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Resonance Raman Study on Proton-Dissociated State of Bacteriorhodopsin: Stabilization of L-like Intermediate Having the All-Trans Chromophore†

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Received June 12, 1985; Revised Manuscript Received November 4, 1985

ABSTRACT: The resonance Raman spectra of light-adapted bacteriorhodopsin in purple membrane were investigated in the alkaline pH region where its Schiff base was kept protonated. Upon raising the pH from 7.0 to 10.5, the spectrum of bacteriorhodopsin excited at 514.5 nm remains essentially unaltered when laser power is low, but distinct features appear due to the formation of a long-lived intermediate as the laser power is raised. The spectral characteristics of the long-lived intermediate are as follows: (1) a small shift or disappearance of the N–H (or N–D) bending mode of the protonated Schiff base, (2) the appearance of new bands at 1188 and 1159 cm^{-1}, and (3) the disappearance of the 1456-cm^{-1} line accompanied with the appearance of several bands at 1470, 1446, and 1397 cm^{-1}. The resultant resonance Raman spectrum resembled that of the L_{543} intermediate, except for the C—C, C=C, and C=N stretching frequencies, which are the same as those of bacteriorhodopsin having the all-trans chromophore. Such an L-like intermediate appears with a midpoint pH of conversion around 9 in parallel to the absorbance change at 296 nm. The pH difference spectrum of bacteriorhodopsin in the UV region was close to the alkaline – neutral difference spectrum of free tryptophan due to the ionization of an amino acid residue in the vicinity of tryptophan. The residue responsible for the ionization at pH 9 is tentatively assigned to the carboxylate with an unusually high pK_a, as pointed out by Engelhard et al. [Engelhard, B., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400]. It is stressed that its ionization stabilizes the L-like intermediate having the all-trans chromophore.

Bacteriorhodopsin (bR)[1] is the only protein in the purple membrane, which is formed as a differentiated patch in the membrane of halophilic bacteria. Retinal is attached to the specific lysine residue of the protein through the protonated Schiff base. The bR molecule possessing *all-trans*-retinal conveys two protons unidirectionally for each photocycle [see the review by Stoeckenius & Bogomolni (1982)] containing several intermediates called K_{610}, KL_{590}, L_{543}, M_{412}, and O_{640} in order of their appearance (Stoeckenius et al., 1979; Shichida et al., 1983). These intermediates are characterized by their visible absorption and resonance Raman (RR) spectra (Marcus & Lewis, 1978; Terner et al., 1979; Braiman & Mathies, 1980, 1982; Argade & Rothschild, 1983; Smith et al., 1983, 1984; Alshuth & Stockburger, 1986). The light energy once stored in the 13-cis isomer of the retinal chromophore is transmitted to the protein moiety to translocate protons. The structural mechanism for the proton transfer has been a subject of recent spectroscopic studies.

† This work was supported by the Grant-in-Aids for Special Project Research on Molecular Mechanism of Bioelectrical Response to A.M. (59123002) and T.K. (59223015) from the Japanese Ministry of Education, Science and Culture. This work was also supported by the Joint Studies Program (1985) of the Institute fo. Molecular Science (Okazaki, Japan).

[1] Abbreviations: bR, bacteriorhodopsin; RR, resonance Raman; FTIR, Fourier transform infrared; λ_{max}, wavelength for maximum absorption.

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The proton in the protonated Schiff base is detached from it upon the formation of the M_{412} intermediate (Marcus & Lewis, 1978). This is followed by the release of protons into the outer milieu and the deprotonation of a tyrosine residue (Hanamoto et al., 1984). Recently, Engelhard et al. (1985) have proposed from their Fourier transform infrared (FTIR) spectra that bR has a protonated aspartic residue, which is deprotonated in the L_{543} intermediate. The pH profiles for the intermediate formation of bR (Kalisky et al., 1981; Rosenbach et al., 1982; Alshuth & Stockburger, 1986) suggest importance of the protein residue ionized around pH 9–11. Similar pH dependency has been observed for the ratio between the slow- and fast-decaying forms of the M intermediate (Hanamoto et al., 1984; Li et al., 1984) and for flash-induced proton release (Li et al., 1984). It appears that the proton attached to the pK_a = 9–11 residue is positively involved in the proton transfer. Therefore, we investigated the pH-dependent RR and absorption spectral properties of bR. Here we report that an intermediate showing an RR spectrum similar to that of L_{543} is stabilized in the all-trans form by ionization of the particular residue having pK_a = 9 and discuss the role of this residue in the proton-transfer reaction.

