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Photolytic Interruptions of the Bacteriorhodopsin Photocycle Examined by Time-Resolved Resonance Raman Spectroscopy†

Iris Grieger and George H. Atkinson*
Department of Chemistry, University of Arizona, Tucson, Arizona 85721
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ABSTRACT: An investigation of the photolytic conditions used to initiate and spectroscopically monitor the bacteriorhodopsin (BR) photocycle utilizing time-resolved resonance Raman (TR^3) spectroscopy has revealed and characterized two photoinduced reactions that interrupt the thermal pathway. One reaction involves the photolytic interconversion of M-412 and M′, and the other involves the direct photolytic conversion of the BR-570/K-590 photostationary mixture either to M-412 and M′ or to M-like intermediates within 10 ns. The photolytic threshold conditions describing both reactions have been quantitatively measured and are discussed in terms of experimental parameters.

Bacteriorhodopsin (BR)[1] has been identified as a protein complex containing the chromophore retinal in the purple membrane (PM)[1] of *Halobacterium halobium*. It functions in the PM to convert absorbed light into the energy required to translocate protons across the membrane and to perform ATP[1] synthesis. These fundamental biochemical activities were first reported by Oesterhelt & Stoeckenius (1973) and have been substantiated subsequently by many workers [for review, see Stoeckenius et al. (1979)].

The maximum absorption of the BR complex occurs at 570 nm, which has led to the designation BR-570. The optical excitation of BR-570 initiates a chemical cycle involving several distinct intermediates [including K-590, L-550, M-412, and O-640 (Stoeckenius et al., 1979)]. Although these intermediates have been only partially characterized, several important features of the molecular bonding and conformation of the retinal chromophore have been elucidated by transient spectroscopies. The early studies of the BR photocycle were derived primarily from transient absorption spectroscopy, while recently, more structurally sensitive data have been obtained from spectroscopies based on vibrational Raman scattering (Lewis et al., 1974; Aton et al., 1977; Marcus & Lewis, 1978; Terner et al., 1979; Stockburger et al., 1979; Braiman & Mathies, 1980; Atkinson, 1981). Vibrational resonance Raman (RR)[1] spectroscopy has been especially useful for examining the bonding and conformations of molecules containing retinal. Specific regions of the RR spectra of the retinal chromophore have been associated with a variety of structural parameters via studies of both biochemically active compounds and synthetically prepared model systems (Eyring et al., 1980a,b; Alshuth & Stockburger, 1981; Massig et al., 1982). These data provide the essential information required to identify the structure of retinal, even in a complex biochemical system such as BR. RR spectroscopy, therefore, is particularly well suited to examine the species that comprise the BR-570 photocycle.

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; ATP, adenosine 5′-triphosphate; RR, resonance Raman; TR^3, time-resolved resonance Raman; cw, continuous wave; SDS, sodium dodecyl sulfate; fwhm, full width at half-maximum.

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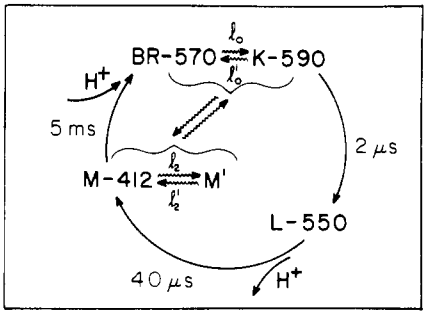

FIGURE 1: Photocycle of bacteriorhodopsin (BR). The l_0 terms denote rate coefficients for photoconversion. Reaction rates refer to PM suspensions at neutral pH and room temperature. The intermediates are given with a subscript to indicate the wavelength of their absorption maximum. The reaction BR-570 ⇌ K-590 → L-550 → M-412 → BR-570 is denoted as the thermal photocycle of BR. Additional pathways due to photolytic interruptions are indicated with braces. The rapid (10-ns) conversion is indicated as occurring only in the presence of radiative excitation (i.e. ⇝). Our data are suggestive on this latter point and are not conclusive.

