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Primary picosecond molecular events in the photoreaction of the BR5.12 artificial bacteriorhodopsin pigment

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ABSTRACT **The picosecond dynamics of the photoreaction of an artificial bacteriorhodopsin (BR) pigment containing a retinal in which a five-membered ring spans the C-12 to C-14 positions of the polyene chain (BR5.12) is examined by using time-resolved absorption and fluorescence and resonance Raman spectroscopy. The ring within the retinal chromophore of BR5.12 blocks the C-13=C-14 isomerization proposed to be a primary step in the energy storage /transduction mechanism in the BR photocycle. Relative to the native BR pigment (BR-570), the absorption spectrum of BR5.12 is red-shifted by 8 nm. The fluorescence spectrum of BR5.12 closely resembles that of BR-570 although the relative fluorescence yield is higher (≈10-fold). Picosecond transient absorption (4-ps pulses, 568–662 nm) measurements reveal an intermediate absorbing to the red side of BR5.12. Kinetic fits show that the red-absorbing intermediate appears within <3 ps and decays with a time constant of 17 ± 1 ps to form only BR5.12. No emission in the 650- to 900-nm region can be attributed to the red-absorbing species. Since rotation around C-12—C-13 and isomerization around C-13=C-14 are prevented in BR5.12, these results demonstrate that motion in these regions of the retinal is (*i*) necessary to form the K-like intermediate observed in the native BR-570 photocycle and (*ii*) not necessary to form a red-absorbing intermediate that has spectral and kinetic properties analogous to those of J-625 in the native BR photocycle. Discussions of the excited and ground electronic state assignments for the intermediate observed in the BR5.12 photoreaction are presented.**

The archaebacterium *Halobacterium halobium* uses sunlight under anaerobic conditions as an energy source for photosynthesis in the purple membrane, which contains a single transmembrane protein, bacteriorhodopsin (BR). Light absorption by BR results in vectorial transport of a proton across the cellular membrane (1, 2). The light-adapted form of BR (BR-570) contains an *all-trans*-retinal chromophore (Fig. 1*A*) that is covalently bound to Lys-216 via a protonated Schiff base. After light absorption, the BR-570 pigment undergoes a series of structural and electrostatic changes that have been identified through transient absorption spectroscopy in terms of at least two photophysical (excited electronic state) intermediates (H and I) and six photochemical (ground state) intermediates (J, K, L, M, N, and O) (1, 2).

The earliest stages of the molecular mechanism by which light energy is stored by BR is of particular importance (2–9). Involving changes in the retinal chromophore, the protein, and/or interactions between the two, these primary molecular events occur within femto/picoseconds of optical excitation in BR and, therefore, encompass the optically populated Franck–Condon (FC) (H) and vibrationally relaxed (I) levels of the excited electronic state of BR-570 (BR*-570). In addition, the J and K photochemical intermediates, having absorption maxima near 625 nm and 590 nm, respectively, are formed within the initial 10 ps (4–9). Transient absorption data have shown that (*i*) BR*-570(H) decays to BR*-570(I) in 100–200 fs (7, 8), (*ii*) BR*-570(I) decays directly to the J intermediate within an ≈500-fs time constant (4–7), and (*iii*) the J intermediate decays to form K with an ≈3.5-ps time constant (5, 6, 9). No additional absorption changes are observed for at least 5 ns (10). The J intermediate has been generally viewed as a ground electronic state species (11), although this assignment has been questioned (12).

Fluorescence spectroscopy has been utilized to characterize the initial BR photocycle intermediates (9, 13, 14). Time-resolved measurements reveal that fluorescence from K*-590 appears with the same ≈3.5-ps time constant found via absorption (9, 13). The maximum of the K*-590 fluorescence spectrum is blue-shifted by ≈17 nm relative to BR*-570 and K*-590 has a 2-fold higher fluorescence yield (13, 14). No fluorescence has been associated with J-625 (9, 13).