Materials and Methods

bR in purple membrane was prepared from the lysate of the cells of *Halobacterium halobium* R_1M_1, which was given by Prof. D. Oesterhelt, with the standard method (Oesterhelt & Stoeckenius, 1974) and stored as suspensions in distilled water at 0 °C. Light-adapted bR in purple membrane (bR_{568}) was exposed to the 0.2 M KCl solution of either 10 mM phosphate buffer (pH 7.0) or carbonate buffer (pH 10.5) just before measurements. N-Deuteration of the Schiff base was performed as follows: bR in H_2O was spun down for 30 min at 50000*g*, and the precipitates were suspended in D_2O. The suspensions were centrifuged again. The same procedure was repeated twice. Neutral or alkaline bR in D_2O was prepared by putting the N-deuterated bR in the corresponding D_2O–KCl buffer solution.

Acetylation of lysine residues in bR was conducted as described previously (Maeda et al., 1982). There remained 14 ± 1% of free amino residues as determined spectrophotometrically after its reaction with trinitrobenzenesulfonic acid (Maeda et al., 1982). Since bR contains no free amino terminus and seven lysine residues (Khorana et al., 1979) and one of them is involved in the binding with retinal, the acetylated bR thus prepared should have no intact lysine residues.

Conventional RR spectra were excited by the 514.5-nm line (ca. 30 mW, focused beam diameter ~0.1 mm) of an Ar^+ ion laser (NEC GLG3200) and measured with a JEOL-400D Raman spectrometer equipped with a cooled RCA 31034a photomultiplier. All the spectra were measured with a spinning cell (diameter = 2 cm, 1800 rpm). The temperature of the spinning cell was kept at about 5 °C by flushing with cold N_2 gas. After the Raman measurements, visible absorption spectra of the sample were examined. The maximum bleaching was 30% for bR at pH 10.5 after 2-h exposure to the laser light. The spectral shape remained unchanged.

For the measurements of flowing samples, an OMA-2 system (PAR 1215) and a diode array detecter (PAR 1420) attached to Spex 1404 double monochromator were used. About 14 mL of the bR solution with A_{568nm} = 1.4 was flowed with Micro Pump (Model 185-415) at the rate of 140 mL/min. Since the cross-section of the rectangular Raman cell is 3 mm × 0.5 mm, a given molecule passes through the laser beam of 0.1-mm diameter with the rate of 155 cm/s and, accordingly, is illuminated by laser light for 64 μs. The sample reservoir was kept at 0 °C. Raman frequencies were calibrated with indene for both kinds of measurements.

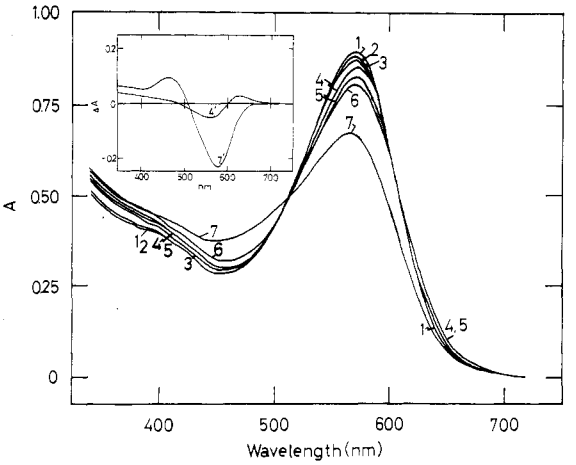

FIGURE 1: pH dependence of the absorption spectrum of bR_{568} in 0.2 M KCl: (1) pH 6.9, (2) pH 8.3, (3) pH 9.3, (4) pH 10.6, (5) pH 11.5, (6) pH 12.6, and (7) pH 13.0. Spectral measurements were done at 3 °C. Difference spectra (4′ and 7′) in the inset were obtained by subtracting spectrum 1 from spectra 4 and 7, respectively.

The pH-difference absorption spectra were measured in reference to the light-adapted bR in purple membrane (A_{570nm} = 0.34) in 5 mM phosphate buffer, pH 7.0, with 0.2 M KCl by means of a double-beam recording spectrophotometer (Hitachi 124). The cuvette chamber was cooled to 3 °C by circulating water, and dry N_2 gas was flushed toward the front and back faces of the cuvettes. The pH values were adjusted by adding 1 or 4 N NaOH solution and determined with Radiometer pH meter M26.

