Transient Resonance Raman Spectra of Neutral and Alkaline Bacteriorhodopsin Photointermediates Observed with a Double-Beam Flow Apparatus: Presence of Very Fast Decaying M_{412}[†]

M. Nakagawa,[‡,§] T. Ogura,[‡] A. Maeda,[∥] and T. Kitagawa*[,‡]

Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki, 444 Japan, Department of Biophysics, Faculty of Science, Kyoto University, Sakyoku, Kyoto, 606 Japan, and Department of Pure and Applied Sciences, The University of Tokyo, Komaba, Meguro-ku, Tokyo, 153 Japan

Received May 23, 1988; Revised Manuscript Received September 21, 1988

ABSTRACT: Time-resolved resonance Raman spectra were observed for neutral and alkaline bacteriorhodopsin (bR) with a double-beam flow apparatus. The delay time (t_d) between the pump and probe beams was changed from 15 to 1200 μs, and the probe-only spectra were observed before and after individual pump–probe spectra in order to rectify the bleaching effect of bR during the measurements. In the 457.9-nm probed spectra, the intensity of the 1566-cm^{-1} band of M_{412} increased until t_d = 190 μs and then decreased with a decay constant (τ_d) of 1.7 ms at pH 7.0. However, at pH 10.5, its intensity was maximum at t_d = 15 μs and decayed monotonously. In the first 300 μs, ca. 40% of M_{412} decayed with τ_d = 0.39 ms, whereas its decay curve at later time was very close to that of neutral M_{412}. The slower decay component in this study corresponds to the so-called fast decay component of M_{412} (M^f). The reversal of positions of the pump and probe beams gave a spectrum identical with the probe-only spectrum, indicating that a possible long-lived intermediate (τ_d > 3 s) contributes little to the present spectrum, although at pH 10.5 the presence of a small amount of bR_{460} was suggested from the probe-only spectrum. Accordingly, the presence of a very fast decaying M species (M^{vf}) was deduced at alkaline pH. In the 514.5-nm probed spectra, the 1190-cm^{-1} band characteristic of the N (or P or L′) intermediate did not grow in conformity with the decay of M^{vf}, suggesting that M^{vf} is not converted into N or the 1190-cm^{-1} band of N is very weak. The two C=C stretching bands of L_{550} at 1551 and 1542 cm^{-1} decayed similarly, and this decay behavior agreed with the rising kinetics of the 1566-cm^{-1} band also with the reported kinetics of the slow rising component of M_{412}. The efficiency for generation of later photointermediates appeared 1.6 times higher at pH 10.5 than at pH 7.0, and the increment is ascribable to generation of M^{vf}.

Bacteriorhodopsin (bR) is a retinal-containing protein found in the purple membrane of a light-harvesting bacterium, *Halobacterium halobium*. This protein transforms light energy into electrochemical energy by transporting protons across the membrane (Oesterhelt & Stoeckenius, 1973), and due to its unique nature for the function, the physicochemical properties of bR have been extensively investigated [see Stoeckenius and Bogomolni (1982) for a review]. Light-adapted bR_{570} has *all-trans*-retinal bound to Lys-216 via a protonated Schiff base, and upon absorbing a photon, it undergoes a cyclic reaction involving trans to cis isomerization at the C_{13}=C_{14} bond and successive deprotonation of the Schiff base. Transient optical spectra (Lozier et al., 1975) indicated the presence of four distinct intermediates, at least, in the photochemical cycle of bR, that is

$$bR_{570} \xrightarrow[ps]{} K_{590} \xrightarrow[2\ \mu s]{} L_{550} \xrightarrow[40\ \mu s]{} M_{412} \xrightarrow[ms]{} O_{640} \rightarrow bR_{570}$$

although some modifications were suggested by others (Ottolenghi, 1980; Birge, 1981; Stoeckenius & Bogomolni, 1982). The ejection of protons from the cell membrane to the outside takes place at a rate comparable to that for the formation of M_{412} (Lozier et al., 1976; Ort & Parson, 1978; Grzesiek & Dencher, 1986), while preceding proton movement between amino acid residues was credited for the formation of L_{550} (Engelhard et al., 1985). The uptake of protons from the other side of the membrane occurs in the relaxation process of M_{412} to bR_{570}. Accordingly, the rising and decaying kinetics of M_{412} would be a key step for the unidirectional proton pump of bR. In order to elucidate this step, we investigated transient resonance Raman spectra of bR by using a double-beam flow apparatus.

Resonance Raman (RR) scattering from bR provides the vibrational spectra of the retinal Schiff base that are sensitive to the surroundings of the retinal–Schiff base as well as to its isomeric structure [see Mathies (1984); Smith et al. (1985a), and Stockburger et al. (1986) for a review]. The Schiff base C=N stretching mode ($\nu_{C=N}$) serves as a diagnostic for its protonation (Lewis et al., 1974) and indeed indicated that only M_{412} among various species in the photochemical cycle has the deprotonated Schiff base (Marcus & Lewis, 1977; Aton et al., 1977; Braiman & Mathies, 1980; Bagley et al., 1982).

