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Spectral Tuning in Bacteriorhodopsin in the Absence of Counterion and Coplanarization Effects*

(Received for publication, August 7, 1995, and in revised form, October 18, 1995)

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The basis for wavelength regulation in bacteriorhodopsin (BR) and retinylidene proteins in general has been studied for decades but is still only partially understood. Here we report the preparation and spectroscopic characterization of BR analogs aimed at investigating the existence of spectral tuning mechanisms other than the two widely accepted mechanisms, weakened counterion interactions and ring/chain coplanarization. We synthesized two novel retinal analogs containing a saturated 13–14 bond, which interrupts the interaction of the protein counterions with the chromophore conjugation system. Furthermore, one of the analogs has a planar polyene system so that the contribution to the red shift of BR by retinal ring/chain coplanarization is also absent. We incorporated these analogs into bacterioopsin and discovered a sizable amount of red shift, which can be accounted for by interactions between the polar or polarizable groups of the protein and the retinal polyene chain. Our results suggest that the wavelength regulation in BR is achieved by synergistic chromophore/protein interactions including ring/chain coplanarization, excited state stabilization by polar or polarizable protein side chains located along the polyene chain, and weakened counterion interactions near the Schiff base positive charge.

Bacterioopsin (BO)† tunes the absorption maximum (λ_{max}) of a retinal protonated Schiff base (PSB) from 440 nm in methanol to 568 nm in bacteriorhodopsin (BR). The mechanism of wavelength regulation in BO and in retinylidene proteins in general has been a subject of intensive investigation. Nakanishi et al. (1) introduced the term opsin shift (OS) to refer to the energy difference (in cm⁻¹) between the absorption maximum of PSB in methanol and that in the protein. Three factors have been suggested to contribute to the 5100 cm⁻¹ OS of BR. (i) The conversion of the twisted conformation of the β-ionone ring/polyene chain of the retinal PSB in solution (2) to a coplanarized conformation (3) induced by the protein contributes 1200–1300 cm⁻¹ (4). This mechanism has been widely accepted. (ii) A weakened PSB[counterion association, either by increasing interionic distance or the salvation of counterions, may further shift the absorption maximum to longer wavelengths (5, 6). (iii) Negative charges, or in a more general sense, the polarizable groups and permanent dipoles distributed along the conjugated chain may stabilize the excited state and cause a red shift (7).

There have been diverse estimates of the counterion contribution to the OS of BR. 1,12-Dihydro-BR analog, the chromophore structure of which is very similar to the native one, exhibits an OS of 1400 cm⁻¹ (8). Solid state ¹³C and ¹⁵N NMR studies suggested a value of 2000 cm⁻¹ from the contribution of the counterion (9). Albeck et al. (10) and Hu et al. (11) suggested that a weakened counterion and the ring/chain coplanarity together would produce an OS value in model compounds nearly comparable with that in BR. Site-directed mutagenesis of BR (12, 13) and solid-state NMR studies (14) indicated that both Asp-85 and Asp-212 are involved in forming a complex counterion environment. In model compound studies, introduction of a second negative charge near the Schiff base produces a blue shift of 600–950 cm⁻¹ (15). In a mutant BR (DB5N/D212N) lacking both protein counterions, halide functioned as a surrogate counterion so that the protein complexed with chloride exhibited the same OS (–5100 cm⁻¹) as in native BR (12, 13). Although model PSB was measured in the polar solvent methanol, which may cause significant solvation of the Schiff base counterion (15), the association of an anion to a proton cation may still be different from the model system. In DB5N/D212N BR, one can argue that the halide may be more or less separated from the PSB compared with solution model compounds. One possible situation is that the halide is well separated from the Schiff base charge and causes an OS by a similar “weakened counterion” mechanism as in the native BR. This assumption does not contradict to the correlation between the size of halide (Cl⁻, Br⁻, I⁻) and the amount of red shift in the mutant BR (12, 13) if one assumes a water molecule is bound between the PSB positive charge and the counterions. However, this correlation also fits the assumption that the chloride ion is closely associated with the PSB positive charge. A closely associated counterion was predicted by Honig et al. (16) on the basis of energetic considerations. In contrast to the inorganic counteranion in model PSB studies (15), the dynamic protein structure, in principle, allows associated counterions to fluctuate, thereby allowing them to contribute to the OS of BR.