An understanding of the structures of the J and K intermediates is critical to elucidating the primary energy storage/transduction mechanism for absorbed light in the BR photocycle. Time-resolved resonance Raman (RR) spectroscopy has been used to determine changes in the vibrational degrees of freedom associated with these early intermediates (2, 15–20). Although these studies have yet to firmly characterize the structural nature of J-625 (16, 18, 20), it appears that J-625 contains a highly twisted retinal [deduced from the strong HOOP modes observed in the 900- to 1000-cm^{-1} region (16, 18)]. The retinal structure of K-590 is clearer. RR studies suggest that it contains a 13-*cis*-retinal configuration, indicating that K-590 formation involves 13-*trans* to 13-*cis* isomerization (15, 17).

The role of 13-*trans*- to 13-*cis*-retinal isomerization in the formation of BR photocycle has been tested with an artificial BR pigment containing a cyclopentane ring spanning the C-12—C-13=C-14 region of the retinal (Fig. 1*B*), which prevents isomerization (and even significant motion) at the C-13=C-14 bond (21–23). Flash photolysis studies with time resolutions between 50 ns and 10 s at both room and liquid nitrogen temperatures and Fourier transform IR studies conducted at liquid nitrogen temperature show that an M intermediate is not formed (22) and that proton pumping does not occur (21–23). These studies indicate that 13-*trans*- to 13-*cis*-retinal isomerization is essential for at least the latter stages of the BR photocycle and for proton pumping.

Analogous studies of rhodopsin (Rh) have been reported in which the contribution of C-11=C-12 isomerization to the visual pathway has been evaluated by incorporating the

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Abbreviations: BR, bacteriorhodopsin; RR, resonance Raman; Rh, rhodopsin; PTA, picosecond transient absorption; PTRF, picosecond time-resolved fluorescence; CCT, cross-correlation time; FC, Franck–Condon.

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C-11=C-12 bond within five-, seven-, or eight-membered rings (24–26). Excitation (25 ps) of Rh5, the five-membered ring pigment, produces long-lived (85-ps lifetime) fluorescence assigned to the FC excited electronic state (25). Two studies of Rh7, the seven-membered ring pigment, report at least one red-shifted intermediate (24, 25). The red-shifted absorption appears within the experimental time resolution (10 ps) and decays with a time constant of 20 ± 10 ps (24). Different conclusions are reached concerning whether this intermediate is an excited electronic (24) or ground-state (25) species. Rh8, the eight-membered ring pigment, forms two red-shifted intermediates on the picosecond time scale (26). The second intermediate, attributed to a batho-like species, decays in nanoseconds (26). The different photochemical reactions in the Rh5, Rh7, and Rh8 pigments have been interpreted in terms of the increasing flexibility of the ring around the C-11=C-12 bond (26).

To more clearly elucidate the importance of the C-13=C-14 bond in the native BR photocycle and to the formation of J and K intermediates, picosecond transient absorption (PTA) and picosecond time-resolved fluorescence (PTRF) spectroscopy are measured here from the artificial BR pigment (BR5.12) containing a cyclopentane ring spanning the C-12—C-13=C-14 retinal region (Fig. 1*B*).

EXPERIMENTAL PROCEDURES

The BR5.12 sample is formed by reconstituting the artificial 5.12 retinal analog with BR as described (21). The opsin is prepared from purple membrane fragments treated with hydroxylamine in the presence of light. The retinal oxime is removed by washing with bovine serum albumin (27). PTA and PTRF measurements are performed as described (9, 13). Briefly, the 527-nm second harmonic output from a Nd/YLF laser (Coherent Radiation, Palo Alto, CA) synchronously pumps two cavity-dumped dye lasers (Coherent 700), each operating with a 1-MHz repetition rate. The temporal pulsewidths of both dye lasers are 4 ps (FWHM) as measured by autocorrelation. The cross-correlation time (CCT), defined by the temporal overlap between the two Gaussian dye laser pulses, is 6 ps giving a jitter of ±1 ps. The BR5.12 photoreaction is initiated by the pump dye laser at 573 nm with a power of 6 mW. The wavelength of the probe dye laser pulses (0.4 mW) can be tuned from 560 to 670 nm. The BR sample is examined as a flowing jet at 10°C.

The fluorescence spectrum is measured by using a scanning 1-m spectrometer [1.2-nm band pass (14)]. RR scattering is detected with a triple monochromator and an intensified multichannel detector (19). The overall detection system has a resolution of 8 cm^{-1} (FWHM).