Mathies and co-workers analyzed the RR spectra of about 20 isotope-substituted retinals (Mathies, 1984; Smith et al., 1985b) and of their complexes with bacterioopsin in the all-trans (Smith et al., 1987b) and 13-cis forms (Smith et al., 1987a). Accordingly, the vibrational assignments of Raman bands have nearly been established. It was pointed out (Smith et al., 1985a) that the in-phase C_{12}–D and C_{14}–D in-plane bending mode of a dideuteriated derivative is sensitive to an

[†] This work was supported by Grant-in-Aids of Japanese Ministry of Education, Science, and Culture for Scientific Research on Priority Areas to T.K. (62617521) and for Scientific Research to A.M. (62470151) and also by Joint Studies Program of this institute (63-101).

* Author to whom correspondence should be addressed.
[‡] Okazaki National Research Institutes.
[§] The University of Tokyo.
[∥] Kyoto University.

0006-2960/89/0428-1347$01.50/0

isomeric structure about the C_{13}=C_{14} bond, and from their frequencies, K_{590}, L_{550}, and M_{412} were inferred to have the 13-cis form, while bR_{570} and O_{640} were deduced to adopt the 13-trans form.

Recently, the presence of multiple forms was suggested for M_{412} from the biphasic nature of its decay (Ohno et al., 1981; Li et al., 1984; Groma & Dancshazy, 1986) and of its formation (Hanamoto et al., 1984; Scherrer & Stoeckenius, 1985; Grzesiek & Dencher, 1986), although the two forms cannot be distinguished by resonance Raman (Deng et al., 1985) or by visible absorption spectra. Low-temperature photolysis studies showed that the yield of M_{412} increased when the pH was raised above 10, suggesting a catalytic action of an amino acid residue with pK_a = 10 on the formation of M_{412} (Kalisky et al., 1981). Additional photointermediates called N, P, and R_{350} are proposed in the relaxation process of M_{412} to bR_{570} (Xie et al., 1987; Drachev et al., 1986; Dacshazy et al., 1986). These intermediates are considered to have longer lifetimes at alkaline pH and higher ionic strength. Besides these natural intermediates, it was found in RR studies that an M-like intermediate having the all-trans unprotonated Schiff base was generated upon laser illumination to M_{412} (Stockburger et al., 1979; Grieger & Atkinson, 1985).

In order to clarify intermediates involved in the photocycle of bR before and after M_{412}, we undertook the present study. In our previous study (Maeda et al., 1986) we pointed out that an L-like species with a long lifetime was accumulated in the spinning cell at alkaline pH, but such species was not recognized in the single-beam flow experiments. In this study the double-beam flow technique was adopted to explore RR spectra of microsecond to millisecond intermediates. We found that some M species decays much faster than the conventional fast-decaying M_{412} and does not yield the N intermediate, although its formation is postulated in a new comprehensive interpretation of bR photocycle based on transient absorption spectra by Kouyama et al. (1988).

Experimental Procedures

The purple membrane (PM) was isolated from *H. halobium* R_1M_1 by the standard method (Oesterhelt & Stoeckenius, 1974) and suspended in 10 mM phosphate (pH 7.0) or 10 mM carbonate buffer (pH 10.5) containing 0.2 M KCl so that the final solution could give A_{568} = 1.8 cm^{-1}. Before the measurement of RR spectra, the sample was sonicated for 15 s to make it free from aggregation of the PM sheets and then illuminated by a slide projector for 1 min for light adaptation. Since the yield of intermediates decreased as the bR aged, the measurements were carried out within a month after isolation of PM.

Transient RR spectra were measured with a double-beam flow apparatus described elsewhere (Ogura et al., 1987). The photocycle of bR was initiated by the pump beam (598 nm, 55 mW at sample, beam diameter 80 μm) that was generated by a dye laser (Spectra Physics Model 375) with Rhodamine 6G pumped by an Ar^+ laser (Spectra Physics Model 164). Raman scattering was excited by the probe beam (514.5 or 457.9 nm) from an Ar^+ laser (NEC GLG3200). Its power was set to be as low as possible (1.4 mW at sample; beam diameter 20 μm). The delay time (t_d) between the pump and probe beams was determined by measuring a flow rate of the sample and the center-to-center distance between the pump and probe beams with a microscope installed behind the sample point. Raman spectra were observed with an OMA II system (PAR 1215) and a diode array detector (PAR 1420) attached to a double monochromator (Spex 1404). Raman shifts were calibrated with indene.

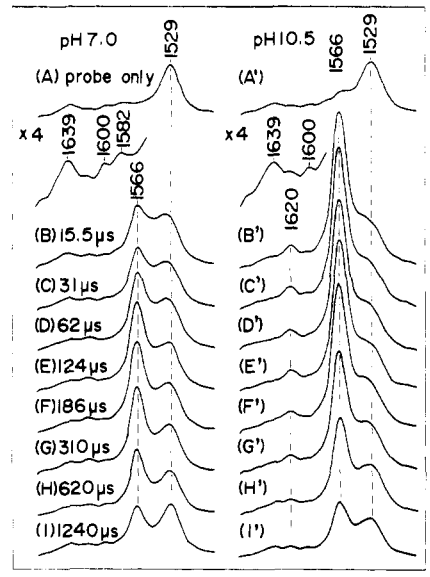

FIGURE 1: Transient RR spectra for the flowing sample of bR at pH 7.0 (left) and pH 10.6 (right). The spectra at the top (A and A') were observed only with the probe beam (457.9 nm, 1.4 mW), but others were obtained in the presence of the pump beam (598 nm, 55 mW). The inserted traces represent the ordinate-expanded (4 times) spectra of (A) and (A'). The delay times were determined by measuring the center-to-center distances of the two beams by using a microscope and are noted at the left side of the individual spectra. Five milliliters of the bR buffered solutions containing 0.2 M KCl was flowed with the rate of 3.23 m/s, and the sample reservoir was kept at 13 °C. The ordinate scales are normalized to spectrum A or A' on the basis of the average intensity of the 1529-cm^{-1} bands in the probe-only spectra observed before and after individual pump–probe spectra. The exposure time of detector was 1.6 s, and the accumulation time was 160 s.