Two important conclusions concerning retinal are noteworthy for the work presented here. First, it is generally accepted that retinal in the light-adapted PM complex is in the all-trans configuration and is bound to the protein via a protonated Schiff base linkage (Oesterhelt & Stoeckenius, 1973; Lewis et al., 1974; Sperling et al., 1977; Stockburger et al., 1979). Second, time-resolved RR (TR^3)[1] techniques have been used by several workers to show that retinal in the M-412 intermediate (Figure 1) is in the 13-cis configuration and is bound to the protein by an unprotonated Schiff base linkage (Pettei et al., 1977; Campion et al., 1977; Stockburger et al., 1979; Braiman & Mathies, 1980; Tsuda et al., 1980; Alshuth, 1982). The pathways associated with the molecular transformation from BR-570 to M-412 are of particular interest since they involve both the isomerization of the retinal chromophore and a change in the protonation environment of the Schiff base linkage.

Studies of the BR photocycle by transient spectroscopies such as TR^3 scattering require that a distinction be made between molecular changes that proceed at thermal energies after photoinitiation from BR-570 and molecular changes that occur only in the presence of exciting radiation (i.e., from vibronically excited states). These questions can be treated experimentally through the precise control of the radiation intensity used both to initiate the photocycle and to monitor the intermediates formed during the photocycle. Ideally, the photolytic initiation involves the excitation of only one ground-state species, namely, BR-570. The rapid (<1-ns) relaxation of vibronically excited BR-570 ensures that the remainder of the photocycle is comprised of reactions that occur on ground-state potential surfaces and therefore are accessible to thermal energies.[2] In this paper, these reactions are termed the thermal cycle (Figure 1: BR-570 ⇌ K-590 → L-550 → M-412 → BR-570) and involve molecular changes that appear to proceed without subsequent irradiation. If spectroscopic results are to be accurately interpreted, then it is well recognized that these ideal experimental conditions are important to emulate. To date, the precise irradiation conditions required to do so have not been well characterized. The BR photocycle is an expecially challenging example because of the strongly overlapped absorption spectra of the primary

[2] This assumes that K-590 is a ground-state species rather than an excited electronic state of BR-570, which is a question that remains open to study.

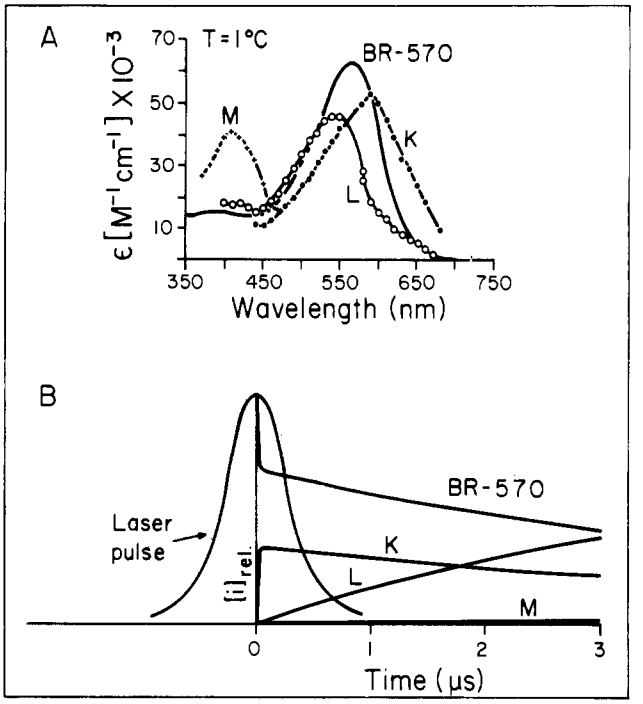

FIGURE 2: (A) Absorption spectra of the intermediates of the BR photocycle at 1 °C [adapted from Lozier et al. (1975)]. (B) Time evolution of the intermediates in the microsecond time regime compared with a nanosecond laser pulse. The relative concentrations of BR-570, K-590, L-550, and M-412 ($[i]_{rel}$) are shown schematically.