RESULTS

Absorption Spectroscopy. Substitution of the native *all-trans*-retinal (Fig. 1*A*) with the artificial retinal (Fig. 1*B*) to form BR5.12 does not significantly alter its absorption spectrum (Fig. 1*C*). Compared to that of the native BR-570 pigment, the BR5.12 absorption spectrum is slightly narrower and the maximum is red-shifted by ≈8 nm to 578 nm.

The absorption coefficients for BR5.12 are estimated by reconstituting BR with an excess of *all-trans*-retinal or retinal 5.12. The resultant optical density of the BR5.12 sample is ≈0.33 that of the native BR-570 sample. Artificial BR pigments generally have reconstitution efficiencies that are <0.5 that of the native BR pigment (28), leading to the conclusion that the absorption coefficients of BR5.12 and BR-570 are similar.

PTA data are recorded by using probe wavelengths in the 568- to 662-nm spectral region and for time delays as long as 110 ps. The BR5.12 photoreaction is initiated by 573-nm excitation for 4 ± 0.3 ps at pulse energies that are selected to be in the linear absorption (nonsaturated) regime for BR5.12. An overview of PTA changes is presented in Fig. 2 for four probe wavelengths. PTA signals measured with probe wavelengths at 568 nm and 600 nm are essentially the same although both are significantly different than those measured at 630 nm and 662 nm. The decreases of the 568-nm and 600-nm PTA signals within the CCT (6 ± 1 ps) reflect optical depletion of the ground-state BR5.12 population during pumping. Subsequently, these signals increase with a time constant of 17 ± 1 ps to a constant zero (≈1%) value. In contrast, the 630-nm signal decreases only slightly within the CCT and remains constant throughout the initial 110 ps. These observations suggest that 630 nm is near an isobestic point for absorption from the stable and transient species present. The 662-nm PTA signal increases within the CCT before decreasing with a time constant of 17 ± 1 ps to a constant zero (≈4%) value. Overall, the 566- to 662-nm PTA data show that one intermediate is formed within the CCT and that it decays with a time constant of 17 ± 1 ps.

Fluorescence Spectroscopy. The fluorescence spectrum observed in the 650- to 900-nm region after low-power 600-nm excitation of BR5.12 (data not shown) closely resembles that measured from native BR-570 (λ_{max} at 748 nm for BR*5.12 and at 740 nm for BR*-570) (14). The fluorescence intensity from BR*5.12, relative to that from BR*-570 (9, 14), is ≈10 times larger (for samples of the same optical density). Based on the conclusion that the absorption coefficients of BR5.12 are not larger than those of BR-570, *vide supra*, the fluorescence yield of BR5.12 is also ≈10 times larger than that of BR-570.

PTRF experiments measure (at a given time delay after the pump pulse) the integrated fluorescence emitted from any excited state(s) populated by the 7-ps probe pulse. The PTRF signal, measured at 750 nm after the 7-ps 573-nm excitation of

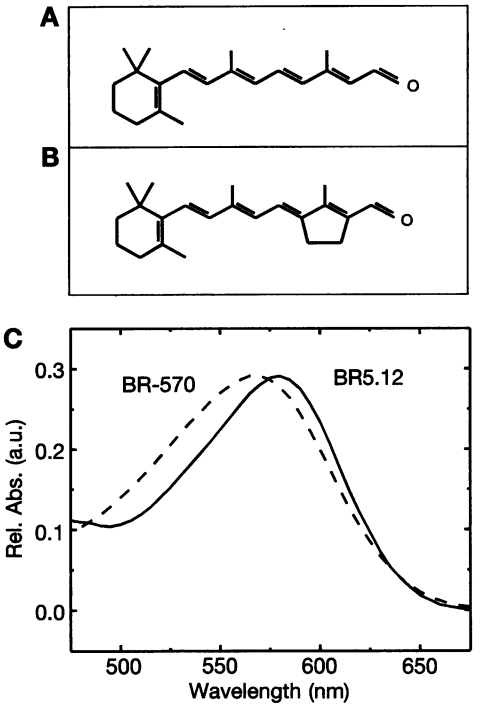

FIG. 1. (*A*) Chemical structure of *all-trans*-retinal appearing in native BR-570. (*B*) Chemical structure of the chemically modified 5.12 retinal having a cyclopentane ring spanning the C-12—C-13=C-14 region of the retinal polyene chain. (*C*) Absorption spectrum of native BR-570 (dashed line, λ_{max} at 570 nm) and the artificial BR pigment BR5.12 formed by reconstituting bacterial opsin with retinal 5.12 (solid line, λ_{max} at 578 nm). Rel. Abs. (a.u.), relative absorbance (arbitrary units).