For convenience of computations, the external point charge model used a point charge to represent either a negative charge in a salt bridge or a negative end of a protein dipole. Mutagenesis studies (17) and two photon spectroscopy studies (18) argue against a discrete charge in the binding site but not against the local electrostatic fields, which would fulfill the conditions of the original point charge model (1). Model studies (19, 20), genetic analysis (21), mutagenesis (22–24), and resonance Raman (25) studies on various rhodopsin pigments all suggest

*This work was supported by Grants GM 27750 (to J. L. S.) and GM 36564 (to K. N.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: BO, bacterioopsin; BR, bacteriorhodopsin; OS, opsin shift; PSB, protonated Schiff base; HPLC, high pressure liquid chromatography; BR(I) and BR(II), BR analogs reconstituted from retinal analogs I and II.
that wavelengths can be regulated by polar side chains along the polyene chain.

To further test for the effective chromophore/protein interactions other than the widely accepted ring/chain coplanarization (3, 4) and counterion mechanisms (9, 11, 14), we synthesized two retinal analogs (I and II shown in Fig. 1) that have a saturated 13–14 bond so that the counterion is isolated from the conjugation system and hence can no longer contribute to the OS. Furthermore, analog II possesses a planar conformation in solution so that β-ionone ring/polyene chain coplanarization will not contribute to the OS.

EXPERIMENTAL PROCEDURES

Synthesis of Retinal Analogs—Analog I (Fig. 1) was prepared by conventional Wittig reaction between (β-ionylidenemethyl) triphenylphosphorane (26) and ethyl 3-methyl-4-oxobutanoate; the latter was obtained by catalytic reduction of ethyl 3-methyl-4-oxocrotonate (Fluka Chemical Co.). Diisobutyaluminum hydride reduction of the resulting isomeric mixture of ethyl 13,14-dihydroretinoate gave analog I, which was further purified by HPLC (8). Analog II (Fig. 1) was prepared by using the triphenylphosphonium bromide derived from 3,7-dimethyl-2,4,6-octatrienol; the latter was prepared by Emmons-Horner reaction of 3-methyl-crotonaldehyde with the anion derived from triethyl 3-methyl-4-phosphonocrotonate, followed by diisobutylaluminum hydride reduction of the resulting ethyl 3,7-dimethyl-2,4,6-octatrienoate and HPLC (8). Synthetic products were characterized by 1H NMR analysis.

UV-visible Absorption Spectroscopy—Absorption spectra were measured on an SLM Aminco DW2000 spectrophotometer at 23 °C. Spectra of retinal analogs and the PS were determined in ethanol. BO was prepared as reported (27). BR analogs were reconstituted in 20 mM 

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RESULTS

Analog I absorbs at 289 nm in ethanol with a diffuse bell-shaped spectrum (Fig. 2A, dotted line) due to C-6-C-7 distortion (28). It binds to apomembranes (27) containing BO and forms BR(I) absorbing at 328 nm with distinct vibrational fine structure (Fig. 2A, solid lines). The OS in this pigment is 4110 cm−1. The red shift and the development of fine structure (Fig. 2A) due to C-6–C-7 distortion indicate that the chromophore is tightly bound and that the analog is forced to adopt a ring/chain coplanarized conformation. Analog II absorbs maximally at 307 nm and exhibits distinct vibrational fine structure in methanol (Fig. 2B, dotted line). The red shift in analog II compared with analog I and its structured spectrum indicate a planar conformation. Although the lack of the native β-ionone ring in II may affect the shift in the resulting pigment when incorporated into BO, a red shift is expected if a spectral tuning mechanism other than the counterion and the

FIG. 1. I, 13,14-dihydro-all-trans-retinal; II, 3,7,11-trimethylodiodeca-4,6,8,10-tetraenal.

FIG. 2. A 5-μl ethanolic solution of compound I (3 × 10−3 M) (A) or compound II (1 × 10−3 M) (B) was added to a 3-ml apomembrane suspension containing BO at pH 7.0 and 23 °C. Absorption spectra (solid lines) were recorded at 2, 14, 30, and 45 min (A) and 2, 3, 5, 9, 12, and 40 min (B) after analog addition as seen from the rise at 328 (A) and 321 nm (B), respectively. Unreconstituted apomembranes to which was added the same volume of ethanol were used as a reference. Spectra of analogs in ethanol are shown as dotted lines.