About 5 mL of the sample solution was circulated to a capillary cell (0.8-mm i.d.) through a silicon tube by a peristaltic pump (Cole-Palmer Model 7553-20). The temperature of the sample was monitored by putting a thermocouple directly into the solution in the reservoir; the sample was kept at 13 °C in a water bath. The flow rate was determined to be 4.76 m/s for the 514.5-nm probe experiment and 3.23 m/s for the 457.9-nm probe experiment from the observed volume rate and the cross section of the capillary cell. Accordingly, it takes ca. 3 s for one turn of sample circulation. Upon variation of the delay time, two independent series of experiments were carried out; one is the ascending order of the delay times, and the other is their descending order. This is to confirm that incomplete light adaptation does not affect the results. Samples were renewed for every series of experiments. In order to rectify an effect of slight bleaching of bR during the measurements, the probe-only spectrum was observed before and after each pump–probe spectrum with identical instrumental conditions, and the average of two spectra was regarded as the corresponding probe-only spectrum.

Results

Figure 1 shows the 457.9-nm probed double-beam transient RR spectra in the C=C stretching region of bR at pH 7.0 (left) and pH 10.5 (right). Only the spectra at the top [(A) for pH 7.0 and (A') for pH 10.5] were observed without the pump beam. In spectra A and A', the in-phase C=C stretching mode ($\nu_{C=C}$) is observed at 1529 cm^{-1} for bR_{570}, and in their ordinate-expanded spectra the C=NH stretching mode ($\nu_{C=NH}$) of the protonated Schiff base is identified at 1639 cm^{-1}. Each pump–probe spectrum was normalized by dividing it with the intensity ratio of the 1529-cm^{-1} band in spectrum A or A' to that in the corresponding probe-only

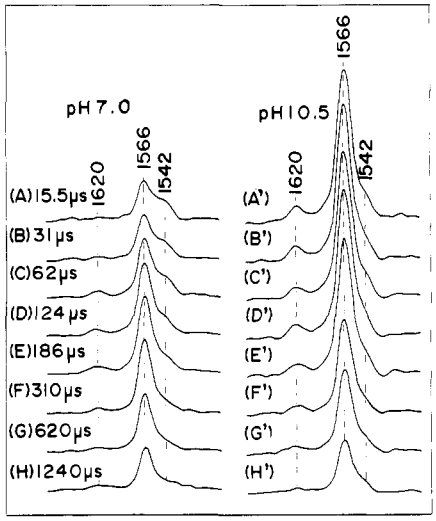

FIGURE 2: 457.9-nm probed difference spectra of bR at pH 7.0 (left) and pH 10.6 (right). These spectra were obtained by subtracting the corresponding probe-only spectra from the individual pump–probe spectra shown in Figure 1. In this calculation an appropriate factor was multiplied with the subtrahend so that the contribution at 1529 cm^{-1} is depleted in the difference spectra, and therefore intensity normalization explained in Figure 1 is reserved. Each spectrum is specified by the delay time denoted at the left.

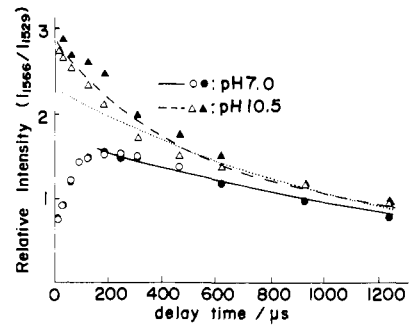

FIGURE 3: Time profile of the 1566-cm^{-1} band, in which its band intensities in Figure 2 are plotted against the center-to-center delay times. The ordinate scale is represented in terms of the maximum peak height of the 1566-cm^{-1} band relative to the maximum peak height of the 1529-cm^{-1} band in the corresponding probe-only spectra, although the spectra in Figure 2 are already normalized by the intensity of the 1529-cm^{-1} band of the probe-only spectrum. The solid and open markers denote the values obtained in the series of the descending and ascending orders of the delay time, respectively. The broken line was calculated from eq 1 by putting $M^{vf} = 1.42$, $\tau_{vf} = 0.39$ ms, $M_f = 1.46$, and $\tau_f = 2.3$ ms, while the solid line was obtained by replacing t with $t - 200$ and $M^{vf} = 0$, $M^f = 1.55$, and $\tau_f = 1.7$ ms. The dotted line indicates a single-exponential decay curve ($M^{vf} = 0$, $M^v = 2.3$, and $\tau^v = 1300$ μs) that was adjusted to best reproduce the data points between 300 and 1200 μs of bR at pH 10.5.

spectrum so as to remove the bleaching effect. In this sense the transient spectra shown in Figure 1 are normalized to spectrum A or A′. The delay times between the pump and probe beams are specified at the left side of each spectrum.