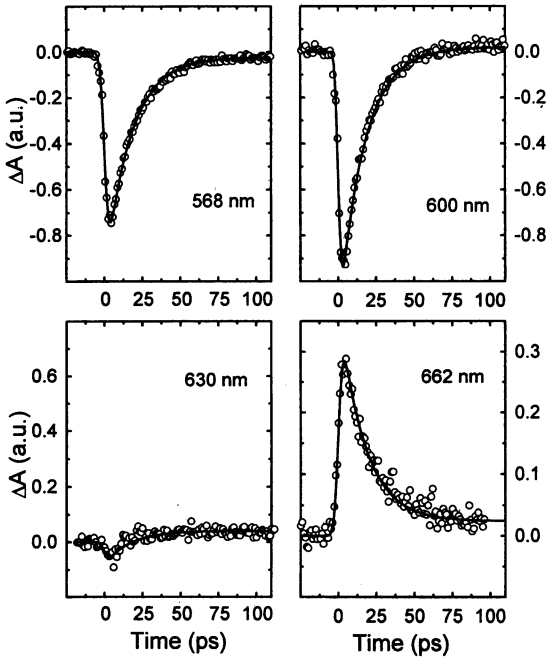

FIG. 2. PTA spectroscopy of BR5.12 (OD_{578} = 1.5 in H_2O at pH 6.5). Excitation occurs with 4-ps pulses at 573 nm and the transient absorption is measured with 4-ps pulses at 568 nm, 600 nm, 630 nm, and 662 nm. PTA data (open circles) are kinetically fit (solid line) by using a model that represents the convolution of the 6-ps CCT with the kinetic function, $\Delta A(t) = A_1\exp(-t/\tau_1) + b$, where $\tau_1 = 17 \pm 1$ ps.

BR5.12, therefore, monitors the ground-state populations formed during the BR5.12 photoreaction.

PTRF data from BR5.12 are recorded independently with three probe wavelengths (600 nm, 628 nm, and 652 nm). For all three probe wavelengths, PTRF data with the same characteristics are observed: a decrease in fluorescence intensity during the CCT followed by increased fluorescence intensity, which returns to the initial level observed from ground-state BR5.12. The kinetics describing the 652-nm PTRF data (Fig. 3) are well represented by a single exponential curve with a time constant of 18 ± 3 ps (in excellent agreement with the decays of 17 ± 1 ps observed by PTA, *vide supra*). The return of the PTRF signal to the ground-state BR5.12 level (>80 ps) is within ±1% (Fig. 3). These observations show that the red-absorbing BR5.12 intermediate exhibits no fluorescence in

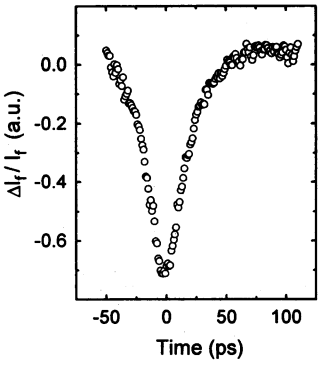

FIG. 3. PTRF of BR5.12. ($\Delta I_f/I_f$) vs. time after excitation with a 7-ps pump pulse at 573 nm is plotted. I_f is the emission intensity at 750 nm initiated by 652-nm 7-ps probe pulses. ΔI_f is the difference between I_f with and without the 7-ps pump pulses at 573 nm. The recovery of the PTRF signal is well described by a single exponential curve with a time constant of 18 ± 3 ps convoluted with the measured 12-ps CCT.