Results

Changes in RR Spectrum at Alkaline pH. The pH-dependent changes in the visible absorption spectrum of light-adapted bR in the purple membrane (bR_{568}) at 3 °C are shown in Figure 1, where the difference spectra between pH 10.6 and pH 7.0 and between pH 13.0 and pH 7.0 are depicted as curve 4′ and curve 7′ in the inset, respectively. The spectral change below pH 10.6 (curve 4) is small, as consistent with the previous observations by Muccio and Cassim (1979) or by Scherrer and Stoeckenius (1984) for dark-adapted bR at room temperature. A small increase of absorbance around 420–500 nm below pH 10.6 is largely due to the increase of turbidity. The deprotonated state of the Schiff base as reflected in the structured absorption band around 460 nm, which was described by Druckmann et al. (1982), becomes prominent only above pH 12 (curve 7). Over the pH range below 12, the absorption spectrum varies in a complicated manner with an accompanied small red shift but without an appreciable deprotonation of the Schiff base. This will be discussed separately (A. Maeda et al., unpublished results).

In Figure 2, RR spectra of light-adapted bR in the purple membrane (bR_{568}) at pH 10.5 (b) and at pD 10.5 (d) are compared with those at pH 7.0 (a) and at pD 7.0 (c). These spectra were obtained by a conventional system with the spinning cell. The spectra at neutral pH (a and c) are almost the same as those reported previously (Terner et al., 1979; Stockburger et al., 1979; Massig et al., 1981; Smith et al., 1984; Alshuth & Stockburger, 1986). The main band of the in-phase C=C stretching mode ($\nu_{C=C}$) is seen at 1528 cm^{-1}.

Its satellite band at around 1550 cm^{-1} comprises ca. 15% of the total intensity for bR in H_2O and 20% for bR in D_2O. These bands are probably due to the L_{543} intermediate with the 13-cis chromophore. The frequency distribution of $\nu_{C=C}$

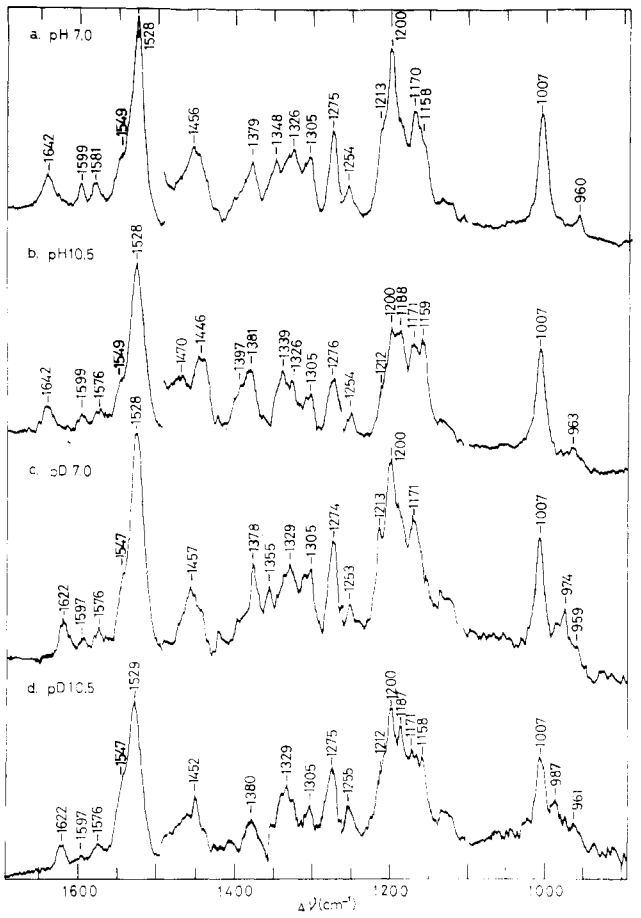

FIGURE 2: CW Raman spectra of bR (a) at pH 7.0 in H_2O, (b) at pH 10.5 in H_2O, (c) at pD 7.0 in D_2O, and (d) at pD 10.5 in D_2O. Instrument conditions: slit width 6 cm^{-1}; scan speed 10 cm^{-1}/min; laser 514.5 nm, 30 mW; 900–1260-cm^{-1} region with time constant 8 s, sensitivity 1000 pulses/s; 1260–1500-cm^{-1} region with time constant 16 s, sensitivity 500 pulses/s; 1500–1700-cm^{-1} region with time constant 4 s, sensitivity 2500 pulses/s. The 1200–1700-cm^{-1} region was measured first, then the 1100–1300-cm^{-1} region, and finally the 900–1100-cm^{-1} region.