DISCUSSION

There are two ways to calculate the OS of BR(II) in the absence of counterion and coplanarization contributions. (i) Subtraction of the amount of red shift caused by coplanarity (λmax, I − λmax, II = 2030 cm−1) from the OS of BR(I), 4110 cm−1, indicates an additional OS of 2080 cm−1 in BR(II). (ii) The difference between OSs of BR(II) and BR(I) is 2690 cm−1. By either calculation there exists additional OS resulting from mechanisms other than the counterion and ring/chain coplanarization. The −600 cm−1 difference between the two methods of calculation is not surprising. The lack of the β-ionone ring may alter the orientation of the analog II in the BR retinal binding site. In other words, a hypothetical prelocked, ring/chain planarized 13,14-dihydroretinal analog may give a higher OS than analog II upon binding to BO. The instability of analog II in the binding site is indicated by two experimental findings. (i) Analog I when bound to BO retards the incorporation of the native retinal by 270-fold while analog II does so by only 11-fold. This effect may result from the lack of the native β-ionone ring on the chromophore and the partially impaired retinal ring/protein interactions in BR(II). (ii) The distinct vibrational fine structure observed in BR(I) is diffused in BR(II), also indicating analog II is more flexible due to the lack of native interactions in the binding pocket. Therefore, we consider 2080 cm−1 as a better estimate of the OS in BR(I) in the absence of counterion and coplanarization effects. The possibility of intramolecular through-space interaction has been previously excluded experimentally by comparing the absorption maxima of dihydro- and tetrahydroretinal analogs and pigment analogs (29). Therefore, this OS demonstrates the existence of
a red shift mechanism in the absence of the counterion and the coplanarization contributions.

We find two possibilities particularly attractive in explaining the source(s) of the additional red shift. (i) An aromatic retinal binding pocket was originally proposed by Braiman et al. (40) and Rothchild et al. (41) based on Fourier transform infrared spectroscopic studies. From the recent atomic resolution BR structural model (30), the retinal chromophore is tightly sandwiched by an aromatic pocket including Trp-182 above, Trp-86 and Trp-189 under, and Tyr-185 on the side of the retinal. In bulk solutions, a polarization-induced shift on absorption results from momentary polarization in a polarizable solvent induced by the transition dipole of the solute (31). Since phototransition produces a large change in retinal dipole moment (32), polarizable solvents red shift the $\lambda_{\text{max}}$ of PSB by 2390 and 2030 cm$^{-1}$ (19, 20). The preorganized aromatic retinal binding pocket in BR would produce more effective stabilization for the Franck-Condon state of the chromophore within its lifetime compared with effects observed in bulk solution. An early study of the energy transfer between retinal and tryptophans indicated the close interactions between them (42). Polarizable aromatic compounds have also been shown to effectively stabilize positive charges (33, 34). However, since tryptophans are expected to play a key role in fixing the backbone of retinal in addition to their role in wavelength determination, tryptophan mutants of BR do not provide a clear test of this mechanism. (ii) Second, fixed polar groups around the polyene chain of retinal may be located in such a way that they can effectively stabilize the excited state or destabilize the ground state of the chromophore. This mechanism is theoretically sound (7, 35), and experimental evidence has begun to accumulate. For example, hydroxyl groups have been shown to play a key role in wavelength regulation in human cone pigments by several groups (21–25).

Incorporating either of the two mechanisms into the BR wavelength regulation scheme can satisfactorily account for our results as well as a number of previous observations of red shifted retinoid chromophores in the absence of PSB formation. Large OS values (3000–4700 cm$^{-1}$) have been observed in non-covalent complexes between BO and retinal analogs with one less double bond (36). The BR reconstitution intermediate, which was shown to be non-covalently bound to BO exhibits an OS of $3100 \pm 200$ cm$^{-1}$ (37). The BR photocycle intermediate M$_{\text{M2}}$ contains a deprotonated Schiff base chromophore, yet its OS is $3350 \pm 150$ cm$^{-1}$. All-trans-retinal cannot covalently bind to the K216C mutant BO protein in a PSB form, but the OS of this non-covalent species is $3330 \pm 150$ cm$^{-1}$ (38). All-trans-retinal can be complexed non-covalently with the dried membrane of BO and a mutant BO K216C and forms a common complex absorbing at 470 nm (OS 4360 cm$^{-1}$) (39). All of these osm shifts greatly exceed the ring/chain coplanarization effect of 1200–1300 cm$^{-1}$ deduced from the $\lambda_{\text{max}}$ difference between native retinal and planar 1,1-dimethyl retinal or the (6-S)-trans-locked retinal.

Chromophore/protein interactions that enable the significant red shift of BR are too complex to be modeled by simple synthetic model compounds. A retinal analog approach is advantageous in that artificial retinals are used to explore the actual real protein binding site. However, we should keep in mind that retinal analogs may be affected differently than the native chromophore. For this reason, the OSS reported here for BR(I) and BR(II), although revealing an effective wavelength regulation mechanism in the retinal binding site, do not quantitatively measure the amount of OS in native BR from this mechanism. Nevertheless our studies demonstrate that some protein/chromophore interactions other than those involved in retinal ring/chain coplanarization and the weakened counte-
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J. Biol. Chem. 1995, 270:29668-29670.
doi: 10.1074/jbc.270.50.29668

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