that the superposition of the Raman spectra due to the 13-*cis* and the all-*trans* PSBs would generate a spectrum qualitatively similar to bR_{480} spectrum in this region [13]. Chromophore chemical extraction reveals that the isomeric composition of both bR_{480} and bR_{540} is not significantly different from dark-adapted bR (unpublished observations), which contains the all-*trans* and the 13-*cis* isomeric retinals in almost molar equivalence. The dark-adapted bR Raman spectrum [12] is quite different from the spectrum of bR_{480} in this region. That the same chromophore isomeric composition can lead to significant changes in the fingerprint region indicates that this region of the spectrum is quite sensitive to the chromophore–protein interaction.

The CD spectrum of bR_{480} in the visible region (data not shown) does not show any exciton coupling band, suggesting that the protein is essentially monomeric and is devoid of quaternary (di- and trimeric) structure. These data are not particularly surprising, since similar results were reported earlier for bR_{540} [7]. The CD spectrum of bR_{480} and native bR in the far-ultraviolet region is shown in Fig. 2 as broken and solid lines, respectively. This region of the spectrum is due to $n-\pi^*$ and $\pi-\pi^*$ transitions of amide bonds in proteins and is particularly sensitive to protein secondary structure. Our native bR spectrum is similar to the spectrum reported for purple membrane sheets [19]. As can be seen, there are some differences between the spectrum of the native bR and that of bR_{480}. The ellipticity at 224 nm, used in the estimation of α-helical content in proteins, is almost identical in the two spectra; the differences appear at lower wavelengths were $\pi-\pi^*$ interactions are observed. The $\pi-\pi^*$ CD contribution is dominated by exciton interactions resulting in Davidov splitting, which gives rise to a positive maximum around 195 nm and a negative around 210 nm. Therefore, experimentally, one normally observes that any alteration or perturbation results in a concerted spectral change in both these maxima, i.e., they either both decrease or increase in magnitude. However, this is not the case here. We find no obvious interpretation for this difference in the spectra; however, the difference is only minimal compared to the complexity of the system (i.e., possibility of scattering artifacts, [19]). We therefore simply ignore this difference for the rest of our discussion. Thus, the formation of bR_{480} does not appear to

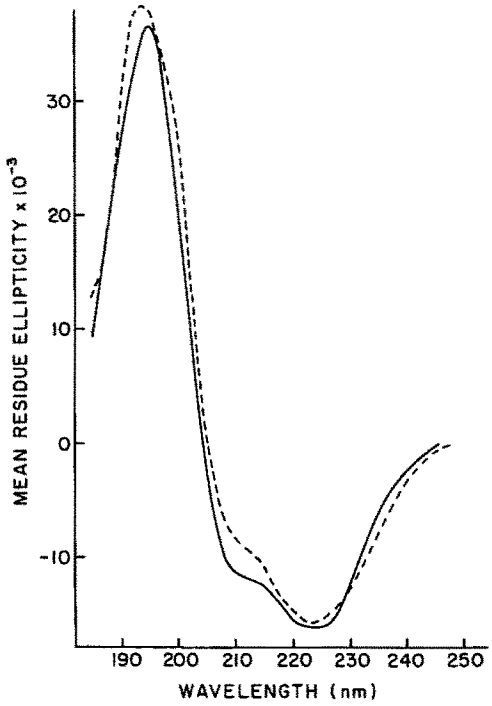

Fig. 2. Ultraviolet CD spectrum of bR_{480} (------) and native dark-adapted bR (———). The path length was 1 cm and the bR concentration was 0.6 μM. The sample pH was 6.

result from any major changes in the protein secondary structure.

The FTIR spectrum (Fig. 3) of air-dried film of bR_{480} is identical to the published spectra of the native bR [20,21]. Not only are the amide I and II observed at the same frequency as in native bR, namely at 1659 and

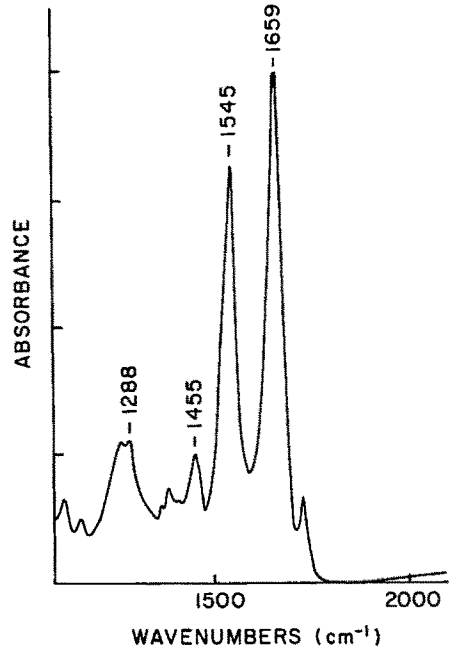

Fig. 3. FTIR spectrum of an air-dried film of bR_{480} in the protein-structure-sensitive region. The bR_{480} concentration prior to air drying was 4 μM and the pH was 6.