can afford a clue for estimating the species appearing in a given RR spectrum in view of its well-known relation with λ_{max} of the visible absorption spectrum of various intermediates of bR (Aton et al., 1977). Under the present experimental conditions (30-mW laser power, 0.1-mm diameter of focused beam), the appearance of L_{543} in the spectrum might be inevitable. It is worth noting that the relative intensity of the satellite band at around 1550 cm^{-1} to the main band at 1528 cm^{-1} remains unaltered upon alkalization to pH 10.5. Moreover, a 1189-cm^{-1} band characteristic of the L_{543} intermediate (Alshuth & Stockburger, 1986) is not obviously seen in the RR spectrum at neutral pH (Figure 2a). Therefore, the difference between the spectra observed at pH 7.0 and 10.5 is not accountable in terms of the increased population of L_{543} intermediate. A major portion of the RR spectrum is derived from the species giving the $\nu_{C=C}$ band at 1528 cm^{-1}. There is no trace of a Raman line around 1565 cm^{-1} where the bR having a deprotonated Schiff base (Druckmann et al., 1982) would give rise to the $\nu_{C=C}$ band.

Characteristics of RR Spectrum at Alkaline pH. The RR spectrum measured at pH 10.5 exhibits several features distinct from those at pH 7.0: the lack of the 1456-cm^{-1} line, the disappearance of the 1348-cm^{-1} line, and the appearance of two lines at 1188 and 1159 cm^{-1} as well as of the shoulder at 1397 cm^{-1}. Alkalization in D_2O causes the shift of the 974-cm^{-1} line to 987 cm^{-1}. These specific features in the spectrum

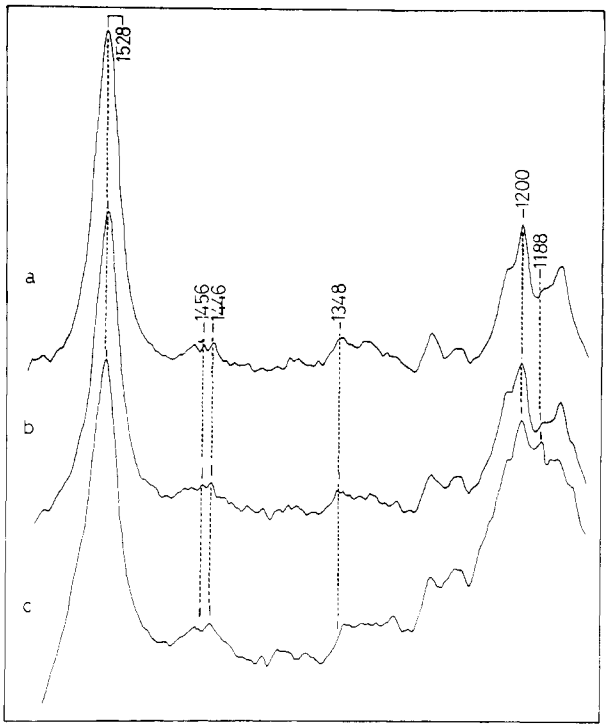

FIGURE 3: RR spectra for the bR samples measured in the flow cell: (a) bR at pH 7.0 measured with 1-mW laser power; (b) bR at pH 10.5 with 1-mW laser power; (c) bR at pH 10.5 with 100-mW laser power. Flow rate was 150 mL/min.

at alkaline pH can also be found in the spectrum of L_{543} (Terner et al., 1979; Argade & Rothschild, 1983; Alshuth & Stockburger, 1986), an intermediate in the normal photocycle. With these respects, the RR spectrum at alkaline pH also resembles the previously reported spectrum of the L-like intermediate at pH 9.6 (Alshuth & Stockburger, 1986), which was obtained by subtracting the RR intensity of the remained bR_{568} from the observed spectrum under the assumption that the RR intensity in the 1510–1525-cm^{-1} region is solely due to bR_{568}.

A distinct feature of the RR spectrum at pH 10.5 from that of L_{543} is an unaltered $\nu_{C=C}$ at 1528 cm^{-1}. Since the 1348-cm^{-1} line in H_2O or the 974-cm^{-1} line in D_2O is considered to be diagnostic for bR_{568}, the lack of these lines in the spectrum at pH 10.5 indicates the absence of bR_{568}. Consequently, the bR species probed at alkaline pH has features resembling both L_{543} and bR_{568} but is not identical with either of them.