intermediates and the rapid formation of the intermediates K and L relative to the pulse width of excitation typically used (Figure 2). The overlapping absorption spectra of BR-570, K-590, and L-550 almost ensure the simultaneous excitation of all three species when the photocycle is initiated. Time-resolved measurements do not provide an easily accessible alternative since the rapid formation of K-590, and even L-550 (schematically illustrated in Figure 2B), requires pulsed excitation of only a few picoseconds to time resolve the excitation of BR-570 only. The initiation of the photocycle with nanosecond and microsecond pulse width lasers, therefore, results in the population of excited electronic states in K-590 and L-550 intermediates as well as BR-570. In such cases, the photochemistry of K-590 and L-550 can be superimposed on the thermal cycle of BR-570, which is often the topic of prime interest. A thorough quantitative understanding of the photolytic conditions that cause such "photolytic interruptions" of the BR photocycle is not available although the topic has been addressed quantitatively in previous studies (Hess & Kuschmitz, 1977; Litvin & Balashov, 1977; Hurley et al., 1978; Ormos et al., 1980; Kalisky & Ottolenghi, 1982).

In this study, TR^3 spectroscopy is utilized to record the formation and disappearance of the M-412 and M′ intermediates in order to reveal the conditions of excitation and monitoring that cause photolytic interruptions of the BR photocycle. Attention is given to the radiative intensities used to initiate the photocycle of BR-570 and generate RR scattering from M-412 and M′ and to the time period after initiation at which the M intermediates appear. The interdependence of these experimental parameters makes it feasible to elucidate new molecular pathways involving both the thermal and photolytic interconversion of BR-570, K-590, M-412, and M′.

Experimental Procedures

Materials. Samples of the PM containing BR were prepared from *H. halobium* S9 strain by the procedure of

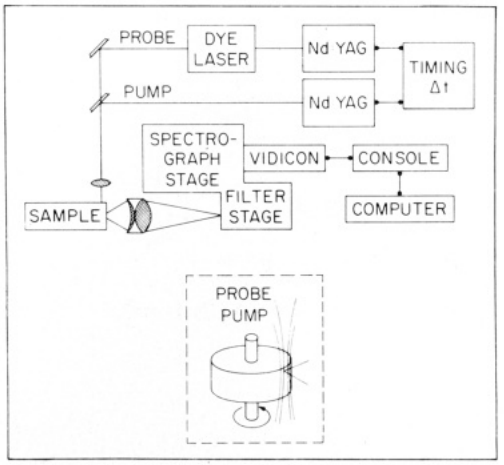

FIGURE 3: Schematic of the time-resolved resonance Raman (TR^3) instrumentation. The pump laser is a frequency-doubled Nd:YAG laser (532 nm) while a Nd:YAG pumped dye laser (420 and 440 nm, respectively) probes the intermediates via TR^3 spectroscopy. A doubly intensified vidicon camera detects the resonance Raman scattering from only the top 0.8 mm of the sample which was contained in a spinning cell.

Oesterhelt & Stoeckenius (1974). These samples gave a ratio of absorbance at 280 nm to that at 568 nm of about 2 and produced only a single band when subjected to SDS[1] gel electrophoresis. Samples were suspended in doubly distilled water (pH 6.6) or in deuterated water (99.75%). Raman spectra were recorded on room temperature samples of the suspension having an optical density of 4 at 565 nm.