At pH 7.0 the $\nu_{C=C}$ band of M_{412} appears at 1566 cm^{-1} in the pump–probe spectra (B–I) but not in the probe-only spectrum (A). However, at pH 10.5, a weak feature is recognized even in the probe-only spectrum (A′). One may argue that this suggests the presence of a small amount of an M-like species as a thermal mixture or as a long-lived M_{412} species (M^s). However, the relative intensity of the 1566-cm^{-1} band to the 1529-cm^{-1} band was unaltered when the laser power of the probe beam was reduced to 0.7 mW. Furthermore, when the pump beam was placed at the downstream side of the probe beam, a RR spectrum identical with spectrum A′ was observed. Therefore, it is unlikely that the 1566-cm^{-1} band of spectrum A′ arose from a photointermediate generated by a probe beam. On the other hand, Druckmann et al. (1982) reported that the unprotonated form of unphotoreacted bR (bR_{460}) gave the absorption maximum at 460 nm and the $\nu_{C=C}$ RR band at 1565 cm^{-1}. Although the pK_a for the Schiff base proton is as high as 13, a small amount of bR_{460} might be present at pH 10.5 and give rise to the RR band at 1566 cm^{-1} in the probe-only spectrum. The presence of a small amount of bR_{460} is practically equivalent to a slight reduction of an effective concentration of bR but does not affect the discussion below.

In the presence of the pump beam at pH 10.5 (B′–I′), the 1566-cm^{-1} band is remarkably intensified and the $\nu_{C=N}$ mode of the deprotonated Schiff base is also recognized at 1620 cm^{-1}. In order to delineate the contribution of M_{412}, difference spectra were calculated by subtracting the corresponding probe-only spectra from the individual pump–probe spectra. In this calculation an appropriate factor was multiplied with the subtrahend so that the 1529-cm^{-1} band of the parent bR_{570} could be deleted in the difference spectrum. The results are shown in Figure 2. Since all the spectra are normalized as explained for Figure 1, the relative intensity of the 1566-cm^{-1} band in Figure 2 measures the relative amount of M_{412} generated.

At pH 7.0 a weak feature appears at the lower frequency side of the 1566-cm^{-1} band until 124 μs (A–D), and as it becomes weaker, the 1566-cm^{-1} band becomes stronger. Accordingly, this side band is attributed to the precursor of M_{412}, namely, L_{550}. Since the rise time of the slow rising component of M_{412}, which dominates at pH 7.0 (90%), is reported to be 94 μs (Scherrer & Stoeckenius, 1985), this observation confirms the correctness of the time scale in the present experiments. Such a feature is less pronounced at alkaline pH and disappears at shorter delay times than at neutral pH. At pH 7.0 the intensity of the 1566-cm^{-1} band increases until $t_d = 186$ μs and then decreases. In contrast, at pH 10.5, the intensity of the 1566-cm^{-1} band is maximum at the earliest delay time in this experiment, and it decreases monotonously as the delay time becomes longer. This clearly indicates that the rising time of M_{412} is less than 15 μs, in agreement with the observations for the fast rising M_{412} reported by Rosenbach et al. (1982) and Scherrer and Stoeckenius (1985). The intensity of the $\nu_{C=N}$ band at 1620 cm^{-1} runs parallel with that of the 1566-cm^{-1} band.

In order to make the kinetic behavior of M_{412} clearer, the peak intensities of the 1566-cm^{-1} band in Figure 2 are plotted against the delay time in Figure 3, where open and closed markers indicate the data from two independent series of experiments, that is, data obtained in the ascending and descending orders of the delay times. The ordinate is scaled in terms of the relative intensity of the 1566-cm^{-1} band to the 1529-cm^{-1} band, although the intensities of the 1566-cm^{-1} bands in Figures 1 and 2 are already normalized by the intensity of the 1529-cm^{-1} band of the probe-only spectrum. The two kinds of data are in agreement within experimental error, indicating that the observed data involve no contribution from degradation of bR or accumulation of any photoproducts.

It is evident that the kinetics of M_{412} are different between at pH 7.0 and 10.5. First, the formation of M_{412} is faster at alkaline pH than at neutral pH and thus agrees with earlier remarks (Lewis, 1978; Kalisky et al., 1981; Rosenbach et al., 1982; Hanamoto et al., 1984; Scherrer & Stoeckenius, 1985). Second, at alkaline pH we see very fast decay of M_{412} in the time range from 15 to 300 μs. A single-exponential decay curve that best reproduces the data for 300–1200 μs of bR at

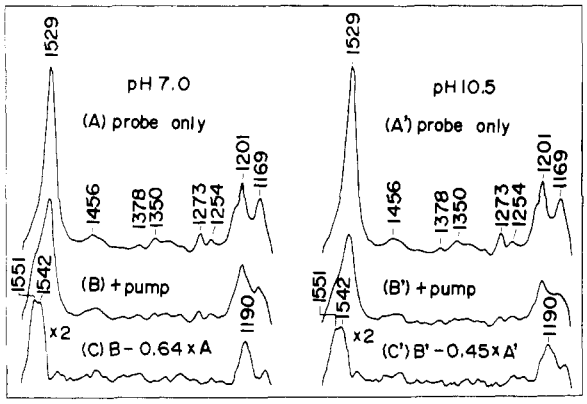

FIGURE 4: Transient RR spectra for the flowing sample of bR at pH 7.0 (left) and pH 10.6 (right). Spectra A and A′ were obtained only with the probe beam (514.5 nm, 1.4 mW), but spectra B and B′ were obtained in the presence of the pump beam (598 nm, 55 mW) at the delay time of 10.5 μs. Traces C and C′ are the difference spectra obtained so as to yield zero intensity at 1529 cm^{-1}; (C) = (B) − 0.64(A), (C′) = (B′) − 0.45(A′).