Long-Lived Intermediate. If the lifetime of an intermediate is prolonged at alkaline pH and the original bR_{568} is not restored in a period of one turn of the spinning cell, the intermediate would be accumulated, and its contribution to the RR spectrum may become predominant. This idea was tested by measurements with a flow system, in which it needs 6 s, at least, until the same molecule is reilluminated by the laser beam.

As shown in Figure 3, the RR spectrum measured at pH 10.5 (b) with a very weak laser power (1 mW) holds the features specific to bR_{568} at neutral pH (a): the presence of 1456- and 1348-cm^{-1} lines and the absence of 1446- and 1188-cm^{-1} lines. Therefore, the RR spectrum of unphotolyzed alkaline bR is the same as that of neutral bR_{568}. The RR line at 1188 cm^{-1} characteristic of the intermediate at alkaline pH appears when the laser power is increased to 100 mW (c). These results strongly suggest that a major portion of the RR spectrum measured with the spinning cell at pH 10.5 (Figure 2b) arises from the accumulated intermediate with a long

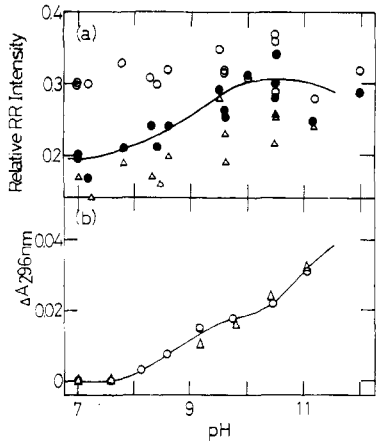

FIGURE 4: (a) pH dependence of the RR intensities at 1200 (open circles), 1188 (closed circles), and 1159 (open triangles) cm^{-1}. The intensities are represented as relative peak height to the 1528-cm^{-1} band. The spectra were measured with the conditions described in the caption of Figure 2. Phosphate buffer was used below pH 8.5, and carbonate buffer was used above it. The pH was adjusted by adding 1 N HCl or 1 N NaOH and determined with a Beckman pH meter ϕ71 before the measurement for the RR spectrum. (b) pH dependence of the difference absorbance at 296 nm with regard to bR at pH 7.0. Open circles and triangles were adopted from two separate series of the experiment.

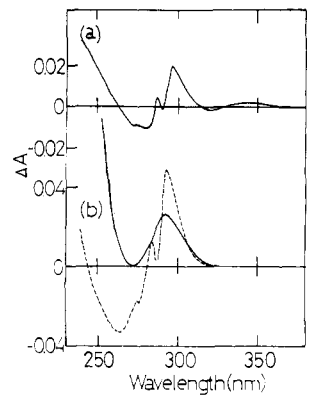

FIGURE 5: (a) Difference between the absorption spectrum of bR at pH 10.5 minus that at pH 7.0. (b) The alkaline – neutral difference spectra of acetyltyrosine ethyl ester (full line) and tryptophan (dashed line), which were obtained as the differences between the neutral solution and the solution with 4 mM NaOH.

lifetime (longer than 30 ms, the period for one turn of the cell).

The RR spectrum of a long-lived intermediate at alkaline pH (Figure 2b,d), however, shows the $\nu_{C=C}$ line at 1528 cm^{-1} as bR_{568}. The C=N stretching mode of the protonated Schiff base ($\nu_{C=NH}$) at 1642 cm^{-1} (a and b) and of its N-deuterated form ($\nu_{C=ND}$) at 1622 cm^{-1} (c and d) is also unaltered. These RR lines are shifted to higher frequencies for the L_{543} intermediate at neutral pH (Terner et al., 1979). This can be considered to be due to the 13-cis structure of the chromophore (Tsuda et al., 1980).