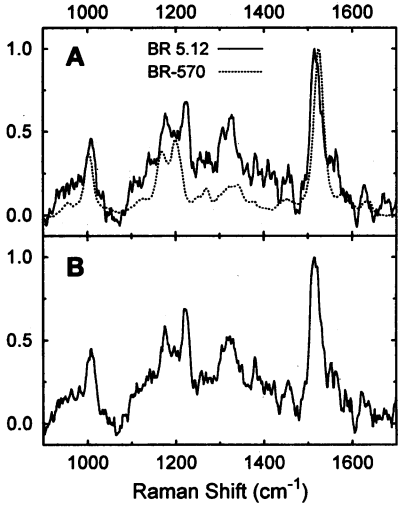

FIG. 4. (*A*) RR spectra of BR5.12 (solid line) and native BR-570 (dashed line) excited with 7-ps pulses at 568 nm (1.7 mW). Comparison of the RR spectra of BR5.12 (OD_{578} = 2) with native BR-570 shows distinct differences in the C—C region attributed to the presence of the five-membered ring; BR5.12 has a C=C band at 1514 cm^{-1}, C—C bands at 1221 cm^{-1} and 1177 cm^{-1}, a CH_3 rock band at 1008 cm^{-1}, and strong bands at 1306 cm^{-1} and 1327 cm^{-1}. (*B*) RR spectrum of BR5.12 recorded after 573-nm excitation (7 ps, 12 mW) for 20 min. The excitation is selected to reproduce the photoexcitation conditions used during several PTA measurements of BR5.12. These results establish that no stable photoproduct other than BR5.12 is formed by the excitation used to measure the PTA data reported here.

the 650- to 900-nm region and decays to a species that has the same fluorescence properties as BR5.12.

RR Spectra. RR spectra of ground-state species (i.e., recorded with pulse energies low enough to prevent significant photochemistry) show that the radiation used for the PTA experiments does not produce irreversible photochemistry but, rather, results in the reformation of BR5.12. The low-power (1.7 mW) RR spectrum of BR5.12 is presented in Fig. 4*A*. To determine whether irreversible photochemistry occurs, the RR spectrum of this BR5.12 sample is recorded again after it has been exposed for 20 min to 12 mW of 580-nm excitation (Fig. 4*B*). There are no discernable differences between the RR spectra before and after the 580-nm excitation (Fig. 4). The absorption spectra of the BR5.12 sample before and after the 580-nm excitation also are the same. Both the absorption and RR data, therefore, indicate that BR5.12 is reformed in high yield.

Artificial BR Pigment Purity. The absorption spectrum, PTA data (absence of 3-ps decay component) and RR spectrum indicate that little (<5%), if any, of the starting samples examined here are composed of native BR-570. Previous spectroscopic studies of artificial BR pigments have been limited by the presence of contaminating amounts of native BR pigments that produce interfering signals.

DISCUSSION

Interest in obtaining experimental evidence clearly establishing the role of structural changes in the C-12—C-13=C-14 region of retinal and, especially, that of C-13=C-14 bond isomerization in the early energy storage/transduction mechanism of the native BR photocycle has motivated not only this work on BR5.12 but also that previously reported (21–23). These earlier BR5.12 studies found neither an M-like intermediate (22) nor proton pumping across the purple membrane (21–23) and, therefore, concluded that neither the later stages of a photoreaction (i.e., M, N, or O intermediates) nor a transmembrane proton gradient exists. Subsequent efforts

have shown that no photocycle intermediates are detectable on time scales longer than 50 ns (i.e., long-lived K and L are not formed) (23). To be experimentally determined, however, is whether J- and/or K-like intermediates are present on the picosecond time scale and, therefore, whether molecular motion in the C-12—C-13=C-14 bonds is required for the formation of either or both J- and K-like intermediates during the primary BR photocycle events.

Absence of K Formation. The results reported here demonstrate that a K-like intermediate is not formed in the BR5.12 photoreaction. The PTA data do show that one intermediate (denoted T5.12) with a red-shifted absorption relative to BR5.12 appears after 578-nm excitation. T5.12 decays with a time constant of 17 ± 1 ps directly and only to BR5.12 and has no detectable fluorescence. T5.12 is not a K-like intermediate, analogous to native K-590, because (*i*) K-590 is associated with the second transient red-shifted absorption to appear on the picosecond time scale after excitation of BR-570 (excluding vibrationally and electronically excited state absorptions) and (*ii*) a substantial well-characterized fluorescence signal is detectable from K-590 (9, 13, 14). Neither criterion is met by T5.12.