pH 10.5 is drawn with a dotted line, and it is apparent that deviations of the data points for 15–300 μs from the curve are evidently beyond the statistical errors deduced from the scattering of points obtained from two independent experiments. Therefore, we fitted the observed points by using a linear combination of two exponential functions:

$$M = M^{vf} \exp(-t/\tau_{vf}) + M^{f} \exp(-t/\tau_{f}) \qquad (1)$$

The broken line in Figure 3 indicates the curve of eq 1 with $M^{vf} = 1.42$, $\tau_{vf} = 0.39$ ms, $M^{f} = 1.46$, and $\tau_{f} = 2.3$ ms, while the solid line indicates the curve for a single-exponential function ($M^{vf} = 0$, $M^{f} = 1.55$, and $\tau_{f} = 1.7$ ms) in which t was replaced by $t - 200$ because the maximum population of M_{412} is achieved around $t = 200$ μs at neutral pH. It is emphasized that at alkaline pH ca. 40% of M_{412} present at $t_d = 15$ μs disappears within 300 μs. This has never been noticed in previous studies (Ohno et al., 1981; Lam & Packer, 1983; Kouyama et al., 1988; Scherrer & Stoeckenius, 1985), because all of them analyzed the spectral changes in a millisecond time period. In those studies the presence of two kinds of M_{412} was postulated; the fast (M^{f}) and slow decay components (M^{s}) had the decay constant of a few milliseconds and 10 ms, respectively, and the decay constant of the slow one lengthens at alkaline pH. In the time range from 400 to 1200 μs, the M_{412} species at both pH 7 and 10.5 seems to have a decay constant of 1.7–2.3 ms, which agrees with that of conventional M^{f}. Consequently, at alkaline pH we have to admit the presence of another M species (M^{vf}) that decays with a decay constant of 0.39 ms, and its population is appreciably large at pH 10.5.

Figure 4 shows the 514.5-nm probed transient RR spectra of bR at pH 7.0 (left) and pH 10.5 (right). Spectra A and A′ were observed without the pump beam, whereas spectra B and B′ were observed in its presence with the delay time of 10.5 μs. The intensities of spectra B and B′ are scaled by the average of two probe-only spectra in the same way as explained for Figure 1. Although the intensities of the 1529-cm^{-1} bands in spectra A and A′ are similar, those in spectra B and B′ are fairly different. This difference was reproducible. Since the pump laser was fairly strong and accordingly bR molecules in the laser beam are considered to be in photoequilibrium between bR_{570} and K_{590}, the observed intensity difference of the 1529-cm^{-1} band should reflect a difference in the rate constant for the first irreversible thermal reaction (probably K_{590} to L_{550}), which leads to an effective change of the photoequilibrium. Anyway, the efficiency for generation of later

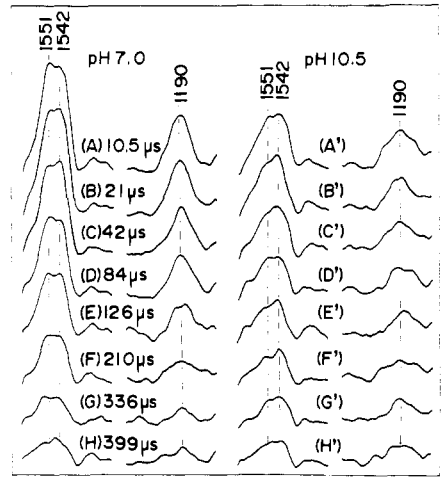

FIGURE 5: 514.5-nm probed difference Raman spectra of bR at pH 7.0 (left) and pH 10.6 (right). Only the spectra in the C=C (1580–1500 cm^{-1}) and C—C stretching regions (1210–1170 cm^{-1}) are depicted for each delay time. The intensities of all spectra are normalized in the same sense as in Figure 2 on the basis of the probe-only spectra observed before and after individual pump–probe spectra.

photointermediates upon light illumination differs between pH 7.0 and 10.5; the greater the intensity reduction of the 1529-cm^{-1} band is, the higher the efficiency for the practical photoreaction is. The intensity ratio of the 1529-cm^{-1} band in the presence of the pump beam to that in its absence was 0.65 for neutral bR and 0.45 for alkaline bR in Figure 4. Spectra C and C′ show the difference spectra calculated from spectra A and B and spectra A′ and B′, respectively, in the same way as in Figure 2 and correspond to spectra of L_{550} at pH 7.0 and 10.5. The C=C stretching modes are observed as a doublet at 1551 and 1542 cm^{-1} and the C—C stretching mode (ν_{C-C}) at 1190 cm^{-1} is observed at both pH values. The intensities of these bands are weaker in spectrum C′ than in spectrum C despite the fact that the efficiency of the photoreaction is higher at alkaline pH. This is probably due to the faster decay of L_{550} at alkaline pH.