Some of RR lines in the 1100–1300-cm^{-1} region are assigned to C–C stretching vibrations (ν_{C-C}). The Raman lines of bR_{568} at 1254, 1213, 1200, and 1170 cm^{-1} have been assigned to the modes involving primarily the C_{12}–C_{13}, C_8–C_9, C_{14}–C_{15} and C_{10}–C_{11} stretching vibrations, respectively (Smith et al., 1983). At alkaline pH, the same lines are held, although a complete absence of the 1213-cm^{-1} line is noted for the RR spectrum of L_{543} (Terner et al., 1979; Alshuth & Stockburger, 1986). Therefore, all the ν_{C-C} frequencies of *all-trans*-bR_{568} as well as its $\nu_{C=C}$ and $\nu_{C=N}$ frequencies remain unchanged upon formation of the long-lived intermediate at alkaline pH. Frequency shifts as well as intensity changes in some of ν_{C-C} have been observed between the RR spectra of *all-trans*- and 13-*cis*-retinal (Curry et al., 1984). These results indicate that the skelton of the chromophore of the long-lived intermediate does not take the 13-cis form like L_{543} but probably the all-trans structure, though it has specific features resembling those of L_{543}.

pH-Dependent RR and Absorption Spectral Changes in the UV Region. The additional two lines of alkaline bR at 1188 and 1159 cm^{-1} (Figure 2b) cannot be attributed to the skeltal C–C stretching modes because of the assigned lines present. Figure 4a illustrates the pH dependences of the RR intensities at 1200 and 1188 cm^{-1}, which are shown conventionally as relative intensity to the 1528-cm^{-1} line. Intensity becomes more variable as the pH increases, owing probably to decreased quality of the spectrum at alkaline pH, yet some trends can be drawn from this figure. The intensity of the Raman lines at 1188 cm^{-1} (b), which is characteristic of the L-like intermediate at alkaline pH, increases with a midpoint pH at ca. 9.0, while the intensity at 1200 cm^{-1} is unaltered.

In the pH region below 10, the pH-dependent increase of absorbance at 296 nm (Figure 4b) appears nearly in agreement with the changes in the Raman intensity at 1188 cm^{-1}. This implies some correlation between the appearance of the long-lived intermediate and the absorption change in the protein residue.

The increase of absorbance at 296 nm is not due to isomerization of the chromophore from the all-trans to 13-cis form, since no changes in the absorbances in the 310–370-nm region, which should be characteristic of isomerization of the chromophore (Lanyi, 1984), appear in the pH-difference spectrum (pH 10.5 minus pH 7.0) in Figure 5a. The difference spectrum exhibits a structure with the maxima at 296 and 286 nm along with a broad negative trough around 275 nm. Such a pattern, which is similar to that described by Scherrer and Stoeckenius (1984), is distinct from the smooth curve for the corresponding difference spectrum of tyrosine (solid line in b) but is rather close to the difference spectrum of tryptophan (broken line in b) at alkaline pH. The pH-dependent spectral change of the tryptophanyl residue is thus caused by a change of an electrical perturbation on the indole ring upon the ionization of the $pK_a = 9$ residue but not due to the dissociation of the indole proton.

The difference in mole absorbance ($\Delta\epsilon_M$) of bR at pH 10.5 is estimated to be 3200 at 296 nm on the basis of the molar extinction coefficient of bR_{568}, which is 63 000 at 568 nm (Oesterhelt & Hess, 1973). This is about four times larger than $\Delta\epsilon_M$ (830) of free tryptophan in H_2O [adopted from the *Handbook of Biochemistry; Selected Data for Molecular Biology* (1970)]. The $\Delta\epsilon_M$ value of free tryptophan increases by a factor of 1.6 in its 2-propanol solution (not shown) and may further increase with more hydrophobic solvent. Then, the number of tryptophan residues required to provide $\Delta\epsilon_M$ of bR would become smaller than four. Either of Trp-12, -86, -182, or -189 may be responsible for them because they might be located close to the chromophore (Plotkin & Sherman, 1984).

Ionization of Protein Residue. As candidates for the $pK_a = 9$ residue, which causes the UV spectral change at 296 nm, cysteine, histidine, and free amino terminus are excluded, because bR is devoid of them (Khorana et al., 1979). Tyrosine is not likely due to the reason described above. Lysine can be a candidate. Hence, acetylation of lysine residues was carried out. The RR spectra of the acetylated bR measured at pH 7.0 and 10.5 (not shown) are almost identical with those shown in Figure 2a,b. This indicates insignificance of the

lysine residue in the alkaline titration of bR. Therefore, only the carboxyl residue with an unusually high pK_a value is conceivable as a candidate for stabilizing the long-lived intermediate at alkaline pH.