1545 cm^{-1}, respectively, but so are the much-debated shoulders around 1640 and 1685 cm^{-1} [19,22]. These shoulders, although not apparent in this extended region spectrum, are clearly visible in the blow-up of the amide I (data not shown). The small peak in the spectrum at about 1740 cm^{-1} is due to the ester carbonyl stretch arising from the small amount of residual lauryl sucrose that was used for delipidation of the sample. These data, in complete agreement with the CD data, show that the formation of bR_{480} does not result from any net change in the protein secondary structure.

Discussion

It was suggested [1] that the blue-shifted absorption maximum of bR_{480}, compared to native bR, resulted from the deprotonation of the Schiff-base linkage between the chromophore and bacterio-opsin. This suggestion resulted from the comparison of bR_{480} with another blue-absorbing pigment form, the high-pH-induced bR_{460}, which had been described and shown to be deprotonated [6]. The equilibrium between bR_{540} and bR_{480} was also likened to that between native bR and bR_{460}, although the pK of the latter equilibrium is 13.5 [6], while that of the former is 2.6 [1]. Even though pK values are known to vary remarkably depending on the changes in the microenvironment of the group involved, a shift in pK of 10 units would still be difficult to reconcile. However, in a systematic study of the effect of detergent structure upon the pK of this equilibrium [1], it was shown that the the pK of this transition could, indeed, be raised by solubilizing the pigment in appropriate detergents. For example, in a mixture of Triton X-100 and lauryl sucrose, the pK was 13, almost the same pK as is observed for the equilibrium between native bR and bR_{460}.

In the light of our finding that bR_{480} contains a PSB, the blue-shifted absorption spectrum of bR_{480} compared to native bR, which was explained on the basis of its putative deprotonated Schiff-base linkage, now needs a new explanation. In order to discuss the effect of such subtle structural changes as mentioned above on the absorption of the pigment, we have summarized the current theoretical understanding of the determinants of color regulation in retinal-based pigments [5,23–26]. It appears that in the case of bR there are three main factors that contribute to the observed opsin shift (the difference in the absorption maxima of the pigment and the PSB of the corresponding chromophore in methanol, expressed in units of cm^{-1} [27]), nameley, (a) an increased counterion distance from the Schiff-base positive charge relative to the PBS, (b) 6-S-*trans* isomerization of the ring with respect to the chain (or ring-chain planarization) from its 6-S-*cis* configuration in PSB, and (c) the presence of a dipole in the vicinity of the β-ionone ring. It has been calculated that moving the

Schiff base counterion by 0.9 Å, from 3 Å (the distance of closest approach between an anion, say Cl^{-1}, and the Schiff-base positive charge in solution) in the PSB to 3.9 Å in bR, would result in an approx. 3000 cm^{-1} gain in the opsin shift of bR [23]. Thus, the contribution from the perturbation near the Schiff-base region to the total observed opsin shift in the case of native bR is believed to be 3000–3500 cm^{-1}. The remaining opsin shift, out of a total of 5100 cm^{-1} observed for bR, is thought to result from a combination of ring-chain planarization, as well as to the interaction of a protein dipole near the ionone ring of the chromophore [24].

As is clear from our ultraviolet CD and FTIR absorption spectroscopic studies, there is no observable difference in the secondary structure of the protein moiety in bR_{480} and native bR. Thus, large-scale protein structural changes can be ruled out as an explanation of the blue-shifted spectrum of bR_{480}. However, there may still exist exist subtle changes and, in fact, our resonance Raman data suggest that they do. The fingerprint region in the resonance Raman spectrum of bR_{480} suggests that the strong chromophore–protein interactions present in native bR have been reduced to a minimum. This conclusion derives from the observation that, while both dark-adapted bR and bR_{480} contain a near equimolar mixture of 13-*cis* and all-*trans* isomeric forms of the chromophore (unpublished observations), the Raman spectrum of bR_{480}, unlike dark-adapted bR, resembles a simple addition of the solution spectra due to a mixture of the PSBs of the respective isomeric chromophores.

The relatively low value of the Schiff-base frequency in bR (approx. 1640 cm^{-1}) and its small shift upon deuteration is thought to be associated with the increased counterion distance from the Schiff-base positive charge [23]. Our measurements show that bR_{480} has a PSB mode at 1668 cm^{-1} and undergoes a 34 cm^{-1} shift upon deuteration. These values are much closer to those observed for the PSBs in solution [13] or for visual pigments [17], which are thought to have a stronger interaction between the protonated Schiff base and the counterion.