The pump–probe spectra similar to spectra B and B′ were observed for various delay times and scaled on the basis of the corresponding probe-only spectra. Then the spectra of transient species were calculated, and their $\nu_{C=C}$ and ν_{C-C} regions for a variety of the delay times are depicted in Figure 5, where the intensities are normalized by the corresponding probe-only spectra as explained for Figure 2. The kinetic behaviors of the $\nu_{C=C}$ and ν_{C-C} bands appear alike at both pHs, and the two bands are generally weaker at pH 10.5 than at pH 7.0. This intensity difference is more prominent at delay times less than 250 μs, being consistent with the behaviors of the lower frequency side bands of $\nu_{C=C}$ in Figure 2. For clarifying the kinetic properties of these bands, the relative intensities of the $\nu_{C=C}$ and ν_{C-C} bands are plotted against the delay time in Figure 6.

In the previous kinetic RR spectra by Marcus and Lewis (1978), the resonance enhancement pattern and kinetic behavior of the 1551-cm^{-1} band were different from those of the 1542-cm^{-1} band, and therefore the two bands were assigned to different photointermediates. We also reproduced their results previously (Ogura et al., 1987), but more careful examination revealed that insufficient light adaptation resulted in dissimilar kinetics of the two bands; the 1542-cm^{-1} band contains the intensity contribution from the 1537-cm^{-1} band of bR_{548} more than the 1551-cm^{-1} band at the initial stage of the flow experiment, but such difference disappears later due to gradual light adaptation by laser irradiation (Nakagawa

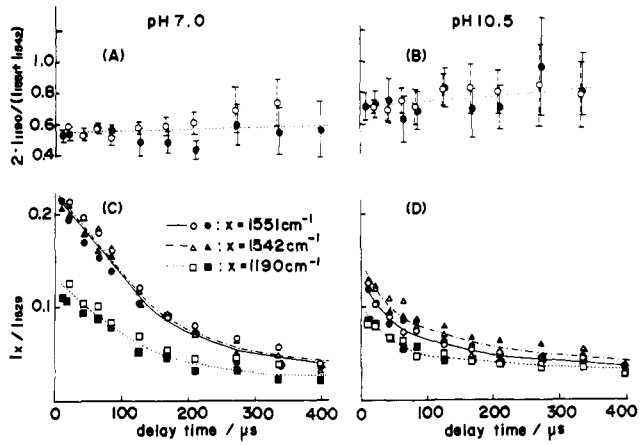

FIGURE 6: Time profiles of the 1551-, 1542-, and 1190-cm^{-1} bands in the difference spectra shown in Figure 5. The left and right sides represent the results at pH 7.0 and pH 10.5, respectively. The meaning of the open and solid markers is the same as for those in Figure 3. (A and B) The relative intensities of the 1190-cm^{-1} band to the average of the 1551- and 1542-cm^{-1} bands are plotted against the delay time. The error bars were estimated for the 1190-cm^{-1} band on the basis of the background noise. The estimated error increases at longer delay times since the 1190-cm^{-1} band becomes weaker. (C and D) The maximum peak heights of the 1551- (circles), 1542- (triangles), and 1190-cm^{-1} bands (squares) relative to the maximum peak height of the 1529-cm^{-1} band of the corresponding probe-only spectra are plotted against the delay time.

et al., 1989). In the present experiment bR was sufficiently light-adapted, and therefore the results from two series of experiments, that is, the ascending and descending orders of the delay times, are in agreement with each other as shown in Figure 6. The intensity ratio of the 1551-cm^{-1} band to the 1542-cm^{-1} band remained unaltered over the time range examined, although both bands diminished in intensity monotonously as the delay time increased. This is apparent in the plots C and D. Therefore, both the 1551- and 1542-cm^{-1} bands are assigned to L_{550} as suggested previously (Argade & Rothschild, 1983; Alshuth & Stockburger, 1985; Smith et al., 1984). This conclusion is unaltered as the pH is raised.

The decay profile of the 1551-cm^{-1} band shown in Figure 6C gives rise to the decay constant of ca. 190 μs for $t_d < 400$ μs, while that in Figure 6D gives also 190 μs for $t_d < 150$ μs. It is stressed that the decay kinetics of the 1551-cm^{-1} band at pH 7.0 is consistent with the rising kinetics of the 1566-cm^{-1} band shown in Figure 3. On the other hand, the presence of two kinetic components are reported for the formation of M_{412} (Hanamoto et al., 1986); the corresponding decay constants of L_{550} at 10 °C are 22 and 152 μs for the fast and slow components, respectively. Accordingly, L_{550}, which contributed to the RR spectra shown in Figure 5, gives rise solely to the slow rising component of M_{412} while the L species corresponding to the fast rising component of M_{412} has already disappeared from spectra A and A′ in Figure 5.

The time dependence of the 1190-cm^{-1} band is also illustrated in Figure 6C,D. One would realize intuitively that the kinetic behaviors of the 1190- and 1551-cm^{-1} bands are alike, but in order to demonstrate it more clearly, the relative intensities of the 1190-cm^{-1} band to the average of the 1551- and 1542-cm^{-1} bands are plotted against the delay time in Figure 6A,B. At pH 7.0, the relative intensity remains unaltered until 400 μs, but at pH 10.5 the value at $t_d = 0$ is slightly larger than that at pH 7.0 and it appreciably increases with the delay time. This may suggest that a species which gives rise to the 1190-cm^{-1} band accumulates slowly, but one can definitely conclude that the intensity increase of the 1190-cm^{-1} band at alkaline pH does not match the intensity decrease of the 1566-cm^{-1} band of M^{vf} shown in Figure 3.