Discussion

Photochemical Reaction at Alkaline pH. It is now evident that deprotonation of the protein residue with pK_a = 9 stabilizes the L-like intermediate. Its chromophore is presumably in the all-trans form because the frequencies of ν_{C-C} and $\nu_{C=C}$ are unaltered from those of bR568. Laser power dependency for its formation suggests that it might arise from a secondary photoreaction of an intermediate in the normal photocycle. The primary intermediate may be the 13-cis L_{543} probed as a satellite $\nu_{C=C}$ band at around 1550 cm^{-1} (Figure 2b,d). No gross changes are noticed for both the visible absorption (Figure 1) and RR spectra (Figure 3) of unphotolyzed bR upon alkalization. Small changes that may occur at alkaline pH will be dealt with as a future problem. The all-trans intermediate thus produced has a quite long lifetime, which should be longer than the period of the spinning cell (33 ms) at least. However, since the absorption spectrum of the sample subjected to extensive Raman measurements keeps the same spectral shape as the original one, a possibility of irreversible denaturation is ruled out. The lifetime of the 13-cis intermediate may also be prolonged at alkaline pH as determined from the time dependence of the $\nu_{C=C}$ line at 1550 cm^{-1} (Alshuth & Stockburger, 1986).

Recently, Engelhard et al. (1985) have proposed from their FTIR spectra that bR_{568} has a protonated form of an aspartic acid residue [Asp(1)]. In their model, the protonated Schiff base is interacting with the dissociated form of another aspartic acid [Asp(2)] in bR_{568} but changes its partner to Asp(1) in L_{543} by expelling its proton. We assume that Asp(1) is the residue with the pK_a = 9, and therefore, the residue is protonated in neutral bR_{568} but deprotonated in bR at pH 10.5. At the moment it cannot be decided whether or not this assumption is compatible with their failure to detect any dissociation of Asp(1) in the pH region up to 9, since some uncertainty is included in the estimation of pH values. Alternatively, another carboxyl residue with a higher pK_a may be responsible for the pK_a = 9 residue. Since the Schiff base is already under interaction with the deprotonated carboxylate [Asp(2)], it is not unreasonable that the appearance of another charged residue at alkaline pH little affects the visible absorption and RR spectra.

In the framework of this model, the deprotonated Asp(1) of L_{543} has a tendency to rebind proton at neutral pH, and therefore, the Schiff base proton will be transferred to it in the next stage. This could result in the formation of M_{412} having the unprotonated Schiff base. In contrast, at alkaline pH, the deprotonated state of Asp(1) or of the particular carboxyl group is stable and would keep the Schiff base in the protonated state for a longer duration. When another photon comes under such circumstances, the chromophore would be isomerized to the all-trans form of the L-like structure having a long lifetime.

Both bR_{568} and the L-like intermediate do not show the deuterium effect expected for the N-syn configuration, which should otherwise affect the RR spectrum in the ν_{C-C} stretching region (Smith et al., 1984). Furthermore, in our normal-mode calculations of retinal Schiff base, the syn–anti transition of the C=N bond causes frequency shifts of several bands above 1100 cm^{-1}, and all these bands are sensitive to N-deuteration (T. Kitagawa et al., unpublished results). This is contradictory to the observed results. Therefore, anti configuration with respect to the C=N bond should be preserved in the L-like intermediate. Nevertheless, the all-trans chromophore of the L-like intermediate should have something different from the all-trans chromophore of bR_{568}. The difference would include the interaction between the Schiff base proton and Asp(2), judging from the absence of the N–H bending mode in the L-like intermediate. This might take place if the carboxylate of Asp(2) were displaced from its original position due to a conformation change of the protein.

Several pH-dependent phenomena having their pK_a in the same pH region are reported. Most interesting among them is the decreased amount of L_{543} in the photo steady state at −90 °C (Kalisky et al., 1981). The absence of L_{543} in the photocycle of the alkaline bR is also reported from flash photolysis experiments (Rosenbach et al., 1982). A failure to detect L_{543} in these studies is probably due to the formation of the L-like intermediate, which should have almost the same absorption spectrum as bR_{568} in view of the same $\nu_{C=C}$.

The pH dependence of the population ratio of the slow- to fast-decaying forms of M_{412} (Hanamoto et al., 1984; Li et al., 1984) is similar to that in flash-induced proton release (Li et al., 1984). This would be correlated with the pH dependence of the formation rate of the long-lived intermediate. It should be emphasized that the presence of a protonated residue of pK_a = 9 and its interaction with the chromophore in the L_{543} intermediate are important for establishing the normal photocycle.