As discussed above, a 1 Å change in the distance between the Schiff base nitrogen and its counterion, as a result of protein structural change, would be expected to result in a decrease in the opsin shift of approx. 3000 cm^{-1}. This would correspond to a hypsochromic shift in the absorption maximum of the native bR to approx. 480 nm, in excellent agreement with our observation (the exact numerical coincidence is likely to be fortuitous, however).

Such a change in the counterion distance can be envisioned to arise as a result of slight opening-up of the protein structure, making the chromophore binding site, particularly the Schiff-base region, permeable to the solvent. Indeed, solvent accessibility studies using hydroxylamine (unpublished observations) show that retinal chromophore in bR_{480} is substantially more reactive than in bR_{540}. It, thus, seems possible that the protein counterion of the Schiff base in the native bR is replaced by the dipoles of the solvent in bR_{480}. We do not observe any protein secondary structure change involved with the formation of bR_{480}. Thus, this change in solvent permeability must result from higher-order structural changes. Since bR_{480} lacks quaternary structure, as revealed by the absence of exciton band in the visible CD, the change in the structure associated with the formation of bR_{480} has to result from tertiary structural changes due to the deprotonation of some structurally significant residue. This change may be subtle, involving slight and local changes in only a few atomic coordinates. However, such a change can result in the exposure of the Schiff-base region to the solvent, as well as to any ions in solution, which may replace the protein counterion of the protonated Schiff base.

Alternative explanations of the observed hypsochromic shift in the absorption maximum of bR_{480}, such as a stronger interaction of the protein counterion with the Schiff base upon bR_{480} formation, or generation of another negative charge in the vicinity of the Schiff base as a result of the pH titration without invoking a protein structural change, cannot be ruled out by the spectroscopic data alone. The simplest explanation, consistent with the reactivity of bR_{480} toward hydroxylamine, however, suggests changes in the protein tertiary structure. Should these changes occur, they could weaken chromophore interactions with important side-chain residues, like typtophane-182, whose substitution by phenylalanine shifts pigment absorption to 480 nm [28].

In delipidated and detergent-free bR, namely bR_{480}, the only titratable groups, beside the Schiff base, are the side-chains of the amino-acid residues. There are not many protein residues that have a pK which is as low as 2.6; the most likely candidates are aspartic and glutamic acids, which have pKs in solution of 3.8 and 4.2, respectively. Because the pK values are sensitive to the local electrostatic fields, it is not very surprising to find shifts of 1–2 units in pK values of the amino acids when they are present in proteins, compared to their solution value. The pK of 2.6 may represent the titration of an aspartate or glutamate residue. It should be noted that while the electrical fields within the native bR molecule arise due to both the lipids (which are mainly acidic in nature) and the protein, that in the delipidated bR are due to the protein alone. It is known that 80% of the intrinsic lipids of bR are acidic [3]. Just on the basis of the surface charge due to the lipids as well as the proteins, the intrinsic pK values can be expected to increase by up to 2 units [29]. Thus, in the native bR, the pK corresponding to the bR_{480} formation may increase drastically due to the contributions

from inaccessibility as well as the surface charge and, therefore, may not be observed. That the apparent pK of this titratable group depends on the accessibility of the aqueous phase, has been recently shown [1].

Thus, depending on the protonation state of a structurally important amino-acid residue, bR appears to assume two distinct tautomeric forms corresponding to the two pigments, bR_{540} and bR_{480}. The pK of this group appears to be very sensitive to the surrounding amphiphiles [1]. In native bR, the strong lipid–protein interactions presumably prevent the titration of this residue by protecting it from the solvent, thereby maintaining the purple color of bR.

In conclusion, our studies provide an example of a pigment resulting from bR which contains a PSB linkage between the chromophore and the protein and yet has an absorption maximum which is significantly blue-shifted compared to the native bR. Deprotonation of the Schiff base is, therefore, not the only mechanism that can explain the blue-shifted absorption maximum in bR-derived pigments. Subtle changes in the protein structure surrounding the chromophore appear equally important. Incidentally, blue-shifted absorption maximum is also observed when bR is solubilized in 60% DMSO [30], or treated with volatile anesthetics [31]. At least in the former case, the pigment has recently been shown [32] to contain a PSB linkage between the chromophore and the protein. Thus, it is clear that a large hypsochromic shift (approx. 100 nm) in the absorption maximum of bR does not necessarily involve deprotonation of the Schiff-base linkage between retinal chromophore and opsin.

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