Instrumentation. The instrumentation used to record TR^3 spectra (shown schematically in Figure 3) was of a pump–probe configuration that was designed to provide versatility with respect to the experimental parameters associated with both initiating and probing the BR photocycle. Two separate pulsed laser systems (a Nd:YAG laser and a Nd:YAG pumped dye laser) were used. Both lasers were operated at 15 Hz and were synchronized electronically to appear at the sample with a variable time delay, Δt. The second harmonic frequency of the Nd:YAG laser (Quanta Ray DCR-1; fwhm pulse width = 10 ns, λ = 532 nm) was used to initiate the BR photocycle. The Nd:YAG pumped dye laser (Quanta Ray DCR-1 and PDL-1; fwhm pulse width = 10 ns, λ = 420 or 440 nm) was used to generate RR scattering from the M-412 (and M′) intermediates. The dye laser operating with stilbene 420 and coumarin 440 was pumped by the third harmonic frequency of the Nd:YAG laser at 347.5 nm. The average power from each laser was measured with a power meter (Scientech, Model 380105) and, together with separate measurements of the repetition rate and pulse width of the laser, was used to calculate peak powers with a precision of ±5%.

The sample was contained in a quartz spinning cell in order to continously present unphotolyzed BR to the excitation region (Stockburger et al., 1979). Radiation from both the initiation and probe lasers was focused into the spinning cell. The beam diameters were 360 μm for the pump laser and 260 μm for the probe laser. This excited area and the spinning rate of the sample cell were correlated in order to assure that the time for the BR sample in the laser beam was determined by the duration of the laser pulse. For time delays $\Delta t \leq 450$ ns, the two laser beams were collinear and were superimposed in the sample that had a flow velocity of 0.5 ms^{-1}. In the other experiments with time delays in the microsecond time regime, the two laser beams were separated spatially. Considering Δt = 200 μs as an example, the beams were separated by 40 μm at a flow velocity of 0.2 ms^{-1}. This alignment ensured that only excited molecules were probed at the given time delay.

The resonance Raman scattering was collected at 90° to the incident radiation and focused onto the 300-μm slit of a triple spectrometer (Spex Triplemate) equipped with two 600 line/mm conventionally ruled gratings and a 2400 line/mm holographic grating. In order to minimize the absorption of laser radiation in the sample, the Raman scattering detected came from within 0.8 mm of the window through which both laser beams entered the spinning cell. A vidicon used in conjunction with two stages of intensification (EG & G, PARC optical multichannel analyzer, Model 1205I ISIT) was used to detect the Raman signal. The vidicon face was positioned in the focal plane of the spectrometer by an adjustable mount, but no optical imaging external to the spectrometer was used. The overall dispersion of the entire detection system was 0.75 cm^{-1} $channel^{-1}$. The detector was interfaced to an on-site computer for data acquisition and analysis.

Methods. The optical conditions used to initiate and probe the BR photocycle were quantitatively measured by means of a photolability parameter, l_i. The formulation of l_i, given by

$$l_i = \Phi_j \, \sigma(\lambda) \, I(\lambda,r) \qquad (1)$$

where Φ_j is the quantum yield for the photochemical process involving the formation of species j, $\sigma(\lambda)$ is the absorption cross section of a particular species at λ, and $I(\lambda,r)$ is the intensity of the exciting radiation at λ in a beam of diameter $2r$, was presented previously for the BR photocycle (Stockburger et al., 1979). Considering the initiation of the BR photocycle as an example, l_0 refers to the formation of K-590 from BR-570. The value of l_i, taken together with the time during which the molecule of interest remains in the excitation beam, t, leads to the definition of a threshold condition for establishing a photostationary mixture: $l_i t > 1$ (Alshuth et al., 1983). The residence time of the sample in the laser beam when a spinning cell is used and the specific focusing parameters required to meet this threshold condition are determined by the pulse width of the laser, namely, 10 ns. Although the pulse width was fixed in this work, the value of l_0 could be changed by increasing the intensity of the excitation laser $I(\lambda,r)$ (eq 1). These parameters can be defined in terms of other photolytically induced reactions such as the conversion of M-412 to M′, where $l_i = l_2$ and $\Phi_2' = 0.5$ (Hurley et al., 1978).