These data, therefore, demonstrate that molecular motion around the C-12—C-13=C-14 region of retinal is required for the formation of a K-like intermediate. Given the earlier room temperature picosecond time-resolved RR data showing that K-590 has a 13-*cis*-retinal (17), the results presented here also suggest that C-13=C-14 *trans* to *cis* isomerization is necessary for K-590 to appear in the native BR photocycle.

Nature of T5.12. Although it is shown here that blocking the C-12—C-13=C-14 bonds prevents the formation of a K-like intermediate, such restrictions in retinal motion do not prevent the formation of a red-absorbing (662-nm PTA data; Fig. 2) intermediate. These PTA data reveal the presence of any intermediate when the C-12—C-13=C-14 bonds are blocked. Given the limited understanding of the molecular processes preceding the appearance of K-590, it is important to characterize the T5.12 structure, especially since the kinetic and spectroscopic properties of T5.12 are analogous to those of J-625 in the native BR photocycle.

The PTA data recorded at 568 nm and 600 nm (Fig. 2) show a depletion of the ground-state population that recovers with a time constant of 17 ± 1 ps. The excellent agreement between the decay of the red-shifted absorption (T5.12) with the recovery of the ground-state population suggests that T5.12 decays directly to BR5.12. This conclusion is strongly supported by the observation that all PTA data return to values near (1–4%) zero (i.e., the absorbance of the BR5.12). In addition, the PTRF and RR data confirm that the only product of T5.12 is BR5.12 and not another species with similar absorbance (i.e., the fluorescence yields, spectra, and the vibrational degrees of freedom are the same as those of BR5.12). Whatever the nature of T5.12, it decays directly and only to ground-state BR5.12.

In analogy to the native BR photocycle, there are two likely assignments for T5.12: (*i*) an excited electronic state populated either via a direct FC transition [BR*5.12(H)] or via subsequent vibrational relaxation [BR*5.12(I)] or (*ii*) a transient ground-state intermediate.

If T5.12 is electronically excited, an assignment as BR*5.12(H) is unlikely since the FC state of native BR decays much more rapidly [<200 fs (8)] than the decay (17 ± 1 ps) of T5.12 reported here, the absorption originating from the T5.12 is much farther to the red than the absorption of the FC state, and the large (170 nm) spectral shift found in the BR5.12 fluorescence suggests that it originates from BR*5.12(I) [as from BR*-570(I) (8)].

If T5.12 is a vibrationally relaxed excited electronic state, it can involve coordinates other than those that lead to BR*-570(I). In BR-570, the transformation from BR*-570(H) to BR*-570(I) is thought to involve a 90° twist around the C-13=C-14 coordinate in retinal that occurs in 100–200 fs (8). Since motion around the C-13=C-14 bond is blocked in BR5.12, the corresponding BR*5.12(I) state must involve changes in other retinal coordinates or the characterization of structural change in the native BR-570 is incorrect. Thus, the assignment of T5.12 as BR*5.12(I) implies that molecular changes associated with the decay of the FC state occur in the retinal outside the C-12—C-13=C-14 bonds. Such an assignment, however, cannot be supported by the fluorescence data. Unfortunately, no fluorescence lifetime data are currently available that would conclusively resolve this issue.

Arguments related to the T5.12 assignment may also be derived from a comparison of the absorption properties of excited electronic states with those of ground-state transitions in both native BR and Rhs. Both BR*-570(H) and BR*-570(I) absorb to the blue side of the BR-570 [indeed, BR*-570(I) absorbs at <500 nm] (4, 5, 8). Rh also has FC and vibrationally relaxed excited state absorptions that appear near or just to the blue side of its ground-state transition (29). Thus, the assignment of the observed T5.12 (with its far-red-shifted >630-nm absorption) to an excited electronic state is inconsistent with the known absorption properties in native retinal proteins.

If T5.12 is a ground-state intermediate, then assignment to either a vibrationally excited (BR′5.12) or a distinct electronic state species needs to be considered. T5.12 is likely not assignable as BR′5.12 since the observed decay of 17 ± 1 ps is much longer than the known vibrational relaxation in native BR-570 (2–7 ps) (19, 20). The assignment of T5.12 as a distinct electronic ground state requires that T5.12 decays directly to BR5.12 and this implies that T5.12 is formed through some retinal distortions outside the C-12—C-13=C-14 region and/or changes in the protein–retinal interactions. Such an assignment does not present any recognizable inconsistencies with respect to known BR-570 properties, but it may require revision of the molecular mechanism currently used to describe the formation of J-625 in the native BR photocycle (*vide infra*).