Discussion

Characterization of the successive intermediate that follows M_{412} is currently under extensive discussion. Drachev et al. (1986) measured light-induced difference absorption spectra in the 500–600-nm region in the millisecond time range and suggested the presence of intermediate P, while Dancshazy et al. (1986) observed a difference peak at 350 nm and called the intermediate R_{350}. Kouyama et al. (1988) characterized an intermediate, N, which has a major absorption maximum at 550–560 nm with lower absorbance than bR_{570} and considered that N, P, and R_{350} are identical. Accordingly, for simplicity, we adopt the notation N for this intermediate. The N intermediate has a lifetime as long as 10 s at alkaline pH and high ionic strength, but its lifetime is deduced to be less than 1 ms at neutral pH. Fodor et al. (1988) determined the chromophore structure of N to be the 13-cis protonated form from time-resolved RR spectroscopy and argued that M_{412} decays directly to N with a lifetime of 4 ms. According to Fodor et al. (1988), the RR spectrum of N has characteristic bands at 1186, 1530, and 1548 cm^{-1}. However, as noted previously, the 1190-cm^{-1} band did not grow in conformity with the decay of the 1566-cm^{-1} band of M^{vf}. Since ca. 40% of M_{412} present at $t_d = 15$ μs is depleted at $t_d = 400$ μs (Figure 3), appreciable intensity increase should be observed at 1190 cm^{-1} in that time range if M^{vf} were converted into N. Consequently, we infer that M^{vf} is not a precursor of N or that the 1186-cm^{-1} band of the corresponding N species is unexpectedly weak. Since the intensity of the 1529-cm^{-1} band of the parent bR_{570} in the normalized pump–probe RR spectra before difference calculation (for example, Figure 4B) increased slightly in the time range 200–400 μs, M^{vf} might be converted into bR_{570} directly or into an unknown species which is less intensity-enhanced in RR spectra upon excitation at 514.5 and 457.9 nm.

In our previous RR study with a spinning cell for alkaline bR (Maeda et al., 1986), an L-like RR spectrum was observed except for the location of the $\nu_{C=C}$ band at 1529 cm^{-1}, and it was ascribed to L′, an intermediate with a long lifetime. The RR spectrum of L′ was remarkably close to the spectrum of N reported by Fodor et al. (1988). One may deduce that the slight increase of the 1190-cm^{-1} band of alkaline bR at longer delay time (Figure 6B) is due to formation of this species. However, when the pump beam (598 nm) was placed at a downstream side of the probe beam (514.5 nm), the observed spectrum was the same as that obtained without the pump beam. Since it takes ca. 3 s for a molecule to run one cycle of the flow system, the N or L′ species would not have a lifetime as long as 3 s under the present solution conditions (0.2 M KCl, pH 10.5) and thus would not be accumulated.

Kouyama et al. (1988) proposed a new relaxation route to N to bR_{570} that includes photoreaction of N to N_L and thermal reversible reaction of N_L to N_M. According to their interpretation about the relaxation from N to bR_{570}, the kinetically defined M^f and M^s (defined from the decay behavior but not from the rising behavior) correspond to M_{412} and N_M, respectively. As explained above, the M species identified in the present experimental conditions did not remain after one cycle of sample flowing. Consequently, we deduce that the 1566-cm^{-1} band shown in Figure 2 arises either from M^{vf} or M^f.

The intensity reduction of the 1529-cm^{-1} band due to incorporation of the pump beam (Figure 4) indicates that the amount of molecules forwarded to the photocycle is 35% and 55% of bR present at pH 7.0 and 10.5, respectively. This value

for neutral pH happens to agree with the reported quantum yield (0.3–0.4) (Goldschmidt et al., 1976, 1977; Becher & Ebrey, 1977; Hurley & Ebrey, 1978; Alshuth & Stockburger, 1985), which is considered to be pH independent between pH 5 and 11 (Renard & Delmelle, 1981; Rosenbach et al., 1982). There is no possibility that an accumulation of a long-lived photointermediate causes the apparent greater intensity reduction of the 1529-cm^{-1} band at alkaline pH. As indicated in Figure 3, the decay behavior of the 1566-cm^{-1} band in the range 400–1200 μs is little altered between pH 7.0 and pH 10.5, implying that the difference between the neutral and alkaline bR exists in the presence of the very fast decay component at alkaline pH. The ratio of the maximum peak height of the 1566-cm^{-1} band at pH 10.5 to that at pH 7.0 in Figure 2 is 1.8, which is noticeably close to the ratio of the efficiencies for generation of later photointermediates at alkaline and neutral pHs (0.55/0.35 = 1.6). It is reported that the amount of the fast rising component of M_{412} (τ = 8.7 μs at 20 °C) increases from 10% at pH 7.0 to 70% at pH 10.5 (Scherrer & Stoeckenius, 1985). Accordingly, it is plausible that M^{vf} corresponds to the fast rising component of M_{412} and most of the increment in the efficiency of L and M formation at alkaline pH comes from the contribution from the species that generates M^{vf}.

References

Alshuth, T., & Stockburger, M. (1985) *Photochem. Photobiol. 43*, 55–66.

Argade, P. V., & Rothschild, K. J. (1983) *Biochemistry 22*, 3460–3466.

Aton, B., Doukas, A. G., Callender, R. H., Becker, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995–2999.

Bagley, K., Dollinger, G., Eisenstein, L., Singh, K., & Zimanyl, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972–4976.

Becher, B., & Ebrey, T. G. (1977) *Biophys. J. 17*, 185–191.