Results and Discussion

RR Spectra of M-412 and M′. The RR spectrum of M-412 has been reported previously from work using cw[1] laser excitation and a flow technique (Stockburger et al., 1979; Braiman & Mathies, 1980). Stockburger et al. (1979) also had suggested that another unprotonated intermediate, absorbing near 412 nm and designated M′, was photolytically formed from M-412 with an $l_2 t$ value of 36. Subtraction methods involving RR spectra recorded with two different values of $I(\lambda,r)$ were used to obtain a RR spectrum of M′. The position of the C=C stretching frequency in the M-412/M′ mixture was reported at 1563 cm^{-1} (Stockburger et al., 1979), but its position in the M′ spectrum alone remained unclear.

The RR spectrum of the M′ intermediate presented here was recorded by using the two-laser pump–probe instrumentation described above with a time delay of 200 μs, probe wavelengths of 420 and 440 nm, and photoconversion parameters $l_2 t$ of 42 and 37, respectively (Figure 4). For these studies, the C=C stretching bands at 1567 (420-nm probe) and 1566 cm^{-1} (440-nm probe) were selected to identify the M-412 species.

It is clear in Figure 4 that the 1567- and 1566-cm^{-1} bands associated with M-412 are accompanied by prominent

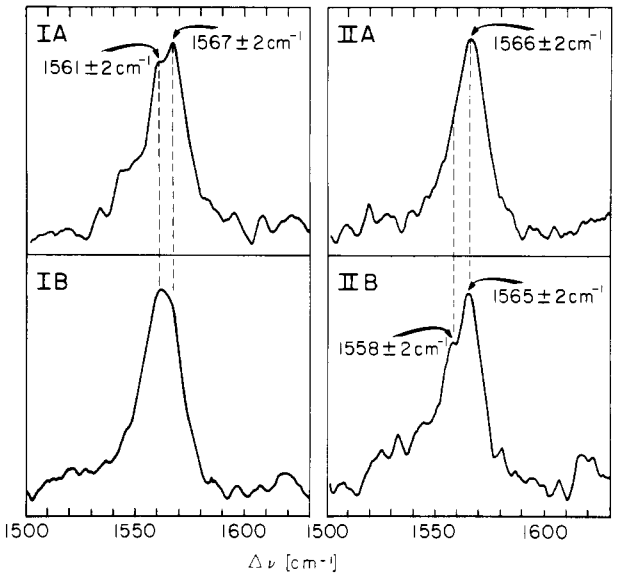

FIGURE 4: TR^3 spectra of the C=C stretching vibration of M-412 and M′. The spectra were taken with a 200-μs time delay between the pump (λ = 532 nm, $I_0 = 3 \times 10^8$ s^{-1}) and probe laser (panels I, λ = 420 nm, $I_2 = 4.2 \times 10^9$ s^{-1}; panels II, λ = 440 nm, $I_2 = 3.7 \times 10^9$ s^{-1}). BR was suspended in either H_2O (panels IA and IIA) or D_2O (panels IB and IIB).

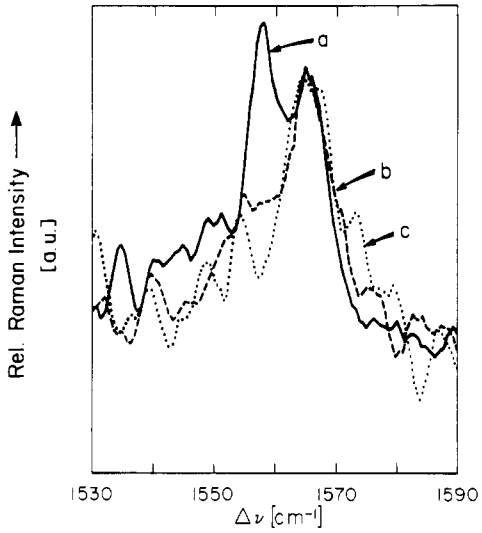

FIGURE 5: TR^3 spectra of the C=C stretching vibration of M-412 and M′ recorded from D_2O suspension at various rate constants for the photoexcitation of M-412: (a) $I_2 = 4.5 \times 10^9$ s^{-1}, (b) $I_2 = 3.3 \times 10^9$ s^{-1}, and (c) $I_2 = 2.6 \times 10^9$ s^{-1}.