RR Spectrum of Intermediates at Alkaline pH. The RR lines specific to L_{543} are supposed to be a result of the interaction of the Schiff base with the dissociated carboxylate residue, probably of Asp(1) described by Engelhard et al. (1985). The RR spectral similarity between the L-like intermediate with the all-trans form and L_{543} with the 13-cis form is most prominent in the frequency region around 1450, 1350, and 1150–1250 cm^{-1} with bR in H_2O and in the 970–990-cm^{-1} region with bR in D_2O. The assignments of these bands are especially important for clarifying the mechanism of proton pumping, in view of the role of the L intermediate in its process.

The 1348- and 974-cm^{-1} lines of bR_{568}, which are shifted at alkaline pH, are assigned to the N–H and N–D bending vibrations of the protonated Schiff base, respectively (Massig et al., 1982). This assignment is supported by an FTIR study by Siebert and Mäntele (1983). Since the N–H bending frequency is much more sensitive to its hydrogen-bonding interaction than the $\nu_{C=NH}$ (or $\nu_{C=ND}$) bending mode without any change of the $\nu_{C=NH}$ (or $\nu_{C=ND}$) frequency is not unreasonable and seems to arise from the changes of the interaction involving the proton on the Schiff base. The N–H bending band at pH 10.5 is presumably located at 1339 cm^{-1}, since this band is somewhat sensitive to N-deuteration. The downshift of the N–H bending mode is explained as a weakening of the hydrogen bonding to the carboxylate (Maeda et al., 1985). Therefore, the frequency shift may reflect the difference between N–H···Asp(1) and N–H···Asp(2) interactions, if one accepts the model described above.

An apparent single band around 1450 cm^{-1} in Figure 2 seems to be composed of two bands at 1457- and 1446-cm^{-1} lines. This splitting is shown clearly in the RR spectrum in Figure 3a. The photoproduct shows the 1470-cm^{-1} line instead of the missing 1457-cm^{-1} band, leaving the 1446-cm^{-1} line (Figure 3c). With the N-deuterated form, the latter band is located at 1452 cm^{-1}. This part of the RR spectrum has never been carefully analyzed so far, but it appears structure sensitive. In our previous study on halorhodopsin (Maeda et al.,

1985), the RR line in this region exhibits an upward shift by 6–7 cm^{-1} in D_2O. The RR line around 1450 cm^{-1} has been assigned to the asymmetric deformation modes of the methyl groups on the basis of the normal-coordinate calculations of *all-trans*-retinal (Curry et al., 1982; Saito & Tasumi, 1983). The RR spectrum of 13-desmethyl-bR (Schiffmiller et al., 1985; A. Maeda et al., unpublished results) lacks such features around 1450 cm^{-1}.

The appreciable effect of deuteration of protein on the CH_3 vibration implies the presence of a strong methyl–protein interaction, which is altered by a conformation change of the protein upon deuteration. Alternative assignment is that the 1450-cm^{-1} line arises from a symmetric stretching mode of a carboxylate, which is resonance enhanced through unknown electronic interactions with the retinal chromophore.

The Raman line at 1397 cm^{-1}, which is appreciably intensified in the photoproduct, is located close to 1387 cm^{-1} of the methyl symmetric deformation modes of *all-trans*-retinal (Curry et al., 1982; Saito & Tasumi, 1983). More closer to it is the 1402-cm^{-1} band of *all-trans*-retinal, which has been assigned to C_{15}–H in-plane deformation (Curry et al., 1984). We cannot distinguish these alternatives at the present.

The two lines appearing in the alkaline spectra at 1188 and 1159 cm^{-1} could be neither the C–C stretching vibrational modes as described under Results section nor the C–N stretching vibrational mode as was suggested by the absence of the effect of ^{15}N substitution on the RR spectrum of bR (Argade et al., 1981). These could be coupled modes of the C–H in-plane deformation with the C–C stretching modes. If both the 1450- and 1397-cm^{-1} lines were assigned to the methyl modes, these two modes might be associated with the C–CH_3 stretching vibrations at C_9 and C_{13} of the retinal, although their frequencies are higher than usually expected. These points remain to be elucidated by further study.

Acknowledgments

We express our gratitude to Drs. M. Stockburger and T. Alshuth of Max-Planck-Institüt fur biophysikalische Chemie, Göttingen, FRG, for sending us their manuscript prior to its publication and also for stimulating discussion on this paper. We thank Professor Tôru Yoshizawa of Kyoto University for his encouragements during this work.

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