Birge, R. R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 315–354.

Braiman, M., & Mathies, R. (1980) *Biochemistry 19*, 5421–5428.

Dancshazy, Zs., Govindjee, R., Nelson, B., & Ebrey, T. G. (1986) *FEBS Lett. 209*, 44–48.

Deng, H., Pande, C., Callender, R. H., & Ebrey, T. G. (1985) *Photochem. Photobiol. 41*, 467–470.

Drachev, L. A., Kaulen, A. D., Skulachev, V. P., & Zorina, V. V. (1986) *FEBS Lett. 209*, 316–320.

Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., & Callender, R. H. (1982) *Biochemistry 21*, 4953–4959.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Fodor, S. P. A., Ames, J. B., Gebhard, R., van den Berg, E. M. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. A. (1988) *Biochemistry 27*, 7097–7101.

Goldschmidt, C. R., Ottolenghi, M., & Korenstein, R. (1976) *Biophys. J. 16*, 839–843.

Goldschmidt, C. R., Kalisky, O., Rosenfeld, T., & Ottolenghi, M. (1977) *Biophys. J. 17*, 179–183.

Grieger, I., & Atkinson, G. H. (1985) *Biochemistry 24*, 5660–5665.

Groma, G. I., & Dancshazy, Zs. (1986) *Biophys. J. 50*, 357–366.

Grzesiek, S., & Dencher, N. A. (1986) *FEBS Lett. 208*, 337–342.

Hanamoto, J. H., Dupuis, P., & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7083–7087.

Hurley, J. B., & Ebrey, T. G. (1978) *Biophys. J. 22*, 49–66.

Kalisky, O., Ottolenghi, M., Honig, B., & Korenstein, R. (1981) *Biochemistry 20*, 649–655.

Kouyama, T., Kouyama, A. N., Ikegami, A., Mathew, M. K., & Stoeckenius, W. (1988) *Biochemistry 27*, 5855–5863.

Lam, E., & Packer, L. (1983) *Arch. Biochem. Biophys. 221*, 557–564.

Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H., & Stoeckenius, R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462–4466.

Lewis, A., Marcus, M. A., Ehrenberg, B., & Crespi, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4642–4646.

Li, Q.-Q., Govindjee, R., & Ebrey, T. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7079–7082.

Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophys. J. 15*, 952–962.

Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S.-B., & Stoeckenius, W. (1976) *Biochim. Biophys. Acta 440*, 545–556.

Maeda, A., Ogura, T., & Kitagawa, T. (1986) *Biochemistry 25*, 2798–2803.

Marcus, M. A., & Lewis, A. (1977) *Science 195*, 1328–1330.

Marcus, M. A., & Lewis, A. (1978) *Biochemistry 17*, 4722–4735.

Mathies, R. A. (1984) in *Spectroscopy of Biological Molecules* (Sandorfy, C., & Theophanides, T., Eds.) pp 303–328, D. Reidel, Dordrecht, The Netherlands.

Nakagawa, M., Maeda, A., Ogura, T., & Kitagawa, T. (1989) *J. Raman Spectrosc.* (in press).

Oesterhelt, D., & Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2853–2857.

Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31A*, 667–678.

Ogura, T., Maeda, A., Nakagawa, M., & Kitagawa, T. (1987) in *Primary Processes in Photobiology* (Kobayashi, T., Ed.) pp 233–241, Springer-Verlag, Berlin.

Ohno, K., Takeuchi, Y., & Yoshida, M. (1981) *Photochem. Photobiol. 33*, 573–578.

Ort, D. R., & Parson, W. W. (1978) *J. Biol. Chem. 253*, 6158–6164.

Ottolenghi, M. (1980) *Adv. Photochem. 12*, 97–200.

Renard, M., & Delmelle, M. (1981) *FEBS Lett. 128*, 245–248.

Rosenbach, V., Goldberg, R., Gilon, C., & Ottolenghi, M. (1982) *Photochem. Photobiol. 36*, 197–201.

Scherrer, P., & Stoeckenius, W. (1985) *Biochemistry 24*, 7733–7738.

Smith, S. O., Myers, A. B., Pardoen, J. A., Winkel, C., Mulder, P. P. J., Lugtenburg, J., & Mathies, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2055–2059.

Smith, S. O., Lugtenburg, J., & Mathies, R. A. (1985a) *J. Membr. Biol. 85*, 95–109.

Smith, S. O., Myers, A. B., Mathies, R. A., Pardoen, J. A., Winkel, C., van der Berg, E. M. M., & Lugtenburg, J. (1985b) *Biophys. J. 47*, 653–664.

Smith, S. O., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1987a) *J. Phys. Chem. 91*, 804–819.

Smith, S. O., Braiman, M., Myers, A. B., Pardoen, J. A., Mulder, P. P. J., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987b) *J. Am. Chem. Soc. 109*, 3108–3125.

Stockburger, M., Klusmann, W., Gattermann, H., Massig, G., & Peters, R. (1979) *Biochemistry 18*, 4886–4900.

Stockburger, M., Alshuth, T., Oesterhelt, D., & Gartner, W. (1986) in *Spectroscopy of Biological Systems* (Clark, R. J. H., & Hester, R. E., Eds.) pp 483–535, Wiley, New York.

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 52*, 587–616.

Xie, A. H., Nagle, J. F., & Lozier, R. H. (1987) *Biophys. J. 51*, 627–635.