shoulders near 1561 and 1558 cm^{-1}, respectively. The relative intensities of the 1567/1566 cm^{-1} and 1561/1558 cm^{-1} features were sensitive to the intensities of the probe beam as demonstrated by the spectra presented in Figure 5 for a 440-nm probe wavelength and for various values of I_2. Specifically, the ratio of the 1558-cm^{-1} band intensity to that of the 1566-cm^{-1} band increased markedly with increasing I_2. The intensity of the excitation laser at 532 nm (i.e., $I_0 = 3 \times 10^8$ s^{-1}) and Δt (i.e., 200 μs) remained unchanged while I_2 was varied in these experiments. On the basis of their respective sensitivities to the intensity of the 420- and 440-nm probe beams, the 1561- and 1558-cm^{-1} bands were assigned to an intermediate formed photolytically from M-412, namely, M′. From the linear correlation observed between the C=C stretching frequency and the wavelength of the maximal absorption (Heyde et al., 1971; Honig et al., 1976), one concludes from Figure 4 that M′ has a maximum absorption near 440 nm. This implies that M′ has an unprotonated Schiff base linkage (Honig et al., 1976).

These results demonstrated that the optical conditions used to record the TR^3 spectrum of M-412 also could cause photolytic interruption of the BR photocycle by forming M′, an intermediate not considered part of the thermally accessible reactions of BR-570 (see Figure 1). For example, with λ = 440 nm and an assumed quantum yield for M′ formation from M-412 of 0.5 (Hurley et al., 1978), the conditions used to obtain the spectra in Figure 5 [$I_2 = (2.6–4.5) \times 10^9$ s^{-1} and photoconversion factors I_2t of 26–45] were sufficient to create a mixture of M-412 and M′ containing comparable amounts of the two intermediates.

M-412 ↔ M′ Photoconversion Mechanism. The mechanism for the photolytic interconversion involving M-412 and M′ was examined by precisely altering the experimental parameters used to record the TR^3 spectra of the M intermediates. The excitation conditions of BR-570 at 532 nm ($I_0 = 3 \times 10^8$ s^{-1} and Δt = 200 μs) were held constant in these experiments. The value of I_2 at a 440-nm probe wavelength was selected to generate comparable RR signals from M-412 and M′ in the C=C stretching region. This TR^3 spectrum in an H_2O solvent is shown in Figure 4, panel IIA. With a rate coefficient for M′ formation of $I_2 = 3.7 \times 10^9$ s^{-1}, an I_2t value of 37 was obtained. Under these conditions, the photostationary equilibrium between M-412 and M′ was established within the 10-ns duration of the probe laser pulse (see Figure 5).

In order to characterize the photoconversion mechanism in more detail, the influence of a deuterated solvent was examined. With excitation and probe conditions selected to match those used for recording the TR^3 spectra of the PM sample in H_2O suspension (Figure 4, panel IIA), TR^3 spectra of M-412 and M′ were obtained for PM samples in D_2O. The spectrum presented in Figure 4, panel IIB, shows that the relative intensities of the 1566- and 1558-cm^{-1} bands depend on the deuteration of the solvent. When these experiments are repeated with a probe wavelength of 420 nm, the same type of results are obtained (Figure 4, panels IA and IB).

The changes in the relative intensities of RR bands observed upon deuteration of the solvent reflect a change in the relative concentrations of M-412 and M′ for a specific photostationary mixture (cf. panel IA with panel IB and panel IIA with panel IIB in Figure 4). Protonation, or where appropriate deuteration, must be effectively competing with the photoinduced reactions that control equilibrium concentrations of M-412 and M′. The reprotonation (or redeuteration) of the unprotonated Schiff base linkage of the M intermediates is an obvious competitive reaction to be considered. This reaction would have to occur on the 10-ns time scale in order to effectively compete with the photoinduced interconversion of M-412 and M′. Since the reprotonation of the Schiff base linkage (M-412 → BR-570) in the thermal photocycle occurs in milliseconds, this pathway cannot explain these results. The possible reprotonation of the Schiff base linkage in the M intermediates on a time scale comparable to 10 ns would be consistent with these observations. These results, of course, are only suggestive of a specific mechanism. Nonetheless, the rapid (<10-ns) conversion of the unprotonated M intermediates to a protonated chromophore needs to be considered, especially in view of the data described below which support a rapid (<10-ns) photolytic pathway connecting BR-570/K-590 with M-like (unprotonated) intermediates.