On the Nature of the Fluorescence State in BR-570 and BR5.12. The question has been raised as to whether the fluorescence observed from BR-570 originates from BR*-570(I) or from another excited electronic state (28). The assignment of the fluorescence to BR*-570(I) is based on stimulated fluorescence lifetimes that match the ≈0.5-ps time constant observed for the BR*-570(I) to J-625 transition (7). The assignment of fluorescence initiated by conventional low-intensity illumination to BR*-570(I), however, awaits conformation by either (*i*) a wavelength-resolved spectrum of the stimulated emission that can be compared in detail with that of the spontaneous fluorescence or (*ii*) measurement of the lifetime of the spontaneous fluorescence showing that it matches the BR*-570(I) to J-625 time constant.

The observation reported here that the ground-state absorption and fluorescence from BR5.12 and BR-570 are similar (*vide supra*) may provide insight into this issue. Excluding a rare coincidence, it appears that spontaneous fluorescence from BR-570 and BR5.12 cannot originate in the same type of I state [since BR*-570(I) is thought to have a 90° twisted retinal along the C-13=C-14 bond, a structure not accessible to BR5.12]. Thus, either fluorescence from both BR-570 and BR5.12 originates in an excited electronic state other than their respective I states, potentially involving motion along retinal coordinates other than the C-13=C-14 bond, or the similarity of fluorescence from the two molecules may imply that the I states are not twisted by 90° about the C-13=C-14 bond [thereby permitting BR*5.12(I) to be a precursor of T5.12].

Implications for Native BR Photocycle. Independent of the assignment of T5.12, its existence alone focuses attention on the relationship between the respective retinal structures of

T5.12 and J-625. If T5.12 and J-625 are structurally identical, then the formation of both intermediates does not require isomerization around the C-13=C-14 bond. This possibility is consistent with the fluorescence results described above. Accordingly, the retinal chromophores in the I and J/T species of BR-570 and BR5.12 maintain the basic *all-trans* structure found in the respective ground states. Thus, the formation of J/T intermediates (and the corresponding red-shifted absorptions) would require motion in retinal bonds outside the C-12—C-13=C-14 region and/or changes in the protein environment. Such a model, however, would still require the J-625 → K-590 transformation to involve motion in the C-12—C-13=C-14 region of retinal (based on K-590 having a 13-*cis*-retinal).

If T5.12 and J-625 are structurally different, the conventional model describing the BR-570 photocycle remains unchanged; namely, the retinal chromophore in J-625 is twisted by at least 90° around the C-13=C-14 bond. The formation of T5.12 in the BR5.12 photoreaction, therefore, must involve molecular motion around retinal bonds other than those in the C-12—C-13=C-14 region. This would produce a ground-state T5.12 that is structurally different than J-625 but spectroscopically similar and that cannot transform into a K intermediate.

Summary Remarks. The results presented here experimentally demonstrate with picosecond time resolution that motion in the C-12—C-13=C-14 region of retinal is required to obtain a K intermediate. Neither molecular motion in retinal outside the C-12—C-13=C-14 bonds nor retinal–protein interactions are effective in forming (stabilizing) a K-like species in the BR5.12 photoreaction. By analogy, it can be concluded that the C-12—C-13=C-14 bonds in native BR-570 must be free to change if K-590 is to be formed. Given earlier results from picosecond-time-resolved RR spectroscopy that show native K-590 to have a 13-*cis*-retinal (15, 17), these observations are consistent with the conclusion that *trans* → *cis* isomerization of the C-13=C-14 bond is a necessary condition for the room temperature formation of K-590. The specific role, if any, of rotation around the C-12—C-13 is not yet determined. These conclusion are independent of the exact nature of T5.12 and its red-shifted absorption.

A conclusive identification of T5.12 remains to be made. Some photochemical/photophysical transformations linked to the red-shifted absorption may yet occur even though motion in the C-12—C-13=C-14 bonds are restricted. If T5.12 is structurally the same as J-625, even with the C-12—C-13=C-14 region blocked, then the molecular mechanism describing the primary BR events must be altered.

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