BR-570 Excitation. The pathway(s) associated with the formation of M-412 and M′ by the excitation of BR-570 also was (were) examined by TR^3 spectroscopy. The thermal

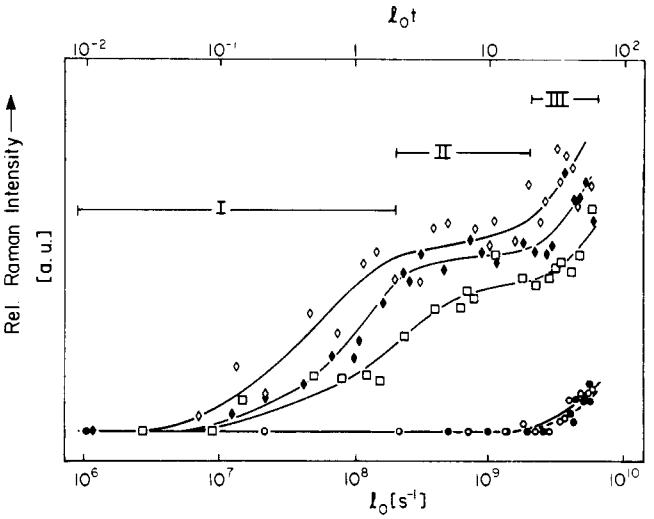

FIGURE 6: Relative intensities (in arbitrary units) of the TR^3 bands of the C=C stretching vibration for M-412 and M′ vs. the photoinduced rate constant l_0 and the amount of photoconversion (l_0t), respectively, of BR-570 at 532 nm. The time delay Δt between the pump and probe laser was varied as follows: 200 μs (◇), 100 μs (◆), 27 μs (□), 450 ns (●), and 10 ns (○). The laser used to generate resonance Raman scattering of M-412 and M′ was operated at 420 nm. See the text for description of regions I, II, and III.

pathway normally described as the BR photocycle (Stoeckenius et al., 1979) was examined by observing the C=C stretching vibrations of M-412 (1567 cm^{-1}) and M′ (1561 cm^{-1}) in TR^3 spectra recorded with a Δt of 200 μs. The wavelength (420 nm) and intensity (l_2t = 35) of the probe radiation were held constant while the intensity of the 532-nm radiation used to initiate the photocycle from BR-570 was varied. The relative intensities of both the 1567- and 1561-cm^{-1} bands from M-412 and M′ are plotted in Figure 6 vs. the l_0 values characterizing the 532-nm excitation.

There are three general regions to note in Figure 6. For l_0 values between 10^6 s^{-1} and 2×10^8 s^{-1} (region I), a steady increase in the RR band intensities for M-412 and M′ was observed. The increasing M-412/M′ concentrations in region I reflect higher absorption by BR-570. As l_0 values reach 2×10^8 s^{-1}, a plateau (region II) is reached which indicates the establishment of a photostationary equilibrium between BR-570 and K-590 (Grieger, 1981; Grieger & Atkinson, 1985). Specifically, the photoconversion of BR-570 to K-590 is balanced by the reverse photolytic reaction, and as a consequence, the formation yield of M-412 and M′ via the thermal pathway does not increase further with increasing l_0 in region II. A new phenomenon was observed in region III where l_0 values exceed 2×10^9 s^{-1}. Here the RR intensities of the 1567- and 1561-cm^{-1} bands rapidly increase again. Transient absorption measurements made at 514.5 nm (Grieger, 1981; Grieger & Atkinson, 1985) and over a 5-ns time interval have revealed a sharp decrease in the sample absorbance for l_0 values similar to those in region III of Figure 6. Both types of measurements suggest that for excitation intensities in region III the photostationary equilibrium between BR-570 and K-590 is significantly altered and that intermediates absorbing in the blue (e.g., M-412 and M′) are formed by a pathway faster than the one provided by the thermal photocycle.

To characterize this rapid formation of M intermediates, TR^3 measurements were recorded with a probe wavelength at 420 nm and for Δt values between 100 μs and 10 ns. All other parameters were held constant at those values described above for the experiments with Δt = 200 μs. Data for the largest (200, 100, and 27 μs) and smallest (450 and 10 ns) values of Δt used are presented in Figure 6. The data for $\Delta t \geq 27$ μs contain the same three regions described in detail above and differ from one another only by exhibiting smaller absolute RR intensities for decreasing Δt values. The latter phenomenon reflects the use of observation periods that are shorter than the time required for the concentration of M-412 and M′ to reach its maximum value via the thermal pathway.

The data for $\Delta t \leq 450$ ns provide unexpected results for excitation intensities in region III, specifically the appearance of a RR band at 1563 cm^{-1}. This RR band can be identified with a mixture of M-412 and M′ on the basis of the results of Stockburger et al. (1979) as well as the results presented above. The low RR signal observed in these experiments prevented high-quality spectra in the fingerprint region from being recorded and thereby precluded the complete characterization of the TR^3 spectrum which could have confirmed this assignment. Nonetheless, the resonantly enhanced nature of the TR^3 spectrum by 420-nm radiation, together with the excellent agreement of the frequency of the 1563-cm^{-1} RR band with that measured for M-412/M′ mixtures, strongly suggests that the intermediate(s) is (are) at least well described as "M-like".

The observation times for $\Delta t \leq 450$ ns are much too short to detect M intermediates formed in the thermal photocycle, and indeed, no RR bands enhanced by 420-nm radiation were observed for l_0 values in regions I and II (Figure 6). The RR signals observed for region III, therefore, must arise from M-412/M′ or M-like intermediates formed via a fast (nanosecond) pathway quite distinct from that found in the thermal photocycle. This nanosecond pathway could originate with the BR-570/K-590 photostationary mixture which can be established within the 10-ns duration of the 532-nm laser pulse. This rapid pathway does not involve L-550, which acts as a precursor for M-412 in the thermal photocycle. These data do not provide information on whether the formation of these rapidly generated M intermediates is accompanied by the protonation changes in the protein residues that are associated with the formation of M-412 in the thermal photocycle (Lewis, 1982; Fukumoto et al., 1984). A more complete characterization of these rapidly generated M intermediates, together with a clearer view of the underlying mechanism, is required to resolve these questions.

CONCLUSIONS

An investigation of the photolytic conditions used to initiate and spectroscopically monitor the BR photocycle utilizing TR^3 spectroscopy has revealed and characterized two photoinduced reactions that interrupt the thermal pathway. One involves the photolytic interconversion of M-412 and M′, two intermediates containing retinal with an unprotonated Schiff base linkage to the protein. The other pathway involves the direct photolytic conversion of the BR-570/K-590 photostationary mixture either to M-412 and M′ or to M-like intermediate(s) within 10 ns. This latter process involves the deprotonation of the Schiff base linkage and the trans to cis isomerization of the retinal chromophore. The photolytic threshold conditions characterizing both reactions have been quantitatively measured (Figures 5 and 6) and are discussed here in terms of experimental parameters.

The results presented here provide a new viewpoint with regard to the interpretation of previous studies of the BR photocycle as well as the design of future studies. The radiative intensities used to initiate and monitor the BR photocycle must be reconciled with the thresholds for photolytically interrupting the BR thermal cycle and for inducing secondary photochemistry. Although long acknowledged, this point has been

quantitatively demonstrated to be of significance for BR-570 and the M-412 and M′ intermediates. Its importance to other parts of the BR photocycle and to spectroscopic studies of photoinduced biophysical reactions in general remains to be